# **Supporting Information for:** Legacy and Novel Per- and Polyfluoroalkyl Substances in Juvenile Seabirds from the US Atlantic Coast

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This document contains 43 pages and 10 figures. 23 tables are contained in an Excel workbook titled “PFAS in Seabird Liver_SuppTables_Revised_Final.xlsx”

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## Chemicals, reagents, and materials

Analytical standards of greater than 99.9% purity, including mass-labeled surrogates, were purchased from Wellington Laboratories (Guelph, ON, Canada). Native Nafion BP2 and PFO5DoDA were provided by Chemours (Fayetteville, NC) in lieu of a commercial source. HPLC grade methanol was purchased from Fisher Scientific (Waltham, MA, USA). Ultrapure water for equipment cleaning was obtained from a Milli-Q system fit with an HPLC water polisher or via HPLC grade water purchased from Fisher Scientific (Waltham, MA, USA). ENVI Carb 2g cartridges were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate was purchased from Fisher Scientific (Waltham, MA, USA)

## Sample collection details

All birds were obtained opportunistically for use in this study, and no birds were killed for the purposes of this study. Birds were collected and stored in accordance with URI Biosafety standards and the Migratory Bird Treaty Act.

Dead Great Shearwater juveniles (*Ardenna gravis*) were obtained as bycatch from the National Oceanic and Atmospheric Administration (NOAA) Northeast Fisheries Observer Program (Falmouth, MA). Dead Herring Gull (*Larus argentatus smithsonianus*) chicks were obtained from the Wildlife Clinic of Rhode Island (Narragansett, RI). Dead Royal Tern (*Thalasseus maximus*), Sandwich Tern (*Thalasseus sandvicensis*), Laughing Gull (*Leucophaeus atricilla*), and Brown Pelican

(*Pelecanus occidentalis*) chicks were obtained as part of routine field and nest surveys conducted by North Carolina Audubon (Wilmington, NC).

Great Shearwaters in this study were self-feeding and had completed their first migration from their remote breeding site in the South Atlantic at approximately 5-6 months old, and are therefore considered juveniles. Great Shearwaters were confirmed to be juveniles based on molt status, presence of the bursa of Fabricius, and stage of gonad development. All birds from Rhode Island and North Carolina were pre-fledging birds under 8 weeks of age, still under parental care at time of death. All individuals were hatch-year juveniles, under approximately six months of age. Great Shearwaters were 5-6 years away from reproductive maturity, while gulls, terns, and pelicans, who mature more rapidly, were at least a year away from reproductive maturity (Tables S3-S4).

## Support for a multi-species, variable age comparison

Species

The literature does not currently support the hypothesis of significantly different uptake, metabolism, or elimination rates between similar seabird species. The species included in this study are similar in trophic strategy and preferred prey items, facilitating an acceptable comparison.

*a) Species-based differences in bioaccumulation capacity*

We point to multiple studies across the literature that suggest foraging preferences, migratory strategies, and other life history details that determine exposure potential

drive PFAS concentrations rather than innate differences in bioaccumulation capacity between seabird species[1,2,11,3–10].

The most valuable support of our sample design comes from studies investigating concentration trends in multiple species surrounding concerted PFAS sources. Lopez-Antia et al. (2017) examined PFOS levels in eggs from three species of bird surrounding a fluoropolymer production site in the Netherlands. The study focused on species that were unambiguously different from one another in terms of size and diet, unlike the similar seabirds included in our study. Great tits are a small passerine (songbird) species that primarily feeds on insects; northern lapwings are small-medium marsh wading birds that feed on small aquatic invertebrates or insects. Mediterranean gulls are opportunist seabirds that feed on fish, bivalves, or crustaceans. The study found no statistically significant differences in egg PFOS levels among the three species, suggesting the common exposure source drove PFOS levels in all birds without clear species-level differences in adult uptake, metabolism, or elimination via maternal offloading. Similarly, Yoo et al. (2008) examined PFAS in eggs from three bird species nesting on Lake Shiwa in Korea, a large, industrially-influenced artificial seawater lake. The study found no significant differences in PFOS and $\sum_5$PFCA between little egret (a large wading bird), little ringed plover (a small shorebird), and parrot bill (a small songbird-like bird) eggs.

Data from controlled studies indicate approximately equivalent accumulation efficiency in adult quails (a game bird) and mallards (a duck) [9]. Domestically reared adult birds

from both species and sexes were exposed to the same PFOS levels via diet. Adult livers and serum were found to contain similar PFOS levels at the completion of exposure; larger differences were apparent between sexes of the same species than between different species. Egg yolk and 14-day old juvenile liver and serum also displayed comparable PFOS levels between both species, without the stark differences between sexes.

Roscales et al. 2019 compared PFAS levels in seabird serum from multiple habitats in or adjacent to the Southern Ocean[11]. Two species were sampled within three colonies across different latitudes and ocean basins. Measurements show larger differences between the same species at different locations, compared to observed differences between different species at the same colony (Roscales et al. 2019, Table 1). This suggests location-based exposure factors, like proximity to PFAS sources or prey PFAS levels, are the primary determinant of PFAS in similar seabird species.

Beyond avifauna, we note that data from marine mammals suggests taxa with a shared phylogeny conserve cellular and tissue machinery driving internal kinetics of PFAS. For example, evidence in marine mammals demonstrates conservation of metabolic pathways at the family level rather than the species level; data to date suggests all odontocete cetaceans are unable to efficiently metabolize FOSA [12–16].

Species-specific toxicokinetic processes and rates are certainly possible and have been demonstrated across contrasting mammal species like rats and humans[17]. However,

based on the available data showing similar tissue residues in seabirds and other types of birds subject to similar PFAS exposures, we suggest further data is required to rigorously support the claim of significantly different accumulation, metabolism, or elimination pathways or rates at a species level in similar birds. The data we have on hand suggests comparisons across multiple species are acceptable, and any major differences in PFAS levels are due to dietary and habitat factors. We highlight this as an important research gap considering the utility of birds as ecosystem sentinels.

*b) Ontogenetic and diet differences that confound comparison between species*

As the above evidence suggests, habitat-related exposure and dietary choices likely drive PFAS levels more than major innate differences in toxicokinetics between seabird species. We assert that the birds included in our study are roughly comparable in diet, all feeding primarily on forage fish and invertebrates (Table S2). All species demonstrate some reliance on Atlantic menhaden *Brevoortia tyrannus*, a key forage fish found along the US Atlantic seaboard from Florida to Nova Scotia. All species are plungers and opportunists rather than pursuit divers. This broad similarity in trophic strategy and position allows our study to highlight and compare differences in PFAS levels due to habitat-driven factors like human inputs and proximity to production sources.

<u>Age</u>

Our study focused on how each habitat/lifestyle contributes PFAS to an associated sentinel predator, and by sampling juveniles and chicks we avoid life history

169 complications associated with older birds to focus on this question. While chick and
170 juvenile stages are distinct in many ways, we do not believe the differences in these life
171 stages significantly compromise PFAS measurements in our study due to the influence
172 of maternal offloading, and the long half-life of PFAS.

173 *a) Both chick and juvenile birds primarily reflect maternal offloading*

174 PFAS are very persistent in the environment and in biota. PFAS transferred from
175 mother to offspring do not readily dissipate over the first few months of development.
176 The lengthy plasma half-life of PFOS in birds (231 days) means that both chicks (~14 –
177 56 days old) and juveniles (~140 – 170 days old) within this study retain and reflect the
178 influence of maternal offloading in liver measurements[18]. Short-term exposure trials in
179 juvenile quail and mallard suggest the liver half-life of PFOS is roughly 2.5 times that of
180 the serum half-life. Using the plasma half-life derived in Tarazona et al. 2015, this
181 equates to an estimated liver half-life for PFOS of 578 days in birds, or 1.6 years. Liver
182 elimination of PFAS via biliary excretion has been demonstrated to be very slow (~1%
183 of supplied dose)[19], and liver metabolism of most PFAAs is thought to negligible.
184 Therefore we reiterate that all individuals of all ages used in this study primarily reflect
185 the influence of maternal offloading. A longer duration of self-feeding by Great
186 shearwaters is unlikely to offset or surpass the PFAS burden received *in ovo*; a study
187 examining prey fish concentrations compared to egg and juvenile liver concentrations in
188 guillemots suggested maternal offloading exposure drastically exceeded dietary
189 inputs[20]. Measurement of shearwater prey (sand lance) from Massachusetts Bay points
190 to similar, low levels of PFAS exposure from offshore prey ($\sum_{12}$PFAAs: ~1 – 8 ng/g
191 whole body, wet weight) (Robuck, unpublished data). We also point to seabird literature

that suggests adults of these species provision their young with the same or similar species that they themselves consume, meaning the dietary additions provided to chicks were similar or identical to those prey items that contributed to PFAS burdens in mothers prior to maternal offloading (Table S2).

The duration of dietary exposure was longer for the older juveniles, who had been self-feeding for several months. More importantly, these birds also had a longer growth period, causing growth dilution of maternally offloaded PFAS in liver.

