Title: Blood Concentrations of Per- and Polyfluoroalkyl Substances are Associated with Lupus-like Autoimmunity in the American Alligator

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Abbreviations: ACN: acetonitrile; CFR: Cape Fear River; CV: coefficient of variation; F: female; Infα interferon alpha type 1; IS: internal standard; LW: Lake Waccamaw; LOD: limit of detection; M: male: PCA: principal component analysis; RBC: red blood cell; SLE: systemic lupus erythematosus; SRM: standard reference material; SVL: snout to vent length; TBARS: thiobarbituric acid reactive substances

20 **Highlights:**

21 • Populations of American Alligator (*Alligator mississippiensis*) from the Cape Fear River
22 in NC have elevated PFAS exposures compared to a nearby reference population

23 • Increased blood PFAS concentrations are associated with increased innate immune
24 activities and hallmark phenotypes of autoimmune disease including increases in
25 autoantibodies

26 • Compared the reference population, expression of interferon alpha responsive signature
27 genes that are predictive of lupus erythematosus in humans was increased ~ 60- to 577-
28 fold in the Cape Fear River Alligators with elevated blood PFAS

Abstract:

Surface and ground water of the Cape Fear River basin in central and coastal North Carolina is contaminated with high levels of per- and polyfluoroalkyl substances (PFAS). Elevated levels of PFAS have also been found in blood of fish and wildlife living in and around the Cape Fear River, and in the blood of human populations reliant on contaminated well or surface water from the Cape Fear River basin as a sources of drinking water. While the public and environmental health impacts of long-term PFAS exposures are poorly understood, elevated blood concentrations of some PFAS are linked with immunotoxicity and increased incidence of some chronic autoimmune diseases in human populations. The goal of this study was to evaluate PFAS exposure and biomarkers related to immune health in populations of American alligators (*Alligator mississippiensis*), a protected and predictive sentinel species of adverse effects caused by persistent toxic pollutants. We found that serum PFAS concentrations in a representative population of alligators from the Cape Fear River were increased compared to a reference population of alligators from the adjoining Lumber River basin. The elevated serum PFAS concentrations in the Cape Fear River alligators were associated with increased innate immune activities and hallmark phenotypes of the human autoimmune disease systemic lupus erythematosus. In addition to significant increases in double stranded-DNA binding autoantibodies in adult Cape Fear River alligators, our qRT-PCR analysis found remarkably high induction of Interferon-α signature genes implicated in the pathology of human autoimmune disease. We interpret the association of increased PFAS exposure with disrupted immune functions to indicate that PFAS broadly alters immune activities resulting in autoimmune disease in American Alligators. This work substantiates and extends evidence from experimental models and human epidemiology studies showing that some PFAS are immune toxicants.

Keywords: autoantibodies; autoimmune disease; crocodilian; immune toxicity; lupus; PFAS

Introduction:

Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic organic chemicals that are global contaminants of both built and natural environments (OECD 2018). Owing to their extensively fluorinated aliphatic backbone, PFAS are chemically stable, resistant to thermal and enzymatic breakdown, and can persist in terrestrial and aquatic environments. Because of their widespread use over the past 70 years, many PFAS and their terminal breakdown products, have become ubiquitous contaminants of the land, water, and air to which humans and wildlife are exposed (De Silva et al. 2021). In the US alone, drinking water supplies for an estimated 200 million people are contaminated with PFAS, with recent analyses estimating the direct annual health-related cost due to PFAS exposure ranges from $37-59 billion in the US and €52-84 billion per year in the European Union (Cordner et al. 2021; Andrews and Naidenko 2020)

Evidence from experimental animal and human epidemiologic studies has linked increased exposure to the most abundant PFAS with immunotoxicity and altered immune functions (DeWitt 2015). Those data led the United States National Toxicology Program to conclude that perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are hazards to the human immune system (DeWitt 2015; NTP 2016). Additional findings have also demonstrated associations between exposures to some PFAS with immunosuppression, with adverse health impacts linked to increased incidence in childhood infection, decreased antibody production in response to vaccination, and increases in severity of COVID-19 (Grandjean et al. 2020; Fenton et al. 2021). There is also evidence linking some PFAS exposures with an increased incidence of chronic autoimmune disorders, including thyroid disease and inflammatory bowel diseases

76 (Fenton et al. 2021). The mechanisms of PFAS-mediated immunotoxicity and their roles in
77 autoimmunity are poorly understood.
78 The Cape Fear River basin, located in central and coastal North Carolina encompasses over
79 9,300 sq. miles of waterways that service ~5.2 million people in rural and urban communities,
80 and is typical of many regions with high PFAS contamination of surface, ground, and drinking
81 water (Fig. 1A). PFAS contamination in the Cape Fear originates primarily from fluorochemical
82 production, manufacturing, wastewater treatment discharges, and the use of aqueous film
83 forming foams (AFFF) as fire suppressants (McCord and Strynar 2019). In 2017, surface water
84 sampling from the Cape Fear River revealed high levels of per- and polyfluoroether acids
85 (PFEA) and fluoropolymer manufacturing byproducts with diverse chemical structures
86 originating from a fluorotelomer and fluoropolymer PFAS production facility active since the
87 mid-1980's (Hopkins et al. 2018; Sun et al. 2016). Both novel PFEAs such as HFPO-DA
88 (GenX), Nafion byproducts, and a variety of other fluorochemicals from PFAS-based aqueous
89 film forming foam used as a fire suppressant by military, municipalities, and regional airports
90 have now been detected in the drinking water and blood samples of residents living near the
91 Cape Fear River (McCord and Strynar 2019; Ruyle et al. 2021; Kotlarz et al. 2020). Recently,
92 total organic fluorine analysis detected extremely high sum PFAS concentrations in excess of
93 100,000 ng/L in the Cape Fear River at drinking water treatment plant intakes suggesting that
94 humans and aquatic ecosystems in the Cape Fear River basin have been experiencing very high
95 levels of total PFAS exposure (Zhang et al. 2019). The potential for impacts on both ecosystems
96 and human populations from this contamination has been confirmed by wildlife and human
97 population based PFAS exposure studies finding increased levels of PFAS in fish, birds, and
98 humans living near the CFR (Guillette et al 2020; Kotlarz et al. 2020; Robuck et al. 2021). The

