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Per- and polyfluoroalkyl substances (PFAS) in plasma of the West Indian manatee (*Trichechus manatus*)

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are ubiquitous, synthetic anthropogenic chemicals known to infiltrate and persist in biological systems as a result of their stability and bioaccumulation potential. This study investigated 15 PFAS, including short-chain carboxylic and sulfonic acids, and their presence in a threatened herbivore, the West Indian manatee (*Trichechus manatus*). Seven of the 15 PFAS examined were detected in manatee plasma. Perfluorooctanesulfonic acid (PFOS) (ranging from 0.13 to 166 ng/g ww) and perfluorononanoic acid (PFNA) (ranging from 0.038 to 3.52 ng/g ww) were detected in every manatee plasma sample examined (n = 69), with differing medians across sampling sites in Florida, Crystal River (n = 39), Brevard County (n = 18), Everglades National Park (n = 8), and four samples (n = 4) from Puerto Rico. With an herbivorous diet and long life-span, the manatee provides a new perspective to monitoring PFAS contamination.

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Keywords

PFAS; PFOS; West Indian manatee; health parameters; Florida; Puerto Rico

INTRODUCTION:

Marine mammals have been utilized as sentinel species to assess biological responses to environmental stressors, such as exposure to contaminants, climate changes and diseases (1–6). Likewise, to humans, wild marine mammals are exposed to a highly variable environment and therefore can act as models to study the exposure and effects of complex chemical burdens. In addition, chemical bioaccumulation through trophic levels, especially in the aquatic environment, allows the employment of fish-eating predators as sentinels to offer additional insight for translation to environmental and ultimately human health (7, 8). For example, exogenous chemical burdens, inclusive of perfluorinated compounds, organohalogens, and flame retardant materials, have been studied in marine mammals such as bottlenose dolphins (*Tursiops truncatus*), killer whales (*Orcinus orca*), and northern fur seals (*Callorhinus ursinus*) (9–12). Attention has been placed on monitoring perfluoroalkyl substances (PFAS) due to an increased presence in the environment, wildlife, and humans (13–15). These commonly utilized synthetic and toxic chemicals are noted to have long half-lives, are resistant to environmental degradation, and bioaccumulate (16). Most notably, they are known to be used in products such as some cookware, paints, and firefighting foams due to their water-repellant properties (17, 18). An increasing number of adverse health outcomes have been associated with PFAS exposure, in both wildlife and humans (15–16, 19–23). A primary focus of PFAS analyses on marine wildlife has been placed on examining predatory species, due to the route of exposure (fish consumption) and concern over bioaccumulation and biomagnification (9, 24–27). However, to date, no studies have examined the PFAS burden in large herbivorous marine mammals, which represents a current gap in PFAS exposure research.

Population and foraging ecology, as well as the habitat status, husbandry, health and morphology of the West Indian manatee (*Trichechus manatus*) have been studied extensively as a result of its wavering status under the Endangered Species Act (ESA) (28–38). However, despite these efforts to learn more about and protect the manatee, there have only been a few studies analyzing their chemical contaminant burden (1, 37–40). The Florida and Antillean manatees (subspecies of the West Indian manatee) live in warm waters, close to the shores of Florida and Puerto Rico (29). Previous studies suggest the resilience of manatees in regard to disease, with little record of widespread outbreaks in the past and a relatively long life-span of about sixty years (41). However, the manatee's history of endangerment is universally acknowledged as a result of human related impacts, such as boat strikes and entanglement in discarded fishing gear (42). In addition, with their close proximity to human populated areas, manatees can ingest, inhale, or come in dermal contact with anthropogenic compounds introduced into coastal waterways leading to increased opportunities of exposure. Because PFAS are ubiquitous, the need to expand efforts describing their presence are necessary to completely determine their biological impact (43).