*b) There is no evidence that specific cellular and tissue machinery known to drive PFAS accumulation differ with age.*

PFAS accumulation is driven by associations with specific proteins in liver and blood, in tandem nonspecific associations with amphiphilic structural lipids[21–23]. We are not aware of any data demonstrating age-based differences in the protein structures or binding efficiency of liver fatty acid binding protein and albumin, the two primary proteins identified to drive PFAS accumulation in liver and blood. Rather, data from *in ovo* studies demonstrates the ability of developing embryos to readily accumulate PFAS in liver, at levels on par with or exceeding maternal liver concentrations[9]. Data from in utero exposure studies in mice reiterate the same[24]. Current, albeit limited, data suggest no appreciable changes in bulk liver phospholipid levels over chick development[25], though rapid changes in storage lipid content occur across all tissues during chick development [26]. Our data in Figure 4 reiterates this – chicks from Narragansett Bay had phospholipid levels similar to older Great Shearwater juveniles (possibly due to similar

PFOS levels) compared to chicks from the CFRE of the same age and from the same family. Elimination rates have been shown to be sex-dependent, but no data exist describing age-specific elimination differences in birds[9,18,27]. Specific mechanisms of PFAS uptake may change on a bulk level with tissue growth as more tissue is available to interact with PFAS, but we believe this would not be apparent in our measurements normalized to ng PFAS/g tissue basis. More importantly, the maternally offloaded burden of PFAS would be diluted with tissue growth. Overall, more research is needed to explore changes in PFAS uptake with age, but data to date do not indicate substantial changes in tissue components driving bioaccumulation of PFAS.

*c) PFAS are not stored in fat*

PFAS are not stored in fat akin to legacy persistent organic pollutants like polychlorinated biphenyls or DDT[28–31], meaning the rapid development of adipose fat stores in juveniles that may impact hydrophobic pollutant levels is not likely to impact organ levels of PFAS in chicks or juveniles.

*d) PFAS demonstrate an inverse relationship with age*

Current literature suggests PFAS do not display the same positive relationships between age like some legacy POPs[32]. Data from several taxa indicate PFAS are significantly higher in juveniles compared to adults [33–37]. Possible explanatory mechanisms for this negative relationship include the influence of maternal offloading combined with the long tissue half-lives of many PFAS, or growth-mediated biodilution. Research examining PFAS in maternal liver, egg, and chick liver have found the highest

PFAS levels in egg, with subsequent chick liver containing PFOS on par with or higher than the mother's liver[9,20]. This suggests that like other wildlife taxa, seabirds also display a negative relationship between PFAS and age. By measuring young birds, we are likely measuring the highest concentrations experienced by these individuals throughout their lifetimes, during a critical development window.

*e) Juveniles are not impacted by reproductive cycles which confound measurements in adults.* Seabirds have been shown to offload significant concentrations of PFAS in eggs[28,29,38].

*f) Using juvenile or immature dead seabirds allows measurement of individuals of a known age and sex.* Most adult seabirds (including all species included in this study) can't be aged or sexed by plumage or morphometric characteristics after their first year of age.

*g) Juveniles demonstrate some of the most predictable ties to a given foraging habitat, helping to constrain dietary and habitat influences on PFAS levels.* For chicks, this is caused by parents engaging in central-place foraging, meaning the adults find food for their chicks within a region adjacent to the nesting colony to facilitate continuous provisioning of chicks. For the Great Shearwater juveniles which were self-feeding, multiple years of tracking data suggest hatch year birds forage in a predictable home range across Massachusetts Bay, the Great South Channel off Cape Cod, and Georges Bank while adults may forage more widely across a larger pelagic region[39,40].

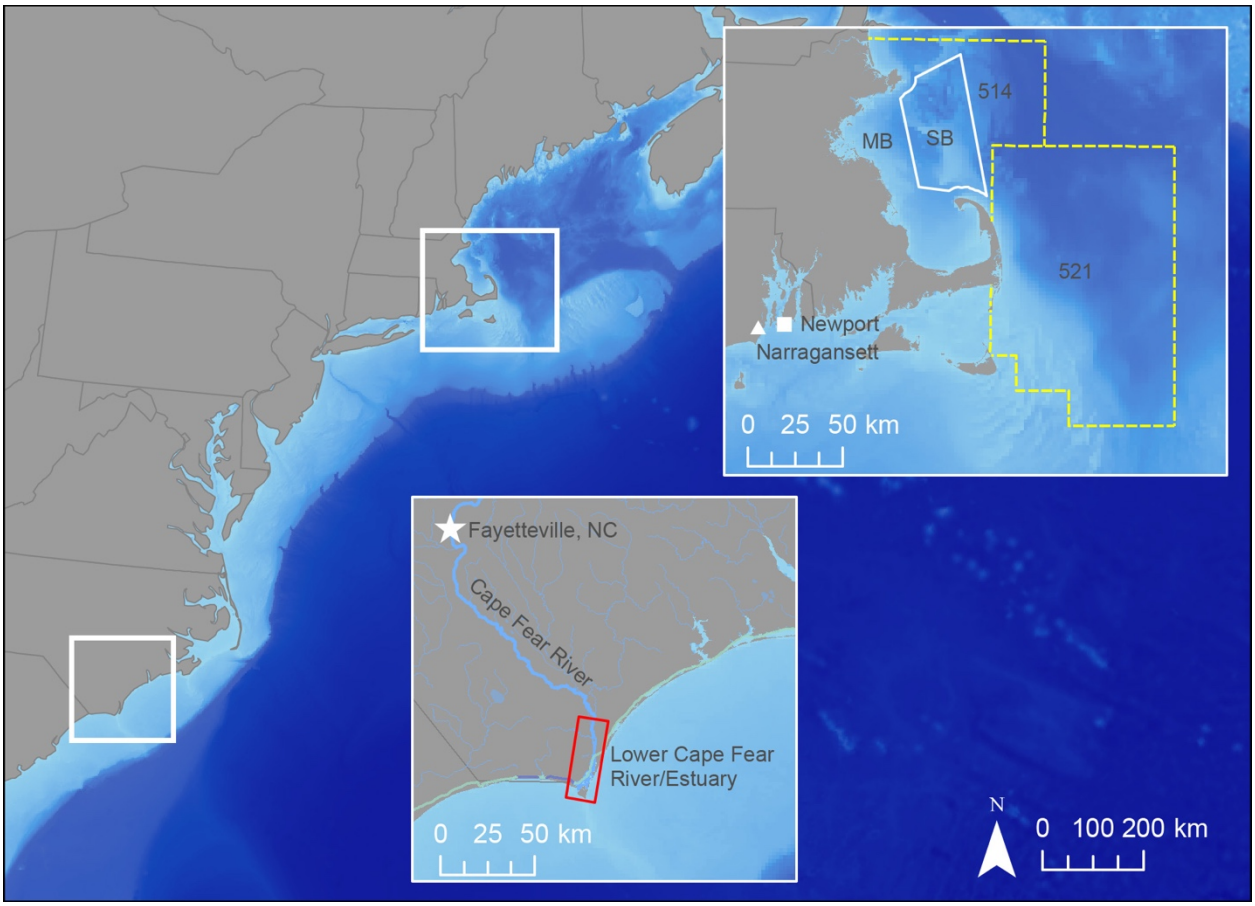



*Figure S1. Map of the US Atlantic East Coast.* Insets provide further detail about collection locations of from each habitat. Great shearwaters were collected as bycatch in Massachusetts Bay and off Cape Cod, designated as NOAA Fisheries Stat Areas 514 and 521. Stellwagen Bank National Marine Sanctuary, a key foraging area for Great Shearwaters, is outlined in white and indicated as "SB" in the top right inset. Herring Gull chicks came from nests located in Narragansett, RI and Newport, RI. The bottom inset shows the Cape Fear River and Estuary system, located in southeastern NC. A Chemours facility is located near Fayetteville, NC, designated on the map as a white star. The red box indicates the lower river and estuary portion of the system; CFRE chicks were hatched on dredge islands near the mouth of the estuary.

## Sample condition, cause of death, and necropsy

Great Shearwaters and Herring Gulls were freshly or very recently dead, and frozen immediately with no decomposition apparent.

The procurement of tissues from juvenile Great Shearwaters caught via fishing bycatch is extremely unlikely to impact liver integrity. Juvenile Great Shearwaters were bycaught via gillnet. Lung hemorrhaging and edema was apparent in some bycatch seabirds (indicative of drowning), but there was no evidence of seawater intrusion into the coelomic cavity housing the liver, and no perforations or damage to any other internal organs beyond lung hemorrhaging. As such we consider bycatch birds as excellent sample specimens, in good condition with no apparent liver degradation or trauma.

Tern, Laughing Gull, and Brown Pelican chicks were recently deceased; chicks spent a maximum of 24-48 hours exposed to the elements prior to collection due to the colony monitoring schedule. Some decomposition was apparent externally on a few individuals. This decomposition was entirely external, apparent via reduced eye integrity, mouth mucus membrane color, and skin pallor. No CFRE individuals displayed evidence of scavenging or substantial autolysis internally.

Phosphotidylcholines, the primary type of phospholipid measured with our bulk assay, have demonstrated stability up to 48 hours after death at 24.1 C, 50% humidity, and atmospheric pressure, suggesting the lipid measurements from CFRE chicks produced

within our study are likely free from majorly compromising impacts related to post-death putrefaction, autolysis, or pH diminution[41,42].