99 public and environmental health impacts of long-term exposures resulting from these
100 undocumented PFAS discharge are largely unknown.
101 The aim of this study was to evaluate the impacts of long-duration PFAS exposure on
102 American alligators (*Alligator mississippiensis*), a top trophic carnivore at the human/wildlife
103 interface of both rural and urban environments (Beal and Rosenblatt 2020; Somaweera et al.
104 2020). Alligators, renowned for their robust and highly protective immune systems, are an
105 especially important aquatic biomonitoring and predictive sentinel species of adverse outcomes
106 resulting from integrated effects of persistent toxic chemicals (Crain and Guillette 1998;
107 Guillette et al. 2000; Finger and Gogal 201; Pérez and Wise 2018). Alligators living along the
108 Cape Fear River are non-migratory apex predators with a life span exceeding 60 years that co-
109 utilize human habitats contaminated with PFAS (IUCN 2018). Building from previous
110 biomonitoring studies demonstrating utility of American alligators as effective indicators of
111 environmental PFAS contamination (Bangma, Bowden, et al. 2017; Bangma, Reiner, et al.
112 2017). With a focus on immune health endpoints, we predicted that populations of American
113 alligators would be sensitive sentinels of cumulative adverse health effects resulting from PFAS
114 exposures common to humans living in the Cape Fear River basin.
115
116 **Methods**
117 ***Animal and sampling procedures***
118 All animal procedures were performed with approval by the North Carolina State University
119 Institutional Animal Care and Use Committee (protocol #18-085-O). Alligators were sampled
120 using active capture methods that employed snatch hooks from locations with direct access to the
121 Cape Fear River (CFR) and the adjacent Lumber River basin (Lake Waccamaw;
122 34.28726947673369, -78.50965117663293) in North Carolina. Cape Fear River sites included:

123 Greenfield Lake (34.2107388211135, -77.93707241283391) Wilmington, NC; Orton Pond in
124 Brunswick County (34.045198995423966, -77.98399224982941); Oak Island, NC
125 (33.91736426475394, -78.12476238375928), and Baldhead Island, NC
126 (33.85295162890547, -77.97713359942439). Sampling occurred in 2018 (July 24 to October 25)
127 and 2019 (April 18 to October 14).

128 Immediately following capture a 10-15 mL a whole blood sample was collected from the
129 post-occipital spinal venous sinus using a sterile 20 g or 18 g needle and a 30 mL syringe
130 (Myburgh et al. 2014). Whole blood samples were transferred to 8 mL serum and lithium
131 heparin coated plasma tubes (Vacutainer, BD). Serum tubes were allowed to clot for 30 minutes
132 at ambient temperature prior to storage on ice. Plasma tubes were inverted gently following
133 collection, then stored on ice until centrifugation. After field collections, blood was centrifuged
134 (1800 x g for 10 min at 4°C) and serum/plasma fractions were immediately aliquoted into
135 Teflon-free cryovials and stored at -80°C until analysis. Following the blood sample collections,
136 alligators were visually examined for general health, and external injuries were noted and
137 photographed. Sex was determined by cloacal examination with total length, snout to vent
138 length, tail girth, head/snout, and rear foot measurements recorded. Following sample/data
139 collection, alligators were released at the site of capture. Typical time from first contact to
140 release of animal was dependent on animal size with the average time to release being < 15
141 minutes.

142 ***Chemicals***

143 Ethanol (Optima®, Lot 183859), ammonium formate (99%, AC401152500), acetonitrile
144 (ACN; Optima®, Lot 184819), and formic acid (99.5%, A117-50) from Fisher Scientific
145 Waltham, MA) was used for extractions and instrument solvent gradient. All water used for
146 extraction, in aqueous buffers, and solutions were prepared in sterile Milli-Q A10 water (18Ω; 3

147 ppb total oxidizable organics), with the purified water analyzed for contamination prior to use.
148 All laboratory glassware was rinsed with sterile Milli-Q water and methanol prior to use. The
149 control material for the instrument methods was National Institute of Standards and Technology
150 (NIST) Standard Reference Material (SRM) 1957 organic contaminants in non-fortified human
151 serum. Chemicals for buffers and other standard laboratory chemicals were also purchased from
152 Fisher Scientific and as indicated below.

153 ***LC-MS analysis of Serum PFAS Concentrations***

154 Matrix matched (serum) calibration solutions (n = 23; ranging from 0.1 ng/mL to 100
155 ng/mL) were prepared from neat standards in charcoal stripped fetal bovine serum (Life
156 Technologies, Grand Island, NY; cat #10437, Lot #1754113; total protein 3.7 g/dL). An internal
157 standard (IS) solution was prepared by diluting a solution containing 10 mass-labeled PFAS in
158 water with 0.1 M formic acid (99.5%, A117-50, Fisher Scientific, Waltham, MA): $^{18}O_2$-
159 PFBS,$^{13}C_4$-PFBA, $^{18}O_2$-PFHxS, $^{13}C_4$-PFOA, $^{13}C_4$-PFOS, $^{13}C_5$-PFNA, $^{13}C_9$-PFDA, $^{13}C_4$-
160 PFBA, $^{13}C_2$-PFHxA, $^{13}C_2$-6:2FTS (Wellington Labs, Guelph, ON). An additional matrix
161 matched quality control (QC) sample was made by combining randomly selected aliquots of
162 sampled American alligator serum (n = 12). The resulting alligator QC sample and NIST SRM
163 1957 (n = 6) was used to test the method for reproducibility and stability (Table S2). Charcoal
164 stripped fetal bovine serum was also spiked with 5 ng/mL of PFAS standards and used as an
165 internal control for accurate measurement (n = 4). Additional control samples included fetal
166 bovine method serum blanks (n = 7), and purified water field blanks (n = 5) made by injecting 4
167 mL of Milli-Q water into vacutainer tubes during field sampling on 5 different days during the
168 sampling period.

169 All control and experimental samples were extracted and analyzed using methods identical
170 to those detailed in our previous studies (Guillette et al. 2020). Briefly, 50 µl of each sample was

171 aliquoted into a 2 mL polypropylene tube to which 100 µl of 0.1 M formic acid and internal
172 standards (12.5 ng) were added. Ice-cold ACN (450 µl) was added to the tube and then vortexed
173 for 3 seconds. Samples were then centrifuged at 12,500 x g for 5 min at room temperature. The
174 resulting supernatant (100 µl) was collected and added to 300 µl of aqueous 0.4 mmol of
175 ammonium formate and then transferred to polypropylene autosampler vials. PFAS were
176 analyzed using a Vanquish UPLC system (Thermo Fisher Scientific, Waltham, MA) equipped
177 with an Accucore C18+ column (2.1 mm x 100 mm x 1.5 µ) at a flow rate of 300 µL/min,
178 injection volumes of 100 µL, and a binary mobile phase gradient composed of 95:5 H_2O:ACN,
179 0.4 mM ammonium acetate and 95:5 ACN:H_2O, 0.4 mM ammonium acetate. A Thermo Orbitrap
180 Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray
181 ionization (HESI) source operated in negative mode was used to detect PFAS. For compound
182 validation, data was collected in data dependent mode with a preferred ion list consisting of the
183 quantitated PFAS standards (Table S1). PFAS quantitation was based on an eight-point
184 calibration curve with three injections per concentration randomly assigned in the sample run of
185 the internal standard normalized integrated peak area of the extracted ion chromatogram of the
186 [M-H]-ion with a 5-ppm mass tolerance. The r^2 of all calibration curves used for analysis were
187 above .97; limits of detection (LOD) were defined as the estimated mean concentration of
188 method blanks plus three standard deviations (Table S2). Multiple replicates of SRM 1957 (n =
189 6) were compared to the values on the Certificate of Analysis for the NIST SRM 1957 standards
190 and were within 15.89 % of expected values (Table S4).