This study assessed PFAS burden in the West Indian manatee in an effort to determine the presence of these chemicals in a lower trophic mammal. Manatees inhabiting three coastal sites in Florida were examined (Brevard County, Crystal River, and Everglades National Park). The difference among sites provided an opportunity to investigate PFAS burden within differing habitats. Each of the three sites have been identified as common locations for manatees year-round as a result of the relatively warm water temperatures. Crystal River and locations within Brevard County (such as the Banana and Indian rivers) are known designated critical habitats for the West Indian manatee (44). A fourth site consisted of a pilot-size sample set (n = 4) from two places in Puerto Rico, Guayanilla and Cabo Rojo. Considered the most endangered marine mammal in Puerto Rico (45), research concerning manatee health is essential in protecting the current population of manatees. The entire manatee plasma cohort (n = 69) was assessed for the presence and concentration of 15 different PFAS. The PFAS concentrations obtained were then related to the location of the sampling site. Correlations between PFAS values and physical morphology (e.g. sex) were also investigated.

MATERIALS AND METHODS:

Sample collection

Plasma samples from West Indian manatees (n = 69) were collected by researchers from the U.S. Geological Survey (USGS) Sirenia Project between 2003 and 2017 (USFWS Research permit: MA-791721, USGS IACUC permit: USGS-WARC-2016–03, and previous permits). Samples were collected in Florida (n = 65), among the following three sites (Supplemental Fig 1 and Supplemental Fig 2), Crystal River (CR) (n = 39), Brevard County (BC) (n = 18), and Everglades National Park (EP) (n = 8). Four additional samples were collected in Puerto Rico (PR, Supplemental Fig 1 and Supplemental Fig 3) in Guayanilla (n = 3) and Cabo Rojo (n = 1). Each manatee was captured and sampled utilizing established sampling procedures (40) and then released. Hematology and morphometrics (length, weight, sex, and body condition indices (BCI); Supplemental Table 1) were obtained by research biologists and veterinarians referring to previously outlined methods (31, 46).

PFAS Standards and SRM 1950

Samples were analyzed for the following 15 PFAS by using a previously published protocol: perfluorobutyric acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTA), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), perfluorooctanesulfonic acid (PFOS), and perfluorooctanesulfonamide (PFOSA) (47, 48). An internal standard (IS) solution was made (Cambridge Isotope Laboratories, RTI International, and Wellington Laboratories), and consisted of $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{18}O_2$-PFHxS, $^{13}C_8$-PFOA, $^{13}C_9$-PFNA, $^{13}C_9$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-PFDoA, $^{18}O_2$-PFBS, $^{13}C_4$-PFOS, and $^{18}O_2$-PFOSA (48). In order to identify the PFAS within the plasma, samples were extracted using methodology described in a prior publication (48). The National Institute of Standards and Technology (NIST)

Standard Reference Material (SRM) 1950 Metabolites in Human Plasma (n = 3), that provides concentration values for six PFAS as reference values, was prepared and analyzed along with samples, functioning as a quality control sample (http://srm1950.nist.gov/).

Sample preparation

In brief, the method consisted of utilizing either 1.0 g of each manatee plasma sample, 1.0 g of deionized water (blank) for each analysis (n = 3), or 1.0 g of SRM 1950 for each analysis (n = 3). The IS solution was gravimetrically added in 600 μL increments to the samples, blanks, and SRMs before extraction (49). After equilibrating for 1.5 h, 4 mL of acetonitrile was added to each tube, sonicated for 30 min, and centrifuged for 5 min at 2500 rpm. The supernatant was then pipetted out of the Falcon tubes and transferred into glass vials. A TurboVap LV (Biotage, Charlotte, NC) was used to exchange the acetonitrile solvent to methanol and was evaporated to 2 mL with nitrogen at 35 °C. The sample in the 2 mL vials was purified using a Supelco Supelclean ENVI-Carb (Supelco, Bellefonte, PA) solid phase extraction cartridge. The extracts were then evaporated to 1 mL under nitrogen gas in the TurboVap LV. At 1 mL, the samples were added to autosampler vials and analyzed for PFAS using LC-MS/MS. The analysis of samples was conducted using an Agilent 1100 HPLC (Santa Clara, CA) coupled with an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Each sample was injected onto a Kinetex PFP LC column (50 mm x 3 mm, 2.6 μm; Phenomenex, Torrance, CA). The autosampler temperature was 18 °C. The PFAS were separated using a flow rate of 150 μL/min and a gradient elution scheme (Supplemental Table 2) with mobile phase [A] 20 mmol ammonium acetate in methanol and [B] 20 mmol ammonium acetate in water. Multiple-reaction monitoring (MRM) transitions were used to detect and quantify PFAS, one transition for the quantitation of each PFAS and one transition for validation to confirm identification (49).