Massachusetts and Narragansett Bay birds were otherwise healthy birds that met unfortunate ends. Narragansett Bay birds fell from their nests and broke one or more limbs, while Massachusetts Bay birds drowned or were strangled in fishing nets. There was no indication of other health problems impacting these individuals that could influence contaminant distributions observed.

Cape Fear birds died due to mostly uncertain causes – predation was not the cause of death because all deceased individuals were collected intact. Colony monitoring data from multiple locations suggests nest abandonment as the most common cause of chick death. Seabirds such as terns and pelicans are very sensitive to disturbance. They will readily abandon a nest due to human, predator, or insect disturbance [43–46]– the chick would then die of dehydration or cold stress. Chicks may also die from tidal flooding or starvation. Chilling, dehydration, and tidal flooding are unlikely to impact contaminant levels in chick livers. PFAS are minimally stored in fat, therefore any starvation-induced use of fat stores is unlikely to release PFAS to liver and overall circulation, should starvation have been the cause of death for any of these birds. No widespread starvation event was apparent in the CFRE region in 2017, making this final potential cause of death somewhat unlikely.

All birds were frozen following collection and tissues sampled within two months. Species identification was corroborated by both specimen collector and necropsy prosector. Each seabird individual was partially thawed and necropsied according to standard protocol, documenting morphometric features, organ weights, overall body condition, sex, and stomach contents after van Franeker 2004[47]. Birds were sexed if gonads were visually identifiable, and aged using morphometric, gonad, and bursa characteristics along with nest observations. Multiple tissues including liver were collected from each bird, and all tissue samples were wrapped in solvent-cleaned aluminum foil, stored in polyethylene baggies, and frozen until analysis at -15C. More details about sample collection locations, bird morphometric data, age, sex, and sample condition can be found in Table S3.

## Sample preparation for UPLC-MS/MS

A modified extraction procedure was developed to maximize sample throughput while minimizing matrix effects and extraction losses, incorporating steps employed in several previously published extraction protocols [28,48–50]. A tissue aliquot was weighed into a polypropylene tube and 4ml of methanol added, followed by 10 ng of isotopically labeled PFAS surrogate mix (1ng/µL). Samples were vortexed for 30 seconds, and allowed to equilibrate for 30 minutes. Samples were again vortexed for 30 seconds, sonicated for 20 minutes, and centrifuged at 4,000 rpm for ten minutes. The resulting supernatant was decanted into a fresh polypropylene tube, and the extraction procedure repeated with 4 mL of 2 mM ammonium acetate in methanol. The extract was frozen at -15C for at least four hours to encourage precipitation of additional biological material and then

centrifuged for three minutes under refrigeration, directly followed by decanting the supernatant for clean-up.

The combined extract was cleaned up using Supelclean ENVI-Carb cartridges (2g, 12 mL, 100−400 mesh, Supelco, U.S.A.). The cartridges were cleaned with 8 mL methanol prior to sample introduction. After sample clean-up, each cartridge was rinsed with 2 mL of methanol. Clean-up was repeated if the final extract contained any hint of color. The extracts were dried to 250 ul at 32°C under 5-7 psi $N_2$, and reconstituted to 1 mL using 2mM ammonium acetate in water for a final sample makeup ratio of 3 parts aqueous: 1 part organic extract. The 1 mL extracts were centrifuged at 10,000 rpm for fifteen minutes at 5°C to remove any remaining tissue residues; the final extract minus any pellet solids was transferred to an autosampler vial in preparation for instrumental analysis.

## UPLC-MS/MS analysis

40 µl of sample extract was injected, and chromatographic separation achieved using a 50 mm BEH C-18 column interfaced to a Waters Acquity UPLC. The mobile phase was made up of methanol and HPLC-grade water modified with 2mM ammonium acetate, made fresh before each run. The applied gradient is detailed in Table S10, the flow rate was set at 0.4ml/min, and column temperature set at 45°C.

Detection of PFAS was carried out using UPLC-MS/MS in negative electrospray ionization mode. Multiple reaction monitoring (MRM) was employed, monitoring two transitions for each compound as available, detailed in Table S7 and S9. Desolvation

temperature was set at 400°C, desolvation gas flow at 600 L/hr, source temperature was set at 150°C, and cone gas flow set at 30°C. Optimal transitions and analyte-specific energies were generated using Intellistart software in combination with manually performed direct infusion experiments.

Quantitation was carried out using an isotope-dilution approach; those analytes lacking a matched mass-labelled standard were quantified using a mass-labelled surrogate of similar molecular weight and retention time (Table S7).

## Sample preparation for HRMS

A tissue aliquot was diluted with three parts water and homogenized to a uniform slurry. An aliquot of homogenate was diluted 4:1 with 0.1M formic acid and vortexed for 30 seconds. The denatured extract was further diluted 5:1 with cold acetonitrile and centrifuged for 5 min at 10000 rpm. An aliquot of the supernatant was removed and combined with dilute ammonium formate buffer (2.5mM) to obtain a final sample extract ratio of 3 parts aqueous: 1 part organic extract.

## HRMS analysis

100 ul of sample was injected, and chromatographic separation achieved using a Vanquish UPLC system equipped with an Accucore 100 mm reverse-phase C18 column, at a flow rate of 300 ul/min. Mobile phase constituents included Solvent A (95:5 water: acetonitrile) and Solvent B (95:5 acetonitrile:water). Mobile phase gradient is detailed in Table S11. Nafion byproduct 2 (Nafion BP2, or 2-[1-[difluoro(1,2,2,2-tetrafluoroethoxy)methyl]-1,2,2,2-tetrafluoroethoxy]-1,1,2,2-tetrafluoro-ethanesulfonic

acid) and $PFO_5DoDA$ (2,2,4,4,6,6,8,8,10,10,12,12,12-Tridecafluoro-3,5,7,9,11-pentaoxadodecanoic acid) were identified with authentic native standards, while $PFO_4DA$ (perfluoro-3,5,7,9-tetraoxadecanoic acid), PFMA (2,3,3,3-tetrafluoro-2-(trifluoromethoxy)-propanoic acid), $PFO_2HxA$ (perfluoro-3,5-dioxahexanoic acid), PEPA (Perfluoro-2-ethoxypropanoic acid), PFO3OA (Perfluoro(3,5,7-trioxaoctanoic) acid), NVHOS (1,1,2,2-tetrafluoro-2-(1,2,2,2-tetrafluoro- ethoxy)ethane sulfonic acid), Nafion byproduct 4 (2,2,3,3,4,5,5,5-4-(1,1,2,2-tetrafluoro-2- sulfoethoxy) pentanoic acid), Nafion byproduct 1 (2-[1-[difluoro[(1,2,2-trifluoroethenyl)oxy]methyl]-1,2,2,2-tetrafluoroethoxy]-1,1,2,2-tetrafluoro-ethanesulfonic acid) and PFECHS (perfluoro-4-ethylcyclohexanesulfonate) were detected using previous accurate mass assessment information. PFAS were detected using a Thermo Orbitrap Fusion mass spectrometer using heated electrospray ionization in negative mode (Thermo Fisher Scientific, Waltham, MA, USA). Full scan accurate mass spectra were acquired from 70 to 700 Da with a resolving power of 120,000 Rs for MS1 and at 30,000 Rs for MS2, and a mass accuracy of ±5 ppm. Data-dependent acquisition was carried out to acquire MS/MS of select features at a resolving power of 30000. The ion transfer tube was set at 250°C and vaporizer temp set at 30 °C. Interference from the tissue matrix prevented the use of the Fusion internal lock mass in this experimental method. Data acquisition and analysis was performed using Xcalibur and Compound Discoverer software (Thermo Fisher Scientific, Waltham, MA, USA).

## Quality Assurance and Quality Control

Six-point, processed and matrix-matched calibration curves were prepared, one for each extraction method. Curve preparation entailed taking multiple aliquots of liver

tissue (from the same batch of slurried tissue) through each extraction in its entirety. The matrix-matched approach is key to account for the influence or interference of biological co-eluents on PFAS response and derived concentrations[50,51].

The UPLC-MS/MS curve points were spiked directly before instrumental analysis with appropriate levels of native and mass-labelled standard, ranging from 0.25-100 ng/ml. The HRMS curve points were spiked before extraction, and therefore recovery-corrected all subsequent quantitation; these curve points ranged from 0.05 -10 ng/400µl. The curves were used for quantification of samples prepared with the corresponding extraction method. Organic chicken liver, demonstrated to contain low concentrations of targeted PFAS, was used as the curve matrix. PFOS was consistently found in organic chicken liver samples, and thus the curve was corrected for background levels of PFOS by subtracting the average of measured background samples (n = 6) from PFOS responses measured in curve point samples. All calibration curves used for quantitation demonstrated an $R^2 \geq 0.98$, with most demonstrating an $R^2 \geq 0.99$.

During targeted analysis via UPLC-MS/MS, process blanks were prepared and analyzed with every 10 samples and found to be free of significant contamination; sample concentrations were not blank corrected as a result. Each sample was injected in duplicate, and duplicate injections monitored for stability. Mobile phase blanks (3:1 aqueous:organic) were analyzed between duplicate sample injections to monitor for analyte carryover or contamination.