191 ***Lysozyme assay***

192 The EnzChek® Lysozyme Assay (Thermo Fisher Scientific, Waltham, MA, Cat. E-22013)
193 was used according to manufacturer's protocols to determine lysozyme activity in serum of a
194 subset of American alligator serum samples (Lake Waccamaw: n = 22, Wilmington CFR: n =

195 21). Samples were diluted 1:5 in sample buffer and analyzed in triplicate, the average relative
196 standard deviation (RSD) for QC and experimental samples were 7% and 9%, respectively.
197 *Alligator serum/plasma complement assay*
198 Packed Sheep red blood cells (RBCs, Innovative Research Novi, MI) were aliquoted into 15
199 ml polypropylene conical tubes and centrifuged for 5 minutes at 800 x g. Cell pellets were
200 washed with phosphate buffered saline (pH 7.4; PBS) and then gently suspend in PBS and
201 recentrifuged. Washed cells were resuspended at a final concentration of 2% RBCs in PBS or
202 PBS supplemented with a final concentration of 50 mM EDTA. Cells were rocked gently and
203 150 µl of the cell suspension was rapidly aliquoted into 1.7 ml microcentrifuge tubes containing
204 150 µl of each alligator serum sample (plasma samples were analyzed in parallel and yielded
205 comparable results) and mixed by inversion. All sample aliquoting for each analysis was
206 completed in ≤ 2 minutes. Samples were incubated at room temperature for 20 minutes and then
207 rapidly placed onto ice/water bath for 10 minutes, samples were centrifuged at 3000 x g for 5
208 minutes and then rapidly returned to ice. Supernatant (100 µl) was aliquoted in duplicate into
209 clear 96-well microtiter plates. Absorbance at 540 nm was determined using a Tecan microplate
210 reader. Controls on each analysis plate included a blank control containing 1x PBS only, 1%
211 RBC in PBS, 1% RBC in 1x PBS supplemented with 50 mM EDTA, and a positive control of
212 maximum RBC lysis containing 2% RBCs to which an equal volume of deionized water was
213 added. Percent maximal complement activity for each sample was calculated by subtracting the
214 mean absorbance from the EDTA containing samples from the mean absorbance of the sample
215 lacking EDTA. That difference was divided by mean maximal absorbance of the positive control
216 samples, and the product was multiplied by 100. Each sample was analyzed a minimum of two
217 times. The mean % CV for maximal RBC lysis positive controls was 1.75 ± 1.42 % and for all
218 experimental samples was 3.41 ± 4.43 %.

219 ***dsDNA ELISA Analysis***

220 Individual wells of 96 well microtiter plates were coated overnight at 4°C with 500 ng of
221 double stranded calf thymus DNA suspended in DNA coating solution (Pierce, Rockford IL, cat
222 # 17250). Plates were washed extensively with PBS containing 0.05 % Tween 20 (Fisher
223 Scientific), incubated at room temperature for 2 hours in blocking buffer consisting of PBS
224 supplemented with 10% bovine serum albumin (BSA; fraction V, Fisher Scientific), and then
225 rewashed with PBS containing 0.05% Tween 20. Alligator plasma samples were diluted 1:500
226 and 1:1000 into PBS containing 10% BSA. Diluted samples (100 µl) were added to wells in
227 duplicate or triplicate and incubated overnight at 4° C. Wells were then washed extensively with
228 PBS/0.05% Tween 20, blotted dry by inversion on to paper towels, and then incubated for 1 hour
229 at room temperature with affinity purified polyclonal horse radish peroxidase (HRP) conjugated
230 goat anti-alligator IgG antiserum (Bethyl Labs, Montgomery, TX; Cat # A140-119P; Lot# A140-
231 119P-6) that was diluted 1:10,000 (0.1 µg/ml) in PBS 10% BSA. Samples were extensively
232 washed with PBS 0.05 % Tween 20, blotted dry, and 50 µl of 1x TMB Solution (Invitrogen, Cat
233 # 00-4201-56) was added to each well. The peroxidase reaction was terminated following
234 incubation for 30 minutes at room temperature by the addition of an equal 50 µl volume of 2M
235 sulfuric acid, and the absorbance of each well measured at 450 nm. Control samples on each
236 analyzed plate included calf serum diluted 1:500 (Gibco), blocking buffer only, 1:500 dilution of
237 alligator plasma or calf serum sample lacking HRP-conjugated anti-alligator antiserum, and
238 coated wells lacking DNA. Following optimization of alligator plasma concentrations and
239 alligator antiserum dilutions, specificity of DNA binding was confirmed by observing significant
240 decreases in immunoreactivity in DNA-coated wells that were treated with DNase1 (10 units;
241 RQ1 Dnase, Promega Madison, WI), and in samples of alligator plasma pretreated with 100 µg
242 of calf thymus DNA. Experimental plasma samples were analyzed in duplicate or triplicate on a

243 single plate at 1:500 dilution and on the same plate using an independent dilution of 1:1000, with
244 each analysis independently replicated. The average intra-assay coefficient of variation for all
245 experimental samples was 5.9 ± 1.4% (1:500) and 4.5% ± 1.1% (1:1000). The average between
246 plate coefficient of variation was 7.0 ± 1.7% (1:500) and 6.0 ± 1.5% (1:1000). The average
247 Absorbance 450 nM observed for each sample diluted at 1:500 was 2.05 ± 0.02 times greater
248 than samples diluted at 1:1000.

249 ***Whole blood cell counts and leukocyte differential***

250 Blood smears were prepared from ~10 µl of peripheral whole blood taken from a subset of
251 alligators. Blood smears were prepared in the field and allowed to air dry at room temperature on
252 Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). Slides were stained with Hemacolor®
253 according to the manufacturer's protocols (Sigma-Aldrich, Cat: 1.11661). Peripheral whole
254 blood cell counts were made by evaluation of stained blood smears using 40X and 100X
255 objectives on a Nikon Eclipse 80i microscope (Nikon; Melville, NY). Each slide was examined
256 independently by three different investigators blinded to sampling site, size, and sex. Leukocyte
257 identity was determined by cellular morphology and staining characteristics (Sykes and
258 Klaphake 2015; Stacy, Alleman, and Sayler 2011). A minimum of 400 leukocytes and
259 thrombocytes were counted to the calculate percentage of each cell type. Leukocytes were
260 categorized into lymphocytes, basophils, heterophils, eosinophils, azurophils, and basophils with
261 percent of each cell type determined by dividing the number of each cell type by the total cells
262 counted minus the number of thrombocytes and then multiplying by 100. Variation between
263 individual researcher counts was determined to be less than 7.9% for each cell type.

264 ***TBARS Assay***

265 Plasma lipid peroxidation was analyzed for 100 µl alligator plasma samples ran in duplicate
266 using TBARS (TCA method) Assay kit (# 700870, Cayman Chemical, Ann Arbor, MI)

267 according to manufacturer's supplied protocols. Malondiadehyde concentrations were calculated
268 by interpolation from a standard curve generated by serial dilution of manufacturer supplied
269 standards.