Quantification

The data produced by the LC-MS/MS was processed for quantitation using Analyst 1.6.2 software. Each PFAS was quantified using a linear equation of the calibration curve. The method detection limits (MDL) were calculated as either the maximum value of the average mass in the extract (ng) plus three times the standard deviation of blanks, divided by the mass of the sample (g) or the lowest calibrant detected, divided by the mass of the sample. The concentrations determined include branched and linear isomers.

Statistical Analysis

IBM SPSS statistical software (version 23) and JMP (12.1.0) were used to identify and investigate correlations between PFAS levels and site differences. Normality was tested in the data by utilizing the Kolmogorov-Smirnov test, in which the Lilliefors correction was implemented as provided by SPSS. Both Pearson and Spearman methods were used to determine correlations in normal (PFNA, PFOS, PFDA, PFUnA, PFOSA, and PFDoA) and non-normal data (PFHxS values), respectively. Kruskal-Wallis testing was utilized to compare differences in PFAS levels among sites that had detections in more than 60 % of the samples, and a Dunn all pairs posthoc was conducted on identified significant relationships.

For the Kruskal-Wallis, samples that were below the limit of detection were reassigned a value of zero to ensure that they would be tied in ranking statistical tests.

RESULTS AND DISCUSSION:

Each manatee plasma sample analyzed (n = 69) contained at least two PFAS of detectable abundance (PFNA and PFOS), consistent with a previous study done with American alligators from the Southeast US (47). Beyond the PFNA and PFOS burden, a number of individuals also had PFHxS, PFUnA and PFDA (percent frequency of detection, 94 %, 88 %, and 81 %, respectively, as shown in Table 1). Several PFAS, such as PFOSA and PFDoA, were infrequently measured, only detected in 6 % (n = 4 samples) and 19 % (n = 13 samples) of the total number of samples (n = 69) respectively (Table 1). The following PFAS were not detected in any of the manatee plasma samples: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFTriA, PFTA, and PFBS. Many of these congeners are characterized as short-chain perfluoroalkyl acids and have been found to be less bioaccumulative than long-chain analogues such as PFOS (14). Thus, their infrequency of detection in this particular study supports these prior results. However, the lack of PFOA in the manatee samples presents an interesting point of further investigation, due to its categorization as a long-chain perfluoroalkyl acid with a high tendency to bioaccumulate in other species (14). PFAS concentration values for each manatee are shown in Supplemental Table 3

PFOS was determined to be the highest PFAS in manatee plasma by concentration among all sites sampled, reaching a maximum of 166 ng/g wet mass for one manatee in Brevard County (Table 2 and Supplemental Table 3). Median PFAS values were also compared by site in which PFHxS, PFDA, PFOSA, and PFDoA display a median less than the method detection limit or zero (Table 2). Throughout all of the manatee samples, PFOS was the most abundant PFAS detected, comprising at least 75 % of each sites' overall PFAS burden (Supplemental Fig 4).