Five samples consisting of chicken liver spiked with 2 ng of all native compounds were also prepared and used to calculate accuracy and precision metrics for UPLC-MS/MS. Accuracy ranged from 18-154% with a mean accuracy of 92%. Precision, calculated as percent relative standard deviation (% RSD), ranged from 7 – 150 %, with a mean % RSD of 28% (Table S13). Precision and accuracy were particularly variable for neutral sulfonamide acetic acids, those compounds lacking an identical mass-labelled surrogate like PFDS, and the $C_{13}$ and $C_{14}$ PFCAs.

Although the matrix-matched curves account for matrix effects in quantitation efforts, matrix effects were calculated for illustrative purposes following methods described by Chambers et al. 2008[51]. Matrix calculations indicated variable enhancement or suppression of each analyte, with an average ion suppression of -20% and an ionization enhancement of 31% (Table S14).

Equation 1: $$\%\ Matrix\ Effects = \left(\left(\frac{Post\text{-}extraction\ spiked\ matrix}{Post\text{-}extraction\ spiked\ solvent}\right) - 1\right) \times\ 100$$

Method detection limits for UPLC-MS/MS ranged from 0.5 – 4.1 ng/ml based on spiked replicate samples multiplied by the Student's t-value appropriate for a single-tailed 99th percentile (Table S15). Method recovery ranged from 14 -112%, with a mean recovery of 61% across all compounds (Table S6).

Equation 2: $$\%\ Recovery = \frac{Pre\text{-}extraction\ spiked\ sample}{Post\text{-}extraction\ spiked\ sample} \times\ 100$$

During HRMS analysis, duplicate process blanks using formic acid and acetonitrile were prepared daily with each sample set, for a total of 8 process blanks. Process blanks

were used to identify contamination introduced via sample preparation and instrument background signal. Mobile phase blanks were injected between different types of samples to monitor instrumental background noise and any carryover between samples. No contamination of emerging PFAS was apparent in process blanks; HFPO-DA and several other legacy PFCAs displayed high levels of instrumental background noise as displayed in instrumental blanks and process blanks. No significant background noise was apparent for Nafion BP2, PFO5DoDA, or PFO4DA, the three PFEAs of interest reported here using HRMS analysis (Table S12). Method recoveries for HRMS were not tracked; each curve point sample was spiked with appropriate levels of native and internal standard and then taken through the extraction to create a recovery-corrected curve for quantification. Samples were analyzed in four batches, and curve stability monitored between runs. Curve responses at all levels varied less than 4% across all four runs for the three PFEAs reported using HRMS measurements in this analysis.

Quantification of emerging compounds via HRMS was limited to those samples above the linear range of the calibration curve; the lower detection limit was determined by comparison to blank values plus three times the standard deviation of blank responses (Table S12). Reporting of emerging compounds below the curve range or those without authentic standards is limited to raw abundances in comparison to process and instrumental blank values (Table S16).

PFOS concentrations measured via both HRMS and UPLC-MS/MS were compared for parity, and were generally found to be within 30% of UPLC-MS/MS results and all were

480 within an order of magnitude. UPLC-MS/MS results were considered more precise due
481 to lower levels of instrumental background noise along with higher levels of QA/QC and
482 were therefore used for comparison and statistical analyses for all compounds
483 excepting Nafion BP2 and PFO5DoDA.

484

485 **Stable Isotope Analysis Sample Preparation and Analysis**
486
487 Liver and muscle of seabird chicks were lyophilized, and ground to fineness using a
488 mortar and pestle. 2-3 mg of tissue was weighed out into tin capsules for carbon and
489 nitrogen stable isotope analysis, while 3-5 mg of muscle tissue were weighed out for
490 sulfur isotope analysis.

491

492 Weighed samples were measured via IR-MS analysis, and results interpreted as parts
493 per thousand relative to appropriate references. $\delta^{15}N$ and $\delta^{13}C$ were measured using an
494 Isoprime 100 Isotope Ratio Mass Spectrometer coupled to a Micro Vario Elemental
495 Analyzer (Elementar Americas, Mt.Laurel, NJ). $\delta^{34}S$ was measured by UC Davis Stable
496 Isotope Laboratory using an Elementar Vario ISOTOPE cube interfaced to a SerCon
497 20-22 IRMS (Sercon Ltd., Cheshire, UK).

498

499 The nitrogen ($\delta^{15}N$ ) isotope composition was expressed as a part per thousand
500 deviation (‰ ) from air. Carbon ($\delta^{13}C$ ) isotope composition was expressed relative to
501 Vienna Pee Dee Belemnite where $\delta X = [(R_{sample} - R_{standard})/R_{standard}] \times 10^3$, where X is
502 $\delta^{15}N$ or $\delta^{13}C$, and R is the ratio of heavy to light isotope (15N: 14N, 13C: 12C).

Duplicates were analyzed every 10 samples, and a blue mussel reference material every 15 samples to ensure measurement quality.

## Phospholipid analysis

50 mg of liver was homogenized at 4ºC in 1 ml phosphate-buffered saline using a Beadruptor Elite bead mill homogenizer from Omni International. An aliquot of the homogenate was transferred to a 15ml polypropylene tube, and 3.75 ml of chloroform:methanol (2:1, v/v) was added. The solution was vortexed, and 0.5 ml deionized water added, followed by another 15 sec of vortexing. The extract was then centrifuged at 3000 rpm for 5 min at room temperature, and the organic layer decanted into a pre-weighed glass vial. The organic extract was allowed to evaporate in a fume hood overnight, and the remaining lipid residue was then weighed to ascertain total lipid content. The residue was then re-suspended in 200 µl of 1% Triton X-100 in 100% ethanol and shaken well for at least two hours. 20 µl of the extract was then pipetted into a 96-well plate, inoculated with phospholipid kit working reagent, and assessed colorimetrically using a Spectramax M2 Multi-Mode microplate reader for phospholipid content via comparison to a 4-point curve containing 0-200 µM phosphatidylcholine[52].

## More detail about stable isotope values and relationships

There is limited fine-scale comparability between stable isotope measurements between habitats included in this study, due to the species and food web specific nature of isotope fractionation factors, alongside inevitable and stark differences in isotopic composition at the base of each food web. As a result, here we focus on associations

between PFAS concentrations and stable isotope measurements *within* each habitat, rather than considering isotope ratios across the sample set as a whole. Likewise, we apply previously described trophic level calculations to measurements within each habitat for illustrative purposes, noting that bulk trophic level calculations uncoupled from food-web specific data lack fine-scale insight.

Summary statistics associated with stable isotope analysis (SIA) of each tissue are presented in Table S20; a three-dimensional presentation of all SIA data can be found here [http://rpubs.com/Arobuck/555350].

Herring Gulls from Narragansett Bay evidenced the widest range of $\delta^{15}N$, while Shearwaters from Massachusetts Bay displayed the least variability in $\delta^{15}N$ (Table S21). Derived trophic level estimates reflected the same patterns of variability, with all birds evidencing an estimated trophic level between 3.24 – 4.59 based on liver $\delta^{15}N$ (Table S20, S21). Trophic level estimates assumed a calanoid copepod primary consumer, a realistic assumption for all habitats. $\delta^{15}N$ values and calculated trophic level were not significantly associated with concentrations of most individual PFAS or $\sum_{19}$PFAS in each habitat. PFOS and $\sum_{19}$PFAS were positively associated with $\delta^{15}N$ only in Massachusetts Bay individuals (Table S23). The lack of more significant associations with $\delta^{15}N$ may be related to similar trophic strategies and prey items utilized by birds included in this study, resulting in a limited range of $\delta^{15}N$ (Tables S21, S23). The lack of association likely also relates to the unique partitioning and accumulation pathways governing PFAS distribution in biota compared to legacy POPs [11,53].

Muscle $\delta^{34}S$ was also compared to concentrations of individual PFAS and ∑PFAS. Muscle $\delta^{34}S$ was only associated with PFUnDA in Narra. Bay individuals; no other statistically significant or observationally notable associations were present between $\delta^{34}S$ and PFAS concentrations.

$\delta^{13}C$ and PFAS concentrations were most notably associated in birds from the CFRE. The association between $\delta^{13}C$ and PFAS levels in CFRE chicks is likely a function of the marine and estuarine foraging habits of species included in this study, coupled to the local geomorphology of the CFRE system. Seabird parents foraging in the CFRE or in the adjacent coastal plume likely obtained resources comparatively enriched in $\delta^{13}C$ due to the abundance of *Sporobolus alterniflora* marshes in the CFRE lower estuary. These marshes make up a substantial portion of the undeveloped land area of the lower estuary and serve as habitat and nursery area for ecologically and commercially important fish and invertebrates[54,55]. *Sporobolus alterniflora* typically reflects an enriched $\delta^{13}C$ value of -13.6‰[56], compared to more depleted $\delta^{13}C$ ratios observed in offshore food webs based on particulate organic matter and phytoplankton (-20‰ to -26‰[57,58]) (Fig. S6). *Sporobolus alterniflora* marshes inhabit geomorphically protected inshore lagoon and barrier island environments in the CFRE, and were physically closer to upstream PFAS sources. Therefore, they were subject to less open-water dilution, likely increasing PFAS exposure potential for prey and predators reliant on these marsh systems. Surface water studies support this hypothesis, with predictable inverse relationships between salinity and surface water ∑PFAS apparent in estuarine[59], coastal shelf, and slope[60]

environments. This suggests an increased potential for PFAS exposure in terrestrially-influenced environments like estuaries or brackish marshes. CFRE seabird parents also likely had an incentive to forage as close as possible to estuarine nesting colonies to conserve energy, further encouraging reliance on marsh and estuary resources. Seabird parents utilizing more offshore, marine systems for chick provisioning were likely foraging in environments with increased dilution and mixing, obtaining larger prey with decreased PFAS levels and a more depleted $\delta^{13}C$ signature.