270 ***Quantitative RT-PCR analysis***

271 Total RNA from archived (stored at -80ºC) American alligator whole blood was isolated
272 using the Quick-RNA Whole Blood Kit (Zymo Research, Irvine, CA). One µg of RNA was
273 reverse transcribed using the high-capacity cDNA reverse transcription kit following
274 manufacturer's recommendations (Applied Biosystems; Grand Island, NY). Standard Fast
275 TaqMan PCR amplification was performed in triplicate on a Step One Plus Real-Time PCR
276 System (Applied Biosystems; Grand Island, NY) in a final volume of 20 µL containing ~10 ng
277 of cDNA (1.5 µL of RT product), 1x Universal Master Mix and custom TaqMan expression
278 assay primers specific for each target alligator mRNA (**Table S5, S6**) (Applied Biosystems;
279 Grand Island, NY). Relative expression was quantified using the $2^{\Delta\Delta Ct}$ method, in which ΔΔCt is
280 the normalized value. Alligator Gapdh expression was used as an independent reference gene for
281 normalization.

282 ***Data and Statistical Analysis***

283 Each animal/blood sample was assigned a randomized numeric code by NC State
284 University researchers at the time of collection. Samples were decoded for site and
285 length/size/sex only after PFAS measurement/analysis and biological markers analysis were
286 completed. Blood slides were further deidentified to mask sample identity for whole blood cell
287 counts. Samples used for each analysis were randomly selected based only on availability of
288 sufficient material for analysis, to balance analysis for comparable numbers of males and
289 females, life stage (adult SVL > 90 cm/juvenile SVL < 90 cm), and to control for possible effects

290 on seasonality samples were date matched. When necessary, SVL and sex were included in
291 statistical models as covariates.
292 Data was analyzed and visualized using Prism (version 9.3, GraphPad La Jolla, CA); R
293 statistical programming environment version 3.5.2; SPSS (Version 27.0, IBM, Armonk, NY);
294 JMP Genomics 9 (SAS, Cary, NC). For PFAS concentration data, means and concentration
295 ranges were determined from values above the LOD and interpolated ([PFAS]= LOD/$\sqrt{2}$)
296 values were used when detected PFAS congeners were detectable but below the LOD (LOD =
297 $mean_{blanks} + 3(SD_{blanks})$, non-detects were replaced with a value of zero. A Shapiro-Wilk's test
298 was used to assess normality of data; for data requiring normalization a natural log
299 transformation was used, [PFAS] data was log_{10} transformed, and percentage/ratiometric data
300 were arcsine transformed. Non-parametric statistics were used for data not meeting analysis
301 model assumptions following data transformation. Linear regression and Pearson's correlation
302 coefficient or Spearman's rank correlation coefficient were used to calculate relationships
303 between total PFAS serum concentrations and serum biomarkers. A 2-way analysis of variance
304 (ANOVA) was used to evaluate peripheral blood counts data (cell type, location), and $\sum log_{10}$
305 [PFAS] (size class, location) data and biomarker activities were initially evaluated at each site
306 using multivariate ANOVA (biomarker activity, $\sum log_{10}$[PFAS], and/or location). Principal
307 component analysis (PCA) was performed to dimensionally reduce PFAS concentrations using a
308 restricted maximum likelihood estimation (REML) and components with a minimum eigen value
309 of 1. Ward's general agglomerative hierarchical clustering procedure was performed on serum
310 PFAS congener concentrations (log_{10} transformed) to demonstrate large scale differences in
311 PFAS clustering using JMP Genomics 9. A minimal level of statistical significance for
312 differences in values among or between groups was considered $p \leq .05$.
313

314 **Results and Discussion**

315 We used liquid chromatography and high-resolution mass spectrometry to determine
316 concentrations of 23 different PFAS present in serum samples of 75 adult and juvenile alligators
317 from North Carolina (Guillette et al. 2020). Alligators were sampled beginning in July 2018 and
318 continued through October of 2019 at sites experiencing both point and non-point source PFAS
319 exposures along the Cape Fear River (CFR; n = 49), and from Lake Waccamaw (LW; n = 26), a
320 site < 50 km away and in the adjoining Lumber River watershed with no known fluorochemical
321 production (Fig. 1A). From the targeted list of 23 PFAS analyzed (Table S1), we found 14
322 different PFAS, including long and short chain perfluoroalkyl acids (PFAA), perfluoroether
323 acids (PFEA) and the fluorotelomer 6:2 FTS in these alligator blood samples (Table S2, S3).

324 As anticipated from previous wildlife exposure studies along the Cape Fear River, PFOS was
325 the dominant PFAS detected in alligator serum (Guillette et al. 2020; Robuck et al. 2021) We
326 detected PFOS in 100% of samples analyzed and it accounted for 79.7% and 75.8% of the total
327 PFAS present in alligator serum samples from the CFR and LW respectively. The median
328 number of PFAS congeners detected in serum samples from CFR alligators was 10 (range 4-12),
329 whereas a median of 5 (range 2-9) were detected in LW samples (Table S3). The relative
330 composition of long and short chain PFAAs and PFEAs detected in blood of CFR American
331 alligators were in general agreement with those found in the blood of adults and children
332 exposed to PFAS from drinking water in Wilmington, NC (Kotlarz et al. 2020).

333 Results of our principal component, multiple linear regression, unsupervised hierarchal
334 clustering, and statistical analysis of total serum concentrations demonstrated both increases in
335 PFAA concentrations and an exposure profile enriched for Nafion byproduct 2, PFO_5DoDA,
336 NVHOS, HFPO-DA and 6:2 fluorotelomer sulfonate (6:2 FTS) was characteristic and predictive
337 of samples from the CFR (Fig. 1 B-D). Concentrations and detection frequencies of PFEAs

338 (HFPO-DA, Nafion byproduct-2), PFAS congeners related to upstream production and 6:2 FTS

339 discharge from the Chemours Fayetteville Works facility, were exclusive to, or greatly enriched

340 in the CFR serum samples (Fig. 2C, D; Table S2). By contrast the PFAS exposure profiles

341 observed in blood of LW alligators were characterized by the presence of bioaccumalitive six-

342 carbon and longer PFAAs (Fig.1D). However, the PFEAs PMPA (15%), PDO4DA (23%), and