Surprisingly, PFAS burden in BC manatees is comparable to predatory mammals, despite their low status in the trophic hierarchy (Supplemental Table 4). Manatees were hypothesized to contain low concentrations of PFAS due to their lower trophic status and the fact they are primarily herbivorous (35). In comparison to another marine herbivore, the green sea turtle (*Chelonia mydas*), the BC manatees had a maximum PFOS concentration approximately 43 times greater ((50), Supplemental Table 4). In comparison to a marine omnivore, the leatherback sea turtle (*Dermochelys coriacea*), the BC manatees had a maximum PFOS level approximately 21 times larger (50). The maximum PFOS level of BC manatees were within the range (minimum and maximum range) of reports for predatory marine species, including juvenile Kemp's ridley sea turtles (*Lepidochelys kempii*), minke whales (*Balaenoptera acutorostrata*), and both adult and juvenile bottlenose dolphins (*Tursiops truncatus*), as shown in Supplemental Table 4 (9, 12, 49–51).

As this is the first study examining the PFAS burden in manatees, nothing is known about the health implications of the high PFAS levels in the BC manatees. Further, the exposure route of PFAS for manatees is also unclear, with current hypotheses centered on PFAS accumulation via diet (e.g., select vegetation through sediment) (52, 53). It has been

suggested that certain PFAS, like PFOS and PFDoA, have an ability to bind readily to sediment in aquatic environments and accumulate within underwater vegetation (54). This proposed pathway, along with our findings and the fact that manatees have a long-life span and high-volume diet of vegetation (consuming up to 10% of their body weight a day), suggests that this could be a previously unexplored route of PFAS exposure (29). Future investigations could examine the levels of PFAS in aquatic plant species, such as those consistent with manatee diets (e.g., seagrass), in order to fully understand the level at which herbivorous organisms are exposed via these dietary routes (36).

Site Correlations

As a result of their rather solitary living style, manatee movements tend to be individual based, with general migration patterns dependent on seasonal water temperature. However, once an individual range is established, the animal usually demonstrates high site fidelity and returns to sites previously visited during winter/summer seasons (30). With that knowledge, site-based differences were examined, with BC manatees demonstrating significantly higher levels of PFAS in comparison to the other sites sampled (Fig 1 and Table 2). Specifically, BC manatees had a statistically significant difference in PFOS and PFHxS concentration compared to all locations ($p < 0.001$ for both analytes), suggesting that the high concentrations observed at this site were most likely a reflection of a high-level site-specific point source of PFAS contamination. The BC manatees had approximately 6, 20, and 42 times more PFOS, when compared to CR, EP, and PR manatees, respectively. Additionally, PFNA was significantly higher in manatees from BC than CR or PR ($p = 0.025$ and $p = 0.008$ respectively). The BC manatees had approximately 2, 1.3, and 5 times more PFNA, when compared to CR, EP, and PR manatees, respectively. PFDA, though not detected in every sample, was significantly higher in BC manatees compared to PR manatees ($p = 0.009$). However, this difference in PFDA concentrations was not significant among the other sites and given the small sample size for the PR population, further investigation into site specific PFDA concentrations is warranted. There were no site specific significant differences in PFUnA. Interestingly, high levels of PFAS detected in American alligator (*Alligator mississippiensis*) plasma (compared to other Southeast US sites) have been previously reported in Brevard County (47, 55). A potential source of these chemicals is aqueous film-forming foam (AFFF) usage, which are commonly utilized in locations of fire-training and contain PFAS, such as PFOS and PFHxS (56), which notably are the two compounds at significantly higher concentrations among the manatees at BC compared to all other sites. Thus, the BC manatee population could be investigated further in relation to the potential health effects associated with this PFAS burden.

Morphometrics

Multiple studies have provided evidence for species-based differences in the correlation of sex and PFAS concentration among marine mammals and reptiles (8, 55, 57). The analysis of the manatee samples did not produce any significant correlation of PFAS concentration and sex. One possible reason why our study did not yield any sex-based differences with the manatee could be due to our smaller sample sizes distributed among the four locations, thus limiting the sample numbers needed to determine whether sex was a contributor to differences in PFAS abundance throughout the entire manatee sub-populations.