## Bioaccumulation factors

Liver-water bioaccumulation factors (BAFs) were calculated by dividing liver geometric mean PFAS levels by measured or estimated surface water concentrations adjacent to nesting or collection locations, followed by log transformation. Water values for Massachusetts Bay and Narragansett Bay were derived from unpublished data from the Lohmann lab, while surface water estimates for the CFRE were estimated using data inputs from Zhang et al. 2019, assuming a conservation of mass dilution approach and estimating salinity near the collection site at M18 using salinity data from the Lower Cape Fear River Program (Tables S18-S19)[61,62].

BAFs reported here range from 0.5 – 3.7, with PFOS, PFNA, PFDA, PFO5DoDA, and Nafion BP2 displaying BAFs near or above 2 variably across the three habitats sampled in this study (Fig. S9). BAFs above 3.3 are considered “bioaccumulative” under regulatory protocol designed for persistent organic pollutants[63].

## Miscellaneous Supporting Figures

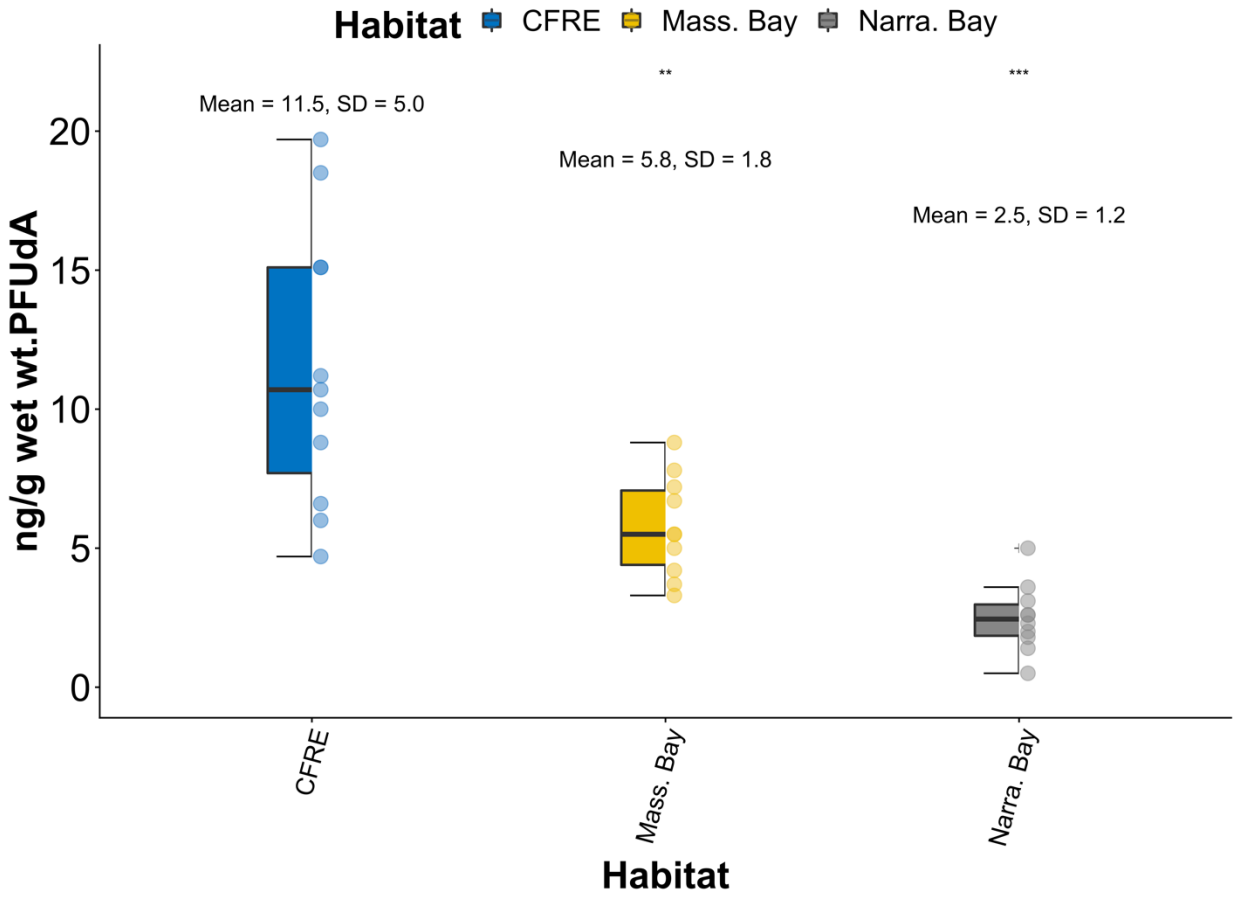
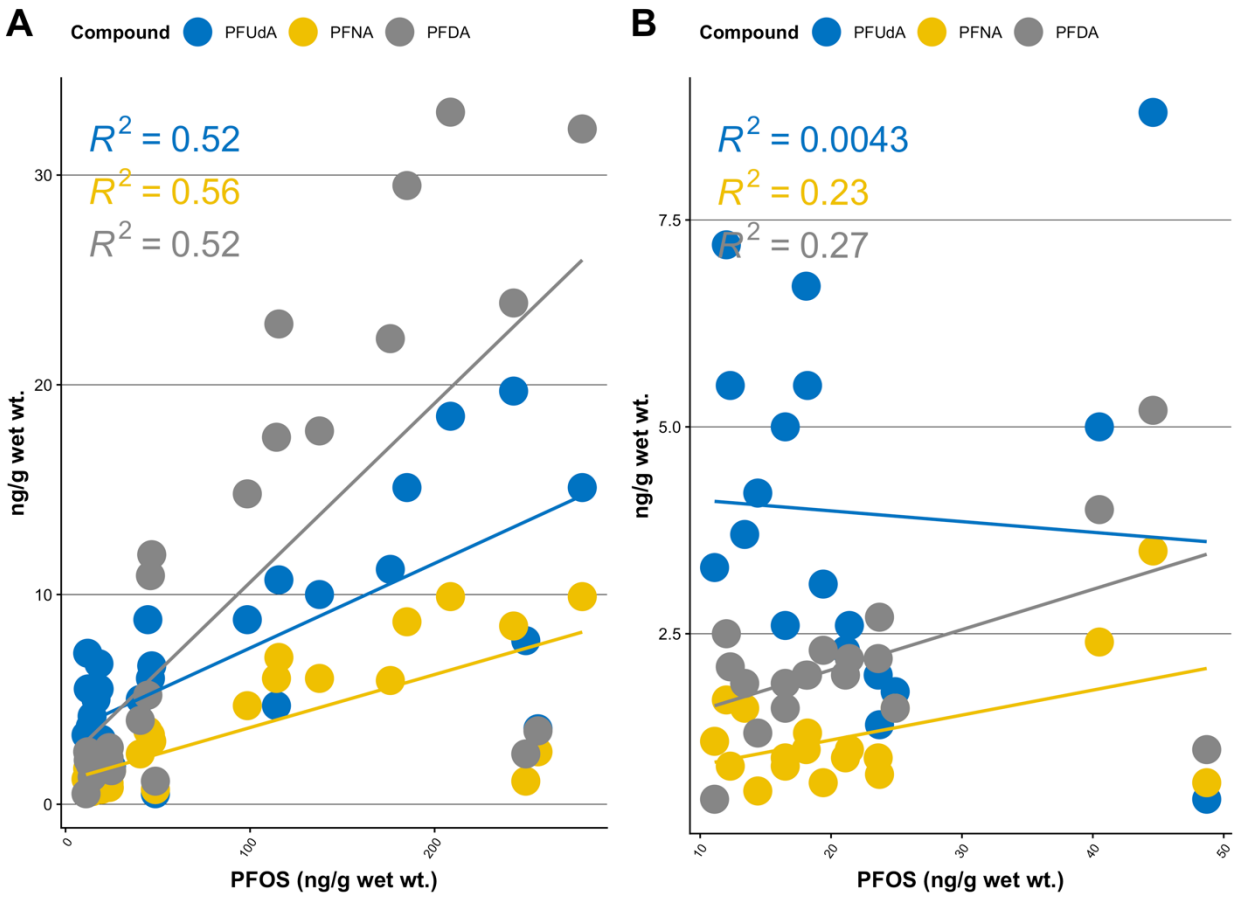



*Figure S2. Concentrations of PFUnDA by habitat.* Habitat mean concentrations of PFUnDA presented as boxplots, with the dark line representing the median, box limits representing the first and third quartiles, whiskers denoting 1.5 times the interquartile range, and crosses denoting outliers. The points reflect measured observations contributing to the summary statistics presented by the boxplot. The asterisks indicate a statistically significant difference between habitat mean PFUnDA compared via the Wilcoxon rank sum test, using the CFRE group as the reference group.