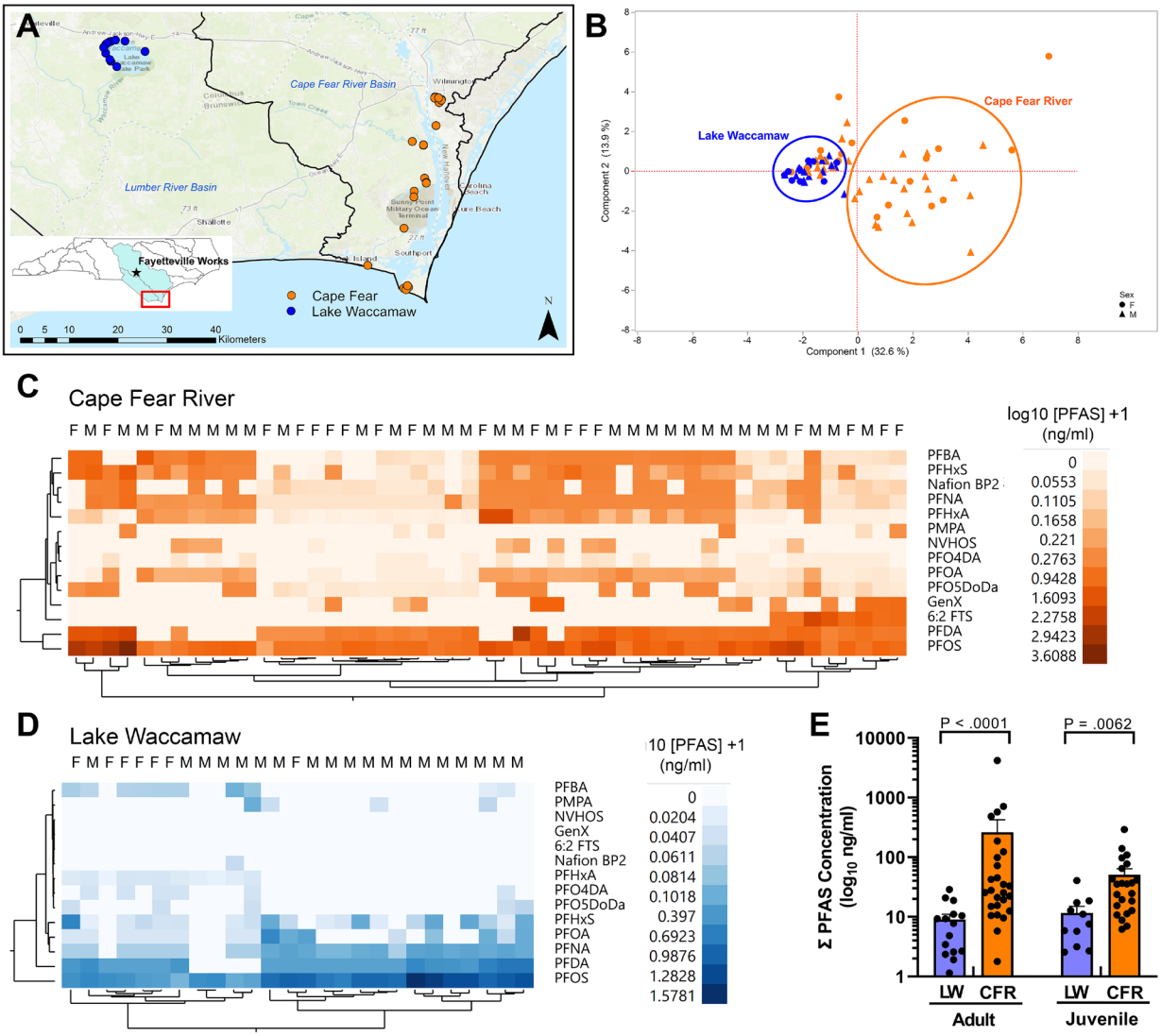

Fig 1. Location of study sites and serum PFAS exposure profiles. (**A**) Location of samples collected at Lake Waccamaw (LW; blue) and from the Cape Fear River basin (CFR; orange). Results of **(B)** principal component and **(C, D)** unsupervised hierarchal clustering of $\log_{10}$ transformed serum PFAS congener concentrations from the CFR and LW. Females and are indicated with a circle in panel (B) or a capital F (C, D) and males are indicated with a triangle or a capital M. (**E**) Mean of $\log_{10}$ transformed PFAS concentrations (ng/ml) found in serum of adult (SVL ≥ 90 cm) and juvenile/sub-adult American alligators from LW (adult n = 15; juvenile n = 11) and the CFR (n = 26; juvenile = 23).

343 PFO5DoDa (19%) were also detectable in some alligator serum samples from LW suggesting
344 that there are low levels of PFEA contamination within the Lumber river basin ecosystem (Fig.
345 1D, Table S2).
346 Concentrations of sum total PFAS detected in serum of CFR alligator was increased
347 compared to alligators sampled from LW (Fig. 1E), two-way ANOVA found a significant effect
348 of location on total serum PFAS concertation ($F(1, 71) = 27.3$, $p < .0001$). We did not detect
349 differences in total PFAS concentrations between juveniles/sub-adults (SVL < 90 cm) and adults
350 ($F(1, 71) = 0.0018$, $p = .965$) at either site, and there was not an interaction between groups ($F(1,$
351 $71) = 0.989$, $p = .323$). Post hoc analysis indicated that serum concentrations of PFAS were
352 significantly increased in both the juveniles/sub-adult ($p = .006$) and adult alligators ($p <.0001$)
353 from the CFR (Fig. 1E).
354 The American alligator is renowned for having a particularly robust and protective
355 antibacterial and antiviral innate immune system (Merchant and Britton 2006; Finger and Gogal
356 2013). Initially focusing on biomarkers of innate immune function, we found that anti-microbial
357 lysozyme activity was significantly increased in adult CFR alligators compared to adult alligators
358 from LW (Fig. 2A). Those findings were similar to our previous findings that high
359 concentrations of PFAS were associated with elevated lysozyme activity in adult striped bass
360 (*Morone saxatilis*) from the CFR (Guillette et al. 2020). By contrast, we did not observe a
361 significant difference between sites for lysozyme activities of juvenile/subadult alligators,
362 although at LW the lysozyme activity of sexually immature animals compared to adults from the
363 same location was significantly increased ($p < 0.0001$; Fig. 2A). The apparent age-related
364 difference in lysozyme activity at LW was unrelated to differences in PFAS exposure, a
365 difference that is likely related to ecological, population density, or other undefined factors at
366 this location. Because of these site-specific differences in lysozyme activity, associations

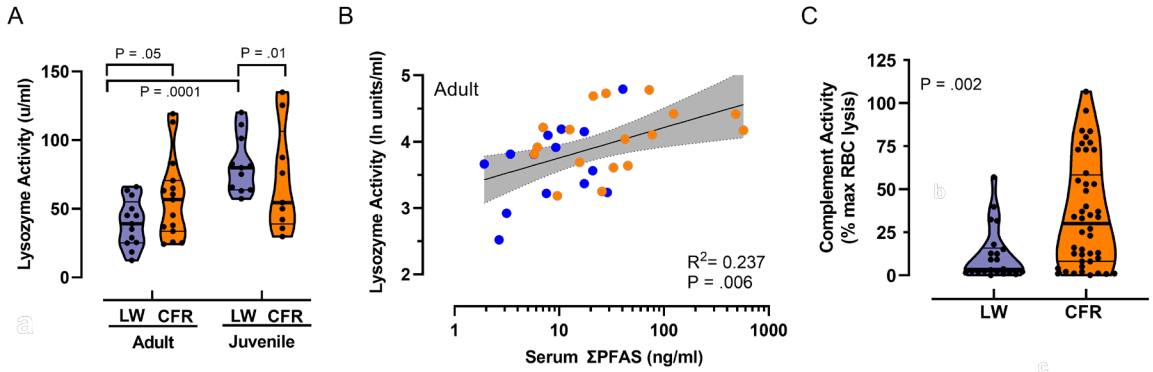

Figure 2. Impacts and relationship of PFAS exposure on innate immune functions. (**A**) Violin plots comparing median lysozyme activity in juvenile (SVL < 90 cm) and adult American alligators (SVL > 90 cm) from Lake Waccamaw (LW; thick lines indicate median; thin lines indicate quartiles) and from the Cape Fear River (CFR). (**B**) Relationship of total PFAS concentrations with lysozyme activity in adult alligators. Solid line is the best fit linear regression, with shaded area represents 95% CI. (**C**) Violin plots of complement activity in plasma samples of alligator from LW and CFR. Shown is the percent of maximal EDTA sensitive sheep red blood cell lysis activity (thick lines indicate median; thin lines indicate quartiles; LW: n = 22, CFR n= 44.