Other hematology and morphometric measurements were considered when analyzing the PFAS data, and included physical morphology (Supplemental Table 1), BCI, TP, and ALT (31–46, 58–60). Examining multiple measurements (straight and curved length, weight, and axial/max girth) with PFAS concentrations provided no significant correlations. Further, BCI was calculated for each manatee, using a previously outlined method, and also was found to contain no statistically significant relationship to PFAS burden (31). Hematology reports were not further investigated in this study, due to the small sample set and immediate focus on determining the presence of these chemicals in manatee species. Additional work should be conducted in order to investigate potential biomarkers for high PFAS burden.

CONCLUSIONS:

This study is the first to highlight the presence of PFAS in an herbivorous marine mammal, the West Indian manatee. Correlations between specific hematology profiles and other health metrics may be investigated further to determine potential biomarkers of health effects from PFAS exposure in manatees. Sites, such as those within Brevard County, Florida, can also be investigated further, due to the elevated levels of PFAS detected in the manatees and other reported species. Additional research about the prevalence and bioaccumulation mechanisms of these chemicals in water, sediments, and plants could also be useful for obtaining a better understanding of their environmental impacts. Knowledge of chemical contaminant impact on manatees, inclusive of PFAS, may have the potential to be used to help improve the protection of sirenian species and further the biochemical understanding of PFAS toxicity mechanisms in wildlife.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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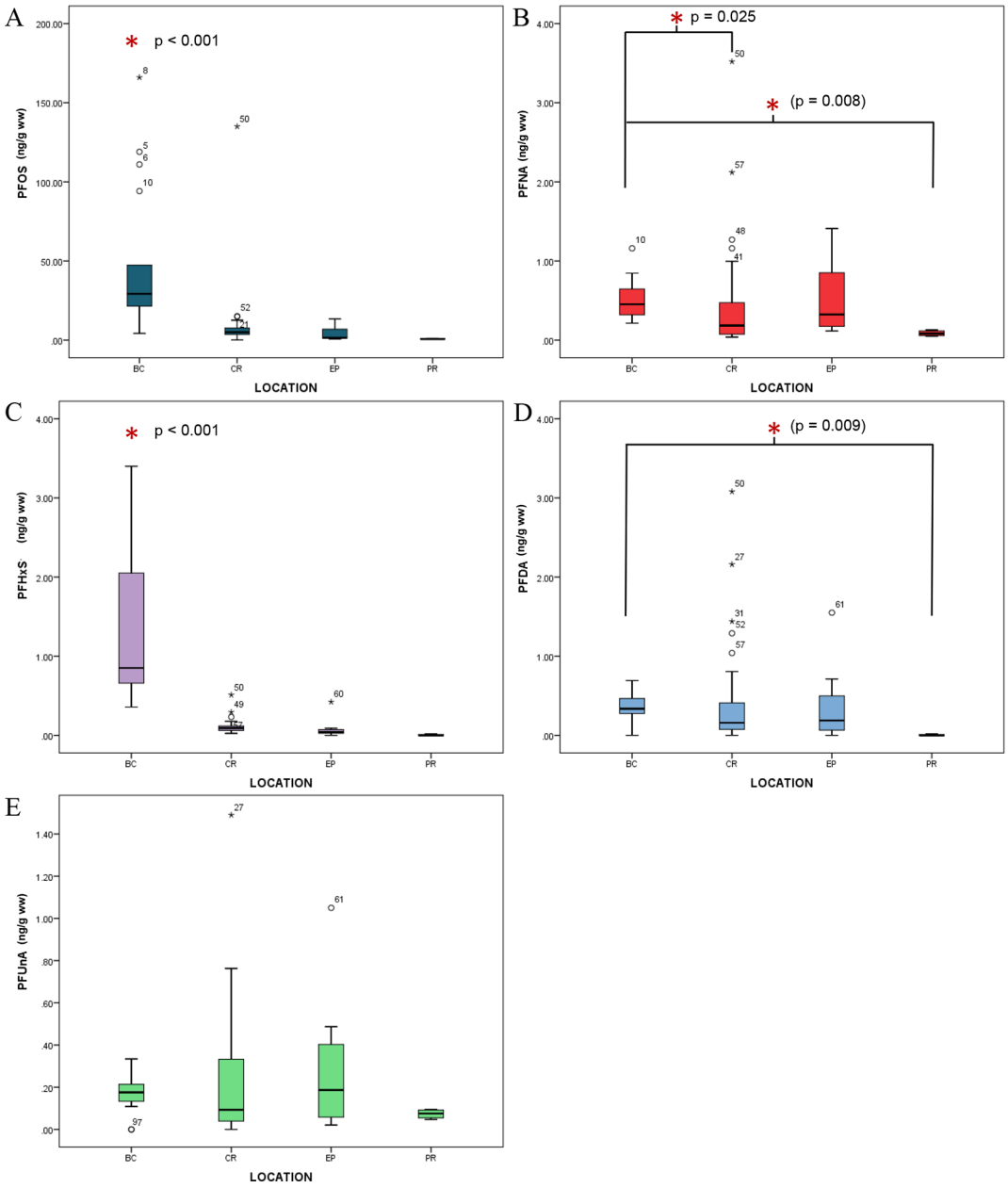