610

611 *Figure. S3. Observed concentrations of PFOS versus concentrations of PFNA,*

612 *PFDA, and PFUnDA* in A) all individuals across three habitats, and B) in only

613 individuals from Narragansett Bay and Massachusetts Bay with two outlier individuals

614 removed. All concentrations were measured via UPLC-MS/MS.

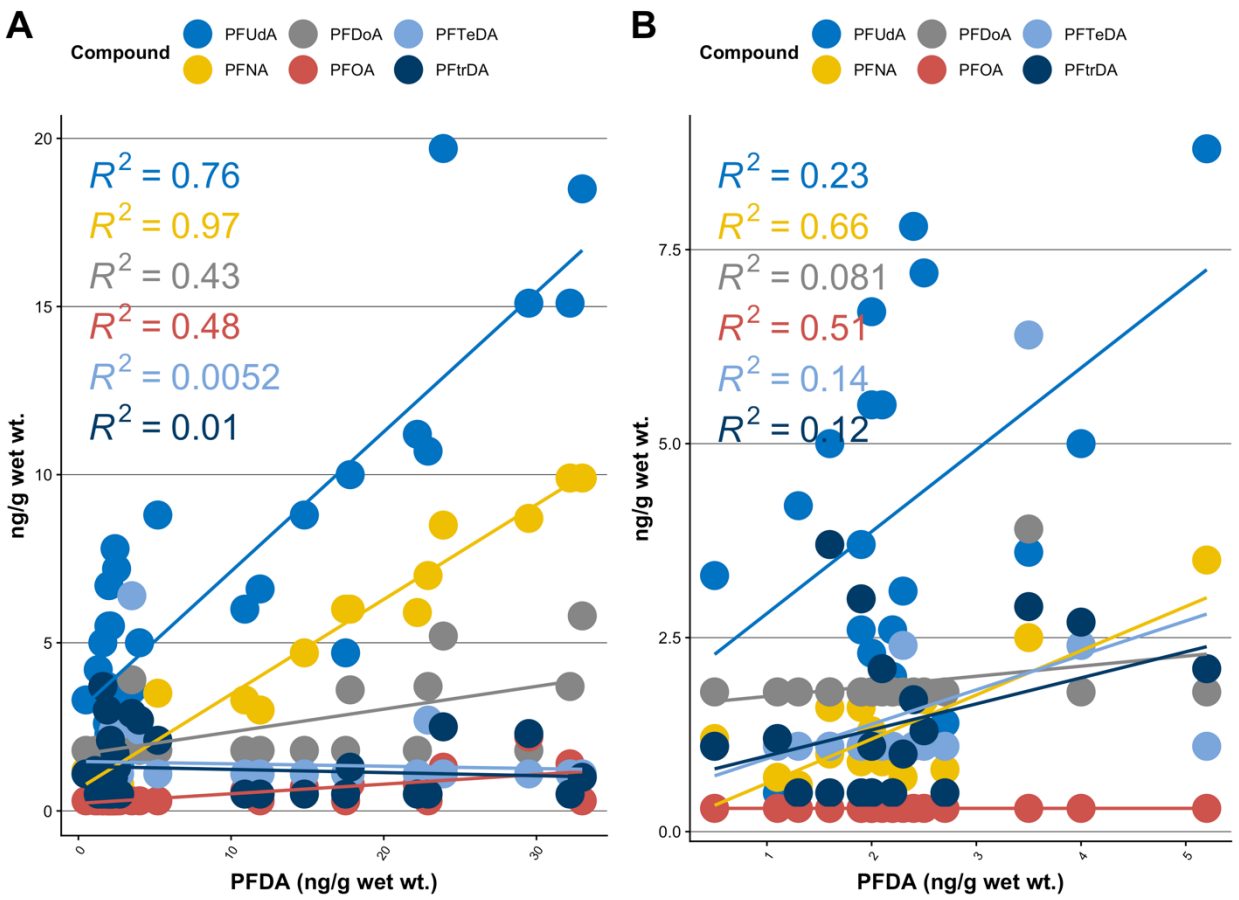
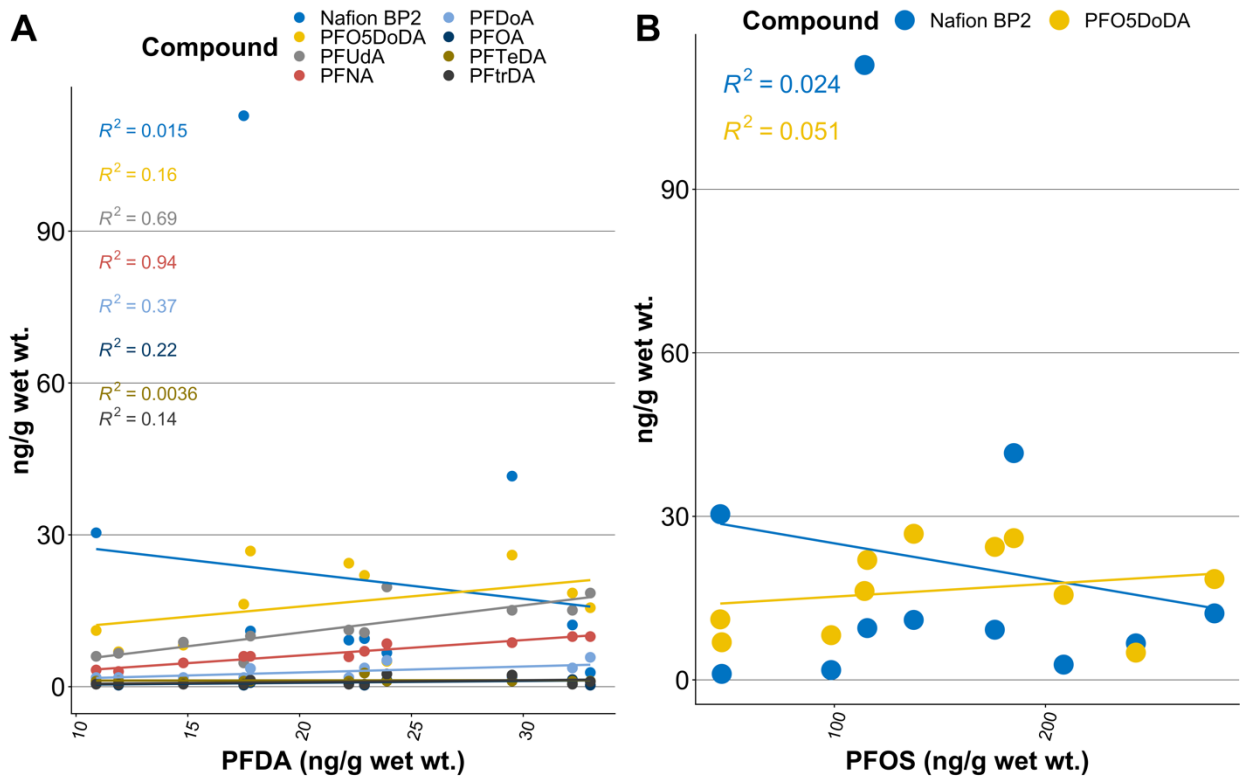



*Figure S4. Concentrations of C8 – C14 PFCAs as a function of PFDA* measured via LC-MS/MS in A) all individuals across three habitats, and B) in only individuals from Narragansett Bay and Massachusetts Bay. Associations between PFCAs as approximated by linear regression slightly decrease without CFRE data included, likely due to the truncation of the data range as a result of more similar concentrations observed in habitats removed from point sources and inclusion of MDL/2 values.