between total PFAS concentrations and lysozyme activity were evaluated only in sexually mature adult alligators. Using Spearman's ranked correlation to assess the relationship between serum total PFAS concentrations and lysozyme activity in adult alligators from both sites, we observed a positive correlation between serum PFAS and lysozyme activity, $r(29) = .45$, $p = .006$ (Fig. 2B). Serum complement activity, a key component of the humoral innate response that has been linked to autoimmunity (Zipfel 2009), was increased in the entire population of sampled CFR alligators (Fig. 2C). As observed for lysozyme, log10 transformed total PFAS concentrations and serum complement activity were found also to be moderately positively correlated, $r(71) = .23$, $p = .05$.

Despite living in environments with near-constant exposure to pathogenic microorganisms, and having especially high burdens of fecal coliform and other pathogenic bacteria (Johnston et al. 2010), crocodilians, including American alligators, rarely suffer from systemic or skin infections from these microorganisms (Barksdale, Hrifko, and van Hoek 2017; van Hoek 2014). However, during our sampling we observed a notably uncharacteristic increase in incidence of

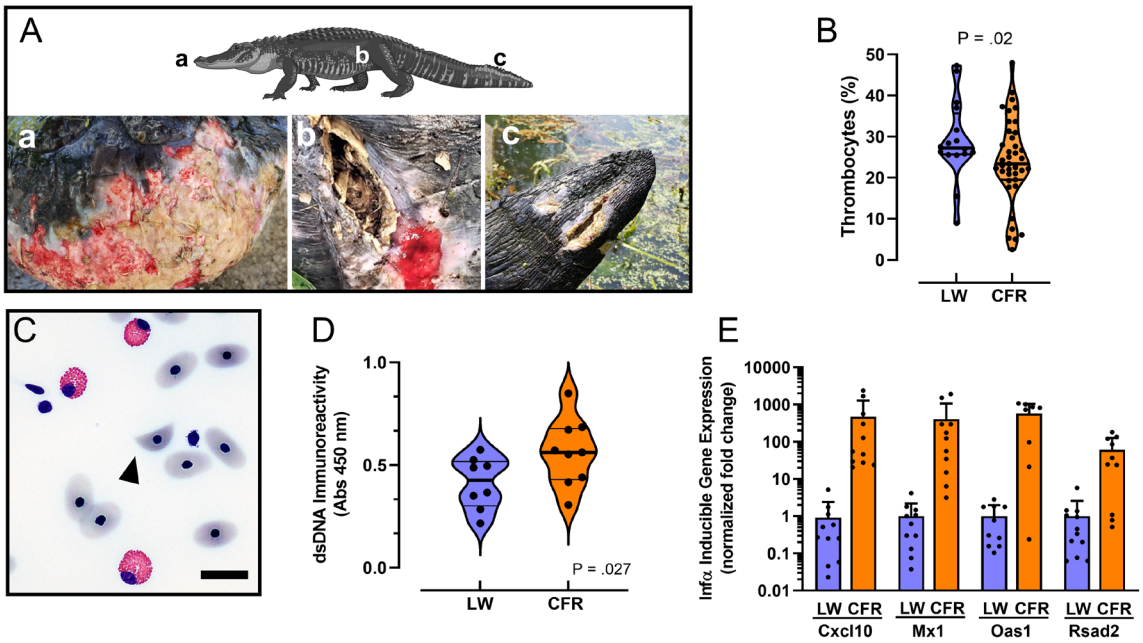

Fig. 3. Autoimmune disease like phenotypes associated with PFAS exposure. (**A**) Examples of cutaneous lesions and slow to heal wounds associated with PFAS exposure in 3 representative animals sampled from the CFR in 2019. Lower case letters indicate body location of lesion shown in each panel. (**B**) Violin plots comparing median thrombocyte numbers quantified from whole blood smears in alligators from Lake Waccamaw (n = 16) and the Cape Fear River (n = 39). (**C**) Representative photomicrograph of a schistocyte (arrowhead) observed in a whole blood sample isolated from an alligator from the CFR. Bar = 10 µm (**D**) Violin plots comparing median anti-dsDNA immunoreactivity in male (n = 4) and female (n = 4) alligator serum samples from each site. (**E**) Quantitative RT-PCR analysis of Infα signature gene expression analysis. Note the X-axis scale is Log_{10} relative fold-change. All increases in expression are significant ($P < .0001$). For violin plots median is indicted by a thick line and thin lines indicate quartiles.

381 skin lesions, slow healing, and infected wounds in ~20% of sampled CFR alligators (Fig. 3A).
382 Those lesions were considered reminiscent of autoimmune disease related vasculitis in humans
383 (Shanmugam et al. 2017). Although fresh and healed wounds, including loss of limbs that were
384 likely related to territorial and mating interactions were observed, similarly infected and slow to
385 heal wounds were not observed in alligators sampled from LW. We also observed decreased
386 clotting in some CFR alligators following blood collection; that phenotype was consistent with
387 our findings of modestly decreased numbers of thrombocytes in whole blood cell counts from
388 CFR alligators (Fig. 3B). Additionally, we found an increased incidence of abnormal red blood

389 cell phenotypes including schistocytes (helmet cells) associated with PFAS exposure in the CFR
390 samples (Fig. 3C, Table S2). Our analysis of whole blood cell counts found no evidence for a
391 general increase in chronic stress; no relationships were detected between sample site or PFAS
392 concentrations and leukocyte differentials, or heterophile/lymphocyte ratios. We also measured
393 relative levels of lipid peroxidation products in plasma which also found no evidence of
394 increased impacts related to general systemic mechanisms of cellular injury and stress in the
395 CFR alligators (Fig. S1).

396 Based on the observed increases in helmet cells and modest thrombocytopenia associated
397 with increased PFAS exposure in CFR alligators, we evaluate other hallmark phenotypes of
398 autoimmune diseases (Crow, Olferiev, and Kirou 2019; Sirobhushanam, Lazar, and Kahlenberg
399 2021). Using an alligator specific anti-double stranded DNA (dsDNA) immunoglobulin (IgG)
400 binding enzyme-linked immunosorbent assay, we evaluated a subset of adult male and female
401 alligators from the CFR and LW and found that dsDNA binding IgGs were significantly
402 increased in CFR alligator plasma samples (Fig. 3D). Differences between males and females
403 were not detected. Finally, our RT-PCR analysis of type 1 interferon (Ifn-α) responsive gene
404 expression in alligator whole blood samples found extremely high expression of four different
405 Ifn-α signature genes implicated in pathology of human autoimmune diseases including systemic
406 lupus erythematosus (SLE) (Crow, Olferiev, and Kirou 2019). Relative to samples from LW, Ifn-
407 α responsive gene expression was induced > 60-fold for *Rsad2* (viperin), and >400-fold for
408 *Cxcl10*, *Mx1*, and *Oas1* in CFR alligator blood samples (Fig. 3E). The finding of extremely high
409 induction of Inf-α signature gene expression in the CFR alligators supports a central role for
410 excessive production of type I interferons in increasing the innate immune functions associated
411 with elevated PFAS exposures. We interpret the greatly increased Inf-α signature gene
412 expression, elevated autoantibodies, vasculitis-like skin lesions, elevated incidence of slow

413 healing wounds, and increases in innate immune activity observed in the CFR population as
414 indicative of an SLE autoimmune-like disease process in American alligators.