Fig 1.

Site differences in concentrations (ng/g ww) of PFAS in manatee blood. Results of Kruskal-Wallis. Asterisk represents significant differences from Dunn all pairs posthoc with significant p-value adjacent. (A) PFOS ($p < 0.001$); (B) PFNA ($p = 0.002$); (C) PFHxS ($p < 0.001$); (D) PFDA ($p = 0.013$); (E) PFUnA ($p = 0.361$).

Table 1.

Summary statistics of PFAS detected above method detection limit (MDL). Concentrations are in ng/g (wet weight).

	PFHxS	PFNA	PFOS	PFDA	PFUnA	PFOSA	PFDoA
Detected	65	69	69	56	61	4.0	13
% > MDL	94	100	100	81	88	6.0	19
Median	0.105	0.233	5.41	0.228	0.123	0.0985	0.0370
Min	< 0.012	0.038	0.13	< 0.009	< 0.010	< 0.011	< 0.009
Max	3.40	3.52	166	3.08	1.49	0.123	0.0950

The total number of samples, n = 69. “Detected” indicates the number of manatees with a concentration for the PFAS above the method detection limit (MDL). “<” signifies the MDL. Wet weight refers to the weight of material inclusive of the presence of water.

Table 2.

PFAS median (ng/g ww) and range for all PFAS measured in manatees across sites

Location		PFHxS	PFNA	PFOS	PFDA	PFUnA	PFOSA	PFDoA
BC (*n* = 18)	% of samples detected in	100	100	100	100	100	0.05	0.22
	Median	0.852	0.453	29.3	0.336	0.176	0.0320	0.0235
	Range	3.04	0.950	162	0.690	0.330	0	0.0400
CR (*n* = 39)	% of samples detected in	100	100	100	100	100	0.02	0.18
	Median	0.0920	0.185	5.05	0.159	0.0930	0.0990	0.0370
	Range	0.490	3.48	135	3.08	1.49	0	0.130
EP (*n* = 8)	% of samples detected in	100	100	100	100	100	0.25	0.25
	Median	0.0405	0.325	1.49	0.188	0.187	0.111	0.0660
	Range	0.420	1.29	12.8	1.55	1.03	0.0200	0.0600
PR (*n* = 4)	% of samples detected in	100	100	100	100	100	0	0
	Median	0	0.0845	0.698	0	0.0755	-	-
	Range	0.020	0.080	0.56	0.020	0.050	-	-

(Brevard County- BC, Crystal River- CR, Everglades National Park- EP, Puerto Rico- PR), n = number of samples the specific PFAS was detected in for each site. Total sample sizes for each site were BC (*n* = 18), CR (*n* = 39), EP (*n* = 8), and PR (*n* = 4).