626

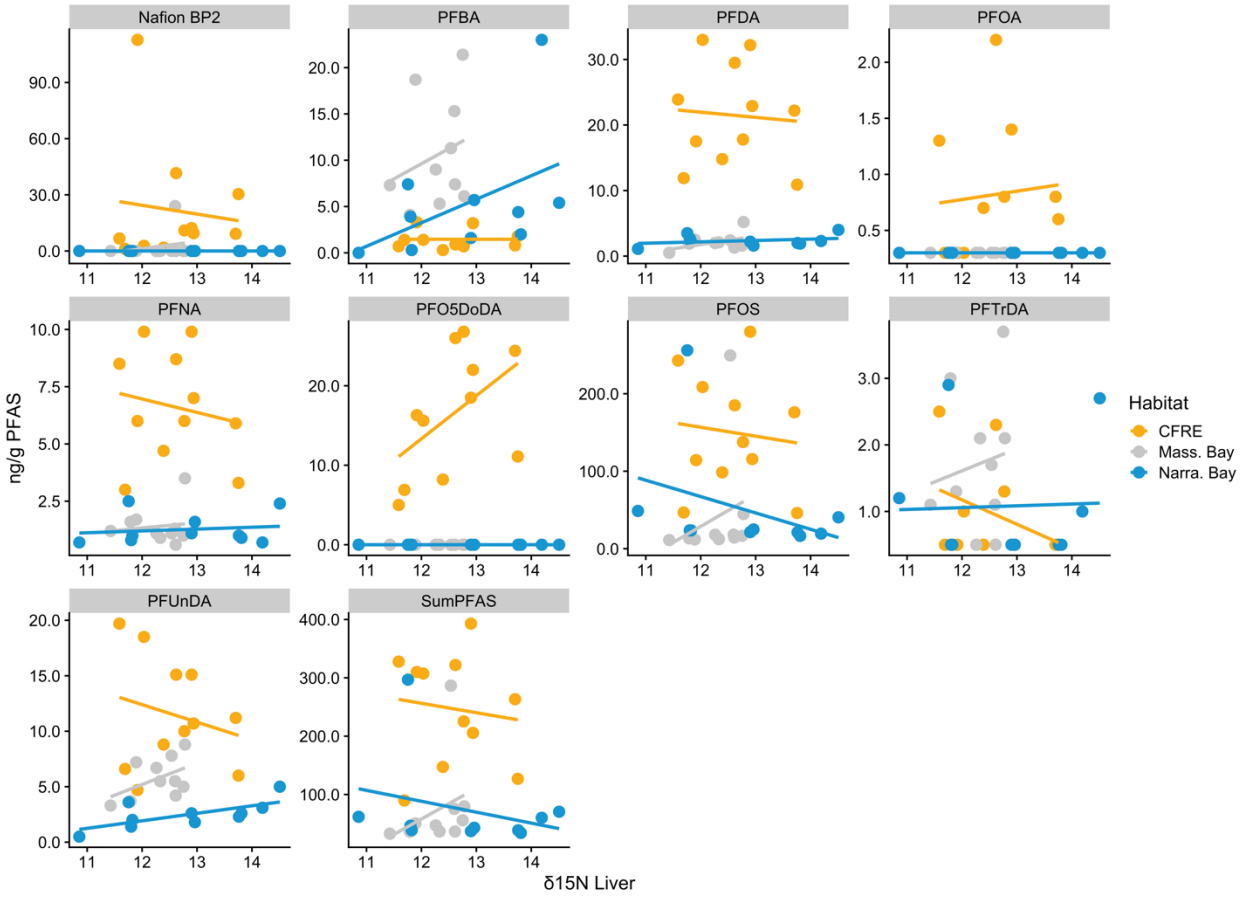
627 *Figure. S5. Observed concentrations of emerging PFEAs versus legacy PFAS in*

628 *CFRE chicks* with A) displaying concentrations of PFDA vs PFEAs and other long-

629 chain PFCAs, and B) displaying concentrations of PFOS vs PFEAs. PFCA and PFSA

630 concentrations were measured via UPLC-MS/MS while PFEAs were measured via

631 HRMS.




632

633 *Figure S6. δ15N vs concentrations of PFAS*. Measured $\delta^{15}N$ in seabird liver and

634 muscle did not relate to concentrations of individual PFAS, or $_{19}\sum$PFAS in seabird liver

635 across any habitat. No correlation was found when the total sample set (n = 31) was

636 assessed in the same way. Muscle $\delta^{15}N$ ratios are presented here; facet plots include

637 only those compounds found in >40% of the sample set.

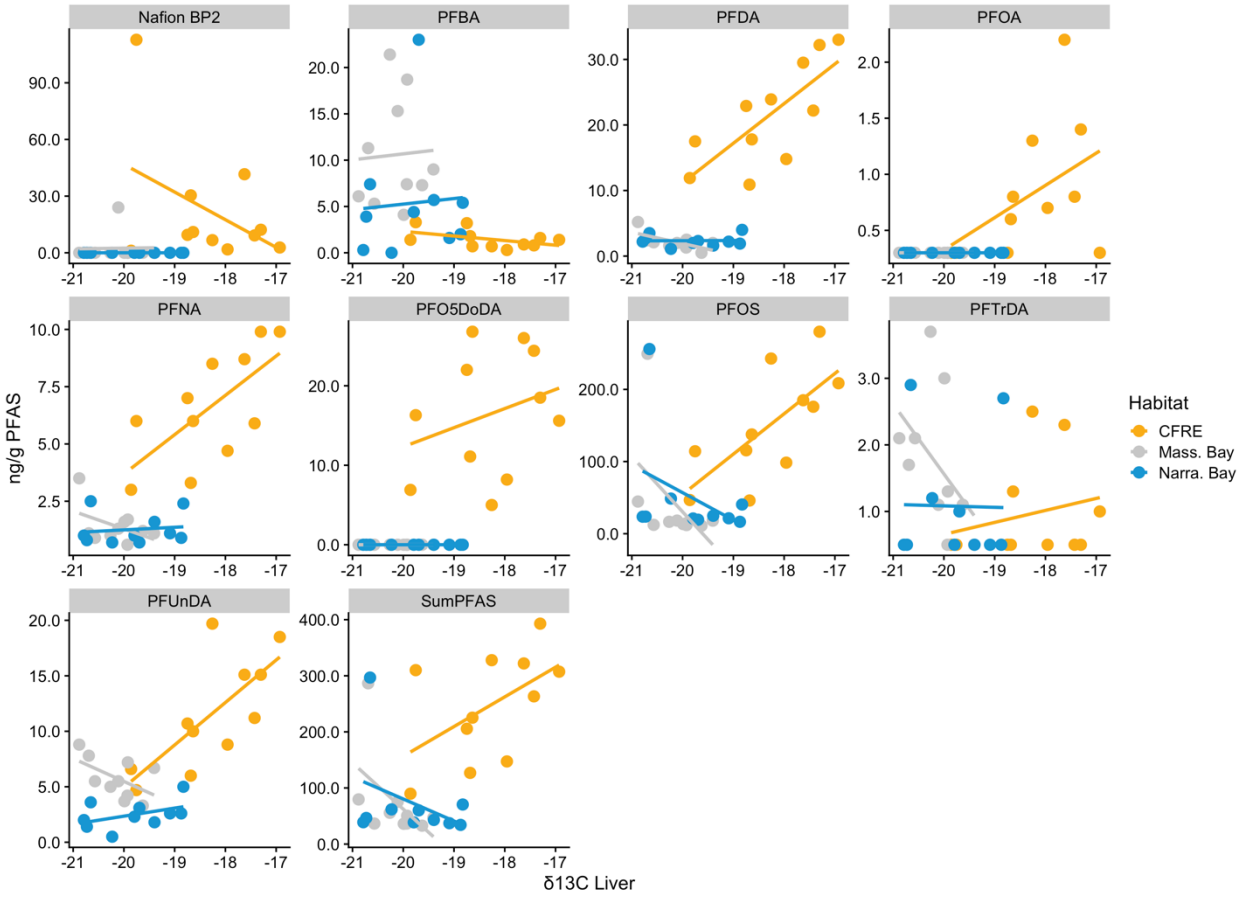
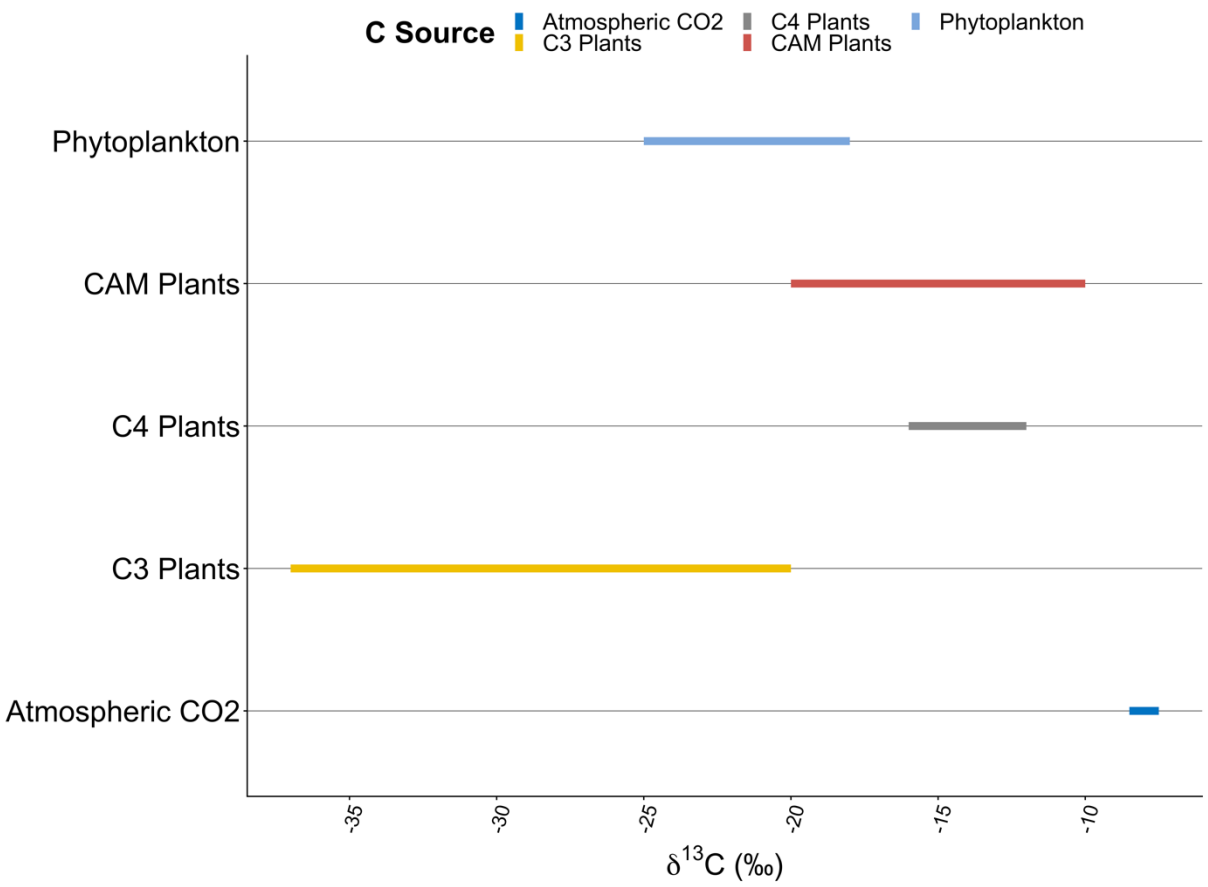