415 ***Conclusions***

416 Overall, our work found strong associations across multiple hallmark phenotypes of SLE-like
417 autoimmune disease and elevated PFAS exposures in American alligators exposed to elevated
418 levels of PFAS from the CFR. The PFAS-associated changes in both the innate immune and
419 adaptive immune system may represent a significant health concern for wildlife and human
420 health that may leave exposed populations at greater risk for infections and autoimmune disease.
421 Uniquely, for this work we implemented an integrated One Environmental Health approach
422 using American alligators as sentinels to more fully understand the consequences of long-term
423 exposure to complex and poorly understood mixtures of PFAS with the aim of improving health
424 of impacted human, animals, and ecosystems (Gibbs 2014; Pérez and Pierce 2018). In addition
425 to being long lived, and sharing their environment with humans, American alligators and other
426 crocodilians, by virtue of having innate immune functions optimized to eliminate microbial
427 pathogens, may serve as an ideal predictive model for detecting autoimmune hazards caused by
428 chemical pollutants. Our findings of adverse immune-health effects in American alligators that
429 have exposure profiles similar to the surrounding human population illuminates and reaffirms the
430 need to reduce exposure and cease production and use of a chemical class that, through its
431 ubiquity and persistence, is a global environmental health concern.

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Writing – review & editing: SMB, TCG, MG, TJ, JM

Data and materials availability: All data, code, and materials used in the analysis will be made available for purposes of reproducing or extending the analysis. All data are available in the main text and the supplementary materials.

Supplementary Materials for

Blood Concentrations of Per- and Polyfluoroalkyl Substances are Associated with Lupus-like Autoimmunity in the American Alligator

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Figs. S1
Tables S1 to S6

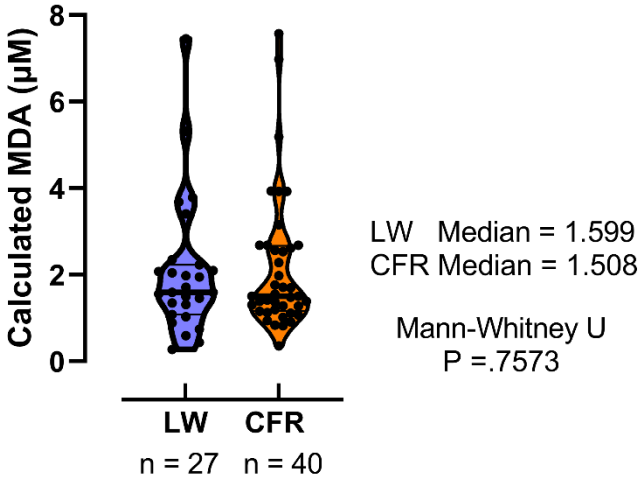
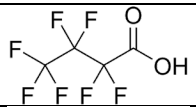
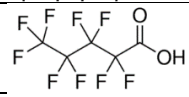
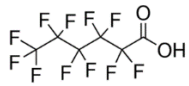
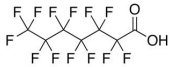
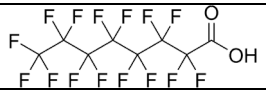
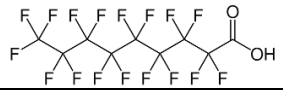
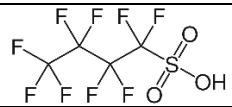
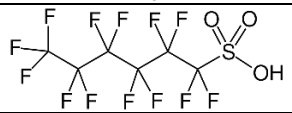
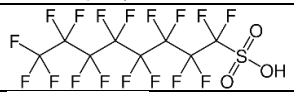
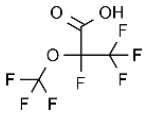


Fig. S1. Analysis of plasma lipid peroxidation: TBARS analysis.

Table S1. Targeted List of 23 PFAS Analytes

Compound Name	Class	Family	Chemical Name	CAS #	Formula	Degree of Fluorination	Ether Bonds	Isomer Type	Functional Group		Structure
									$R\text{-}CO_2H$[a]	$R\text{-}SO_3H$[b]	
PFBA	PFAA	PFCA	Perfluorobutanoic acid	375-22-4	$C_4HF_7O_2$	Per	0	Linear	Y	--	
PFPeA	PFAA	PFCA	Perfluoropentanoic acid	2706-90-3	$C_5HF_9O_2$	Per	0	Linear	Y	--	
PFHxA	PFAA	PFCA	Perfluorohexanoic acid	307-24-4	$C_6HF_{11}O_2$	Per	0	Linear	Y	--	
PFHpA	PFAA	PFCA	Perfluoroheptanoic acid	375-85-9	$C_7HF_{13}O_2$	Per	0	Linear	Y	--	
PFOA	PFAA	PFCA	Perfluorooctanoic acid	335-67-1	$C_8HF_{15}O_2$	Per	0	Linear	Y	--	
PFNA	PFAA	PFCA	Perfluorononanoic acid	375-95-1	$C_9HF_{17}O_2$	Per	0	Linear	Y	--	
PFDA	PFAA	PFCA	Perfluorodecanoic acid	335-76-2	$C_{10}HF_{19}O_2$	Per	0	Linear	Y	--	
PFBS	PFAA	PFSA	Perfluorobutanesulfonic acid	375-73-5	$C_4HF_9SO_3$	Per	0	Linear	--	Y	
PFHxS	PFAA	PFSA	Perfluorohexanesulfonic acid	355-46-4	$C_6HF_{13}SO_3$	Poly	0	Linear	--	Y	
PFOS	PFAA	PFSA	Perfluorooctanesulfonic acid	1763-23-1	$C_8HF_{17}SO_3$	Per	0	Linear	--	Y	
PMPA*	PFAA	PFECA	Perfluoro-2-(perfluoromethoxy)propanoic acid	13140-29-9	$C_4HF_7O_3$	Per	1	Branched	Y	--	

PEPA*	PFAA	PFECA	Perfluoroethoxypropyl carboxylic acid	267239-61-2	$C_5HF_9O_3$	Per	1	Branched	Y	--	
HFPO-DA (GenX)*	PFAA	PFECA	Hexafluoropropylene oxide dimer acid	13252-13-6	$C_6HF_{11}O_3$	Per	1	Branched	Y	--	
PFO2HxA*	PFAA	PFECA	Perfluoro (3,5-dioxahexanoic) acid	39492-88-1	$C_4HF_7O_4$	Per	2	Linear	Y	--	
PFO3OA*	PFAA	PFECA	Perfluoro (3,5,7-trioxaoctanoic) acid	39492-89-2	$C_5HF_9O_5$	Per	3	Linear	Y	--	
PFO4DA*	PFAA	PFECA	Perfluoro-3,5,7,9-butaoxadecanoic acid	39492-90-5	$C_6HF_{11}O_6$	Per	4	Linear	Y	--	
PFO5DoDA (TAFN4)*	PFAA	PFECA	Perfluoro (3,5,7,9,11 pentaoxadodecanoic) acid	39492-91-6	$C_7HF_{13}O_7$	Per	5	Linear	Y	--	
Hydro-EVE Acid*	PFAA	PFECA	Perfluoroethoxsypropanoic acid	773804-62-9	$C_8H_2F_{14}O_4$	Per	2	Branched	Y	--	
NVHOS*	PFAA	PFESA	Perfluoroethoxysulfonic acid	1132933-86-8	$C_4H_2F_8O_4S$	Per	1	Linear	--	Y	
Nafion Byproduct 1*	PFAA	PFESA	Perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid	29311-67-9	$C_7HF_{13}O_5S$	Per	2	Branched	--	Y	

Nafion Byproduct 2*	PFAA	PFESA	7H-Perfluoro-4-methyl-3,6-dioxaoctanesulfonic acid	749836-20-2	$C_7H_2F_{14}O_5S$	Poly	2	Branched	--	Y	
R-PSDA (Formerly Nafion Byproduct 4)*	PFAA	PFESA	Perfluoro-4-(2-sulfoethoxy)pentanoic acid	2416366-18-0	$C_7H_2F_{12}O_6S$	Per	1	Branched	Y	--	
6:2 FTS	FTSA		6:2 fluorotelomersulfonic acid	27619-97-2	$C_8H_5F_{13}O_3S$	Poly	0	Linear	--	Y	

a: Carboxylic acid functional group; b: Sulfonic acid functional group. Perfluoroalkyl acid (PFAA); Perfluorocarboxylic acid (PFCA); Perfluorosulfonic acid (PFSA); Perfluoroalkane sulfonamides (FASA); Perfluoroalkane sulfonamidoacetic acids (FASAA); Perfluoroether carboxylic acids (PFECA); Perfluoroether sulfonic acids (PFESA); Fluorotelomer sulfonic acid (FTSA)

Table S2. Contained in Data File 1.

Table S3. Detected PFAS Concentrations

Location	N =	Sex (F/M)		∑ PFAS	PFBA	PFHxA	PFOA	PFNA	PFDA	PFHxS	PFOS	PMPA	HFPO DA	PFO4 DA	PFO5 DoDA	NVHOS	Nafion BP2	6:2 FTS
Cape Fear River	48	19/29	Freq (%)	100	83	94	85	98	81	90	100	13	27	46	69	25	79	17
			Mean (ng/ml)	166	2.04	3.19	0.38	0.85	18.47	2.16	133	0.08	1.34	0.06	0.37	0.13	0.75	3.95
			Median (ng/ml)	27.90	1.31	0.28	0.26	0.81	3.83	0.66	12.07	<LOD	<LOD	<LOD	0.15	<LOD	0.26	<LOD
			Min (ng/ml)	1.78	ND	ND	ND	ND	ND	ND	0.65	ND	ND	ND	ND	ND	ND	ND
			Max (ng/ml)	4194	26.4	44.4	1.29	8.45	466	27.2	4061	1.46	15.0	0.19	1.55	0.72	7.75	95.2
Lake Waccamaw	27	7/20	Freq (%)	100	37	69	59	89	85	74	100	15	0	22	22	0	4	0
			Mean (ng/ml)	10.2	0.06	0.03	0.21	0.36	1.51	0.26	7.67	0.02	ND	0.02	0.03	ND	0.01	ND
			Median (ng/ml)	7.66	<LOD	<LOD	0.08	0.24	0.84	0.09	5.62	<LOD	ND	<LOD	<LOD	ND	<LOD	ND
			Min (ng/ml)	1.15	ND	ND	ND	ND	ND	ND	0.25	ND	-	ND	ND	-	ND	-
			Max (ng/ml)	40.5	0.26	0.08	2.82	0.91	5.00	3.35	36.9	0.30	-	0.09	0.14	-	0.12	-

F, female; M, male, Freq, detection frequency; LOD, limit of detection; Min, minimum concentration; Max, maximum concentration; ND, not detected.

Table S4. SRM 1957 Analysis

	SRM 1957 (ng/mL)	Certified Value (ng/mL)	% Difference
PFOA	4.812 ± 0.057	5 ± 0.440	3.76
PFNA	0.852 ± 0.018	0.878 ± 0.077	2.96
PFDA	0.328 ± 0.004	0.39 ± 0.12	15.89
PFOS	21.643 ± 0.341	21.1 ± 1.3	2.57
PFHxS	3.652 ± 0.041	4 ± 0.830	8.70

NIST Standard Reference Material 1957, ran in triplicate within each batch of samples (n = 6).

Table S5. Alligator gene list and results of qRT-PCR mRNA analysis

Gene Name	Gene Symbol	RefSeq ID	Fold Change (Mean ± SEM)	p-value
C-X-C motif chemokine ligand 10	Cxcl10	XM_019534257.1	478 ± 241	.001
Interferon-induced GTP-binding protein Mx1	Mx1	XM_019476366.1	405 ± 201	.009
2'-5'-oligoadenylate synthase 1	Oas1	XM_019490991.1	577 ± 162	.001
Radical S-adenosyl methionine domain-containing protein 2	Rsad2	XM_019492667.1	61.1 ± 20.7	.004
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	XM_006258364.3	-	-

Table S6. Primer sequences used for qRT-PCR analysis.

Primer	Primer sequence (5' - 3')
Cxcl10-F_qRT	TCATGCCTCAGCTAATGTAACC
Cxcl10-R_qRT	GTCAGTACAAGCTGCTCTCTTT
Cxcl10-P_qRT	AGTGATGACCGTGCTGAAACACCA
Mx1-F_qRT	CTGGTGAAGGAGTGGGTATTAG
Mx1-R_qRT	CACTCTTGTGATCCCTGGTAAG
Mx1-P_qRT	CCCTGGAAATCAGCTCCCAAGATGT
Oas1-F_qRT	TCAGAAAACGCAGCCTGAAC
Oas1-R_qRT	ATGCTTAGCCACAGAGCGAA
Oas1-P_qRT	TCAAAGGGAGCAAGGG
Rsad2-F_qRT	ACTACAAGTGCGGCTTCTGC
Rsad2-R_qRT	AATTTTCTCCATACCTGCCGCC
Rsad2-P_qRT	TTCCACACGGCCAAGA
Gapdh-FqRT	AAAGTCGGCGTAAACGGATTTG
Gapdh-R_qRT	GACCTCAACTTTGCCACTGC
Gapdh-P_qRT	TATCGGCCGCCTGGT

F, forward primer; R, reverse primer; P, fluorescein (FAM) labeled TaqMan probe