*Figure S7. δ13C vs concentrations of PFAS*. Measured $\delta^{13}C$ in seabird liver and muscle did not relate to concentrations of individual PFAS, or $_{19}\sum$PFAS in seabird liver from Massachusetts Bay or Narragansett Bay. No correlation was found when the total sample set (n = 31) was assessed in the same way. PFDA, PFNA, PFOS, PFUnDA, and $_{19}\sum$PFAS were moderately associated ($p < 0.05$) with $\delta^{13}C$ ratios, only in CFRE chicks. See SI text for further discussion of this limited phenomenon. Muscle $\delta^{13}C$ ratios are presented here; facet plots include only those compounds found in >40% of the sample set.

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651 *Figure S8. $\delta^{13}C$ in key organic and inorganic matrices.*

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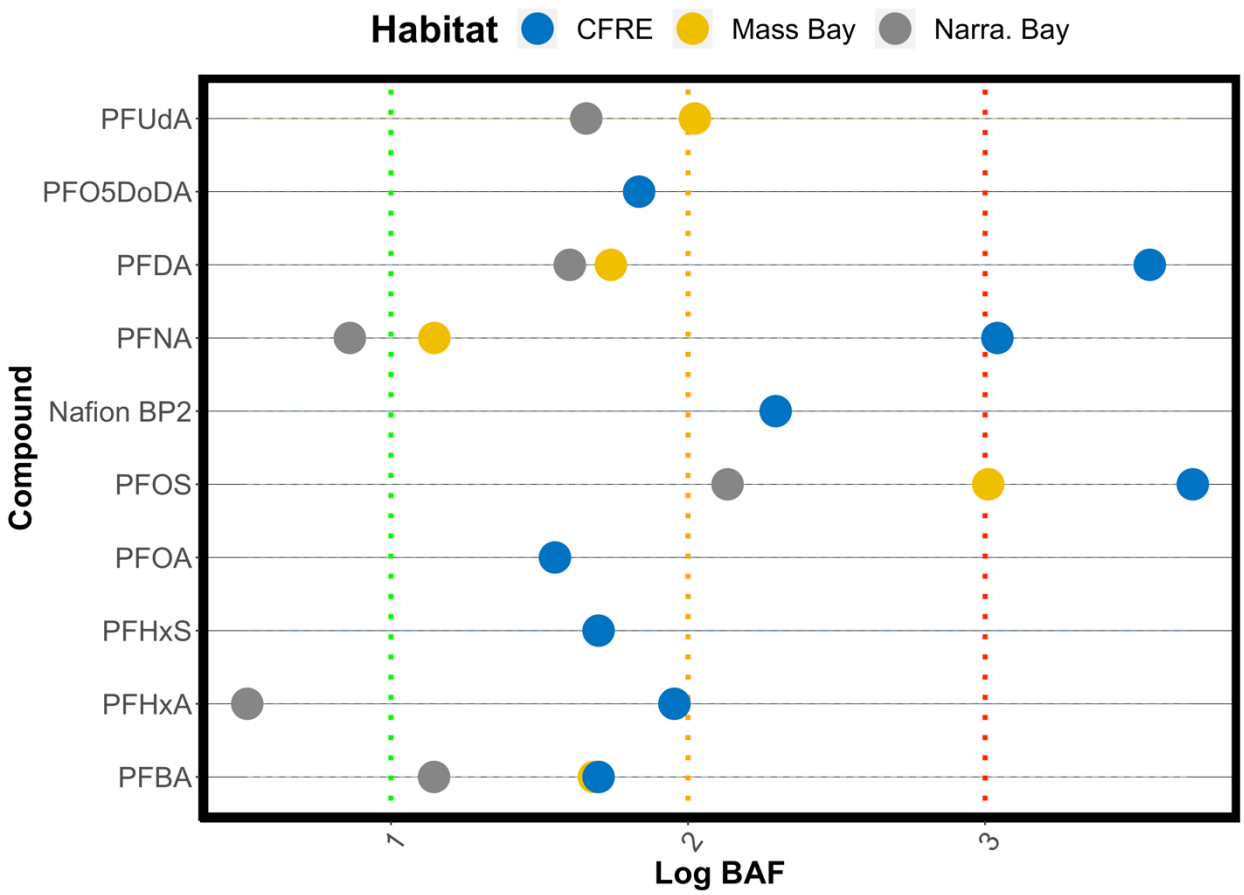


654

655 *Figure S9. Log BAFs for 10 compounds across three habitats.* BAFs were

656 calculated by dividing mean liver concentrations observed in each habitat by observed

657 or estimated surface water concentrations.

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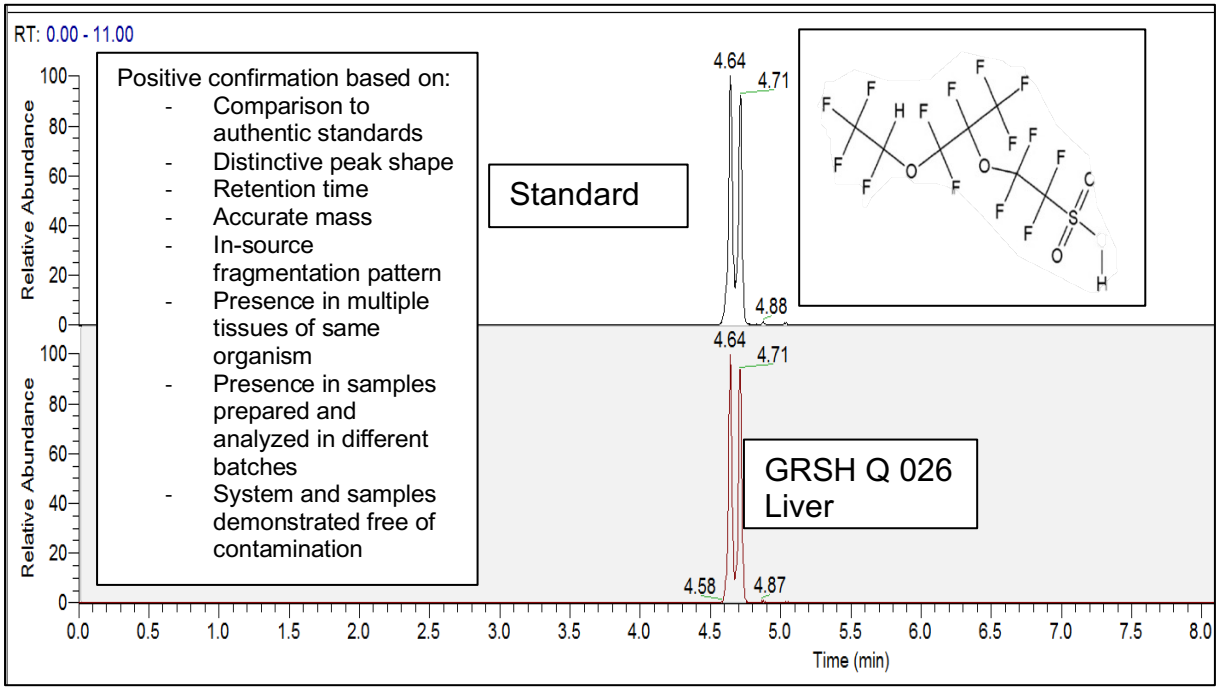


*Figure S10. Chromatograms of Nafion BP2 in native standard and Great Shearwater liver*. Extracted ion chromatogram (EIC) of Nafion BP2, [M-H] ion with m/z = 462.9326 ± 5ppm. The figure shows the authentic standard (top) compared to the EIC observed in a Great Shearwater liver from Massachusetts Bay (bottom). Nafion BP2 was identified in two Great Shearwater juveniles and one Herring Gull chick outside of the CFRE system. Positive identification of Nafion BP2 in these non-CFRE samples was confirmed by comparison to authentic standards, distinctive peak shape, accurate mass, in-source fragmentation patterns, $MS^n$ data when available, retention time, and sample preparation and analysis information.

## Tables

See tabs contained in Excel file “PFAS in Seabird Liver_Final.xlsx”

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