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Contents

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Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of manmade chemicals that have been widely used in a variety of consumer and industrial products, including, for example, non-stick cookware, waterproof clothing, and firefighting foam. These chemicals are highly resistant to degradation and once in the environment, they have a high potential to bioaccumulate in mammals and other wildlife. Studies have linked PFAS exposure to cancer, and hormonal and immune system disorders in many species [1,2]. Two of the most well-known and studied PFAS groups of chemicals are perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA).

There is ongoing research into the harmful effects of PFAS contamination in the environment including, the development of methods to detect and measure PFAS [3] and explore effective remediation treatments for environmental PFAS contamination [1].

Overall, environmental PFAS contamination is a complex and challenging issue that requires further research and careful consideration; therefore, it is important to continue studying these chemicals and developing strategies to detect and address their presence worldwide.

In this article collection, we highlight studies focusing on the performance of US Environmental Protection Agency methods for the analysis of PFAS in water, and the consequences of PFAS present in species of shearwater fledgling, American alligators, and European pond turtles.

By Cecilia Kruszynski De Assis, Editor

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EMERGING CONTAMINANT ARTICLE

Topical Collection on PFAS Analytics and Treatment

AWWA WATER SCIENCE

An interlaboratory study on EPA methods 537.1 and 533 for per- and polyfluoroalkyl substance analyses

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Abstract

The US Environmental Protection Agency (EPA) Methods 537.1 and 533 were developed for analysis of per- and polyfluoroalkyl substances (PFAS) in drinking water. They have been also widely used for source water assessments. However, there are few studies reportedly supporting such applications. The main purpose of this interlaboratory study was to evaluate the performance of these two methods for use with both potable and nonpotable waters. The obtained matrix spike recoveries indicate that both methods are generally applicable for analysis of PFAS in pristine nonpotable water matrices, however, with a notable challenge for effectively extracting long-chain PFAS from some nonpotable water matrices. Another challenge associated with EPA Method 533 is the impacts likely caused by co-extracted common inorganic anions on those PFAS that do not have their own isotopically labeled analogues available. The experimental results indicate that these challenges can be successfully resolved or reduced by enhancing postextraction bottle rinsing and elution procedures.

KEYWORDS

EPA 533, EPA 537.1, PFAS interlaboratory study, potable and nonpotable water

1 | INTRODUCTION

Human exposure to per- and polyfluoroalkyl substances (PFAS) is a worldwide public health issue (Lindstrom, Strynar, & Libelo, 2011; Paul et al., 2009). Reports have estimated that over 3,000 PFAS exist on the global market and have been used in a wide variety of industrial and consumer applications (Wang et al., 2017). Concerns over PFAS contamination have increased substantially through the years due to increased occurrence assessments and studies reporting PFAS detected in global water sources and finished drinking water (DW) (Ahrens, 2011; Crone et al., 2019; Gebbink et al., 2017; Mak et al., 2009). Recent discoveries of legacy and emerging PFAS identified in water sources related to discharges of PFAS manufacturing facilities have greatly increased concerns about the use of fluoroalkyl ether substances as alternatives for long-chain legacy PFAS (Newton et al., 2017; Strynar et al., 2015; Sun et al., 2016; Wang et al., 2013).

In the United States, a national DW occurrence assessment of six PFAS was conducted under the Third Unregulated Contaminant Monitoring Rule (UCMR 3) (USEPA, 2017a), 29 PFAS have been included in the recently proposed fifth round of UCMR (USEPA, 2019a), and the US Environmental Protection Agency (EPA) established the DW lifetime health advisory levels at 70 ng/L for perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) (USEPA, 2016). At the state levels, the pace of action on PFAS issues varies greatly from state to state. Several states have established,

recommended, or proposed DW compliance standards (e.g., maximum contaminant levels, notification levels, human health–based values, trigger levels, or action levels) for individual PFAS or as sums of selected PFAS. A few other states have also developed statewide action plans. Source water evaluation for PFAS is also a current topic (AWWA, 2020; Nakayama et al., 2010; Schultz et al., 2004). DW supplies have become increasingly vulnerable to PFAS contamination from industrial discharges (Hu et al., 2016; Nakayama et al., 2007), firefighting activities (Backe et al., 2013; Place & Field, 2012), wastewater (WW) discharges (Schultz et al., 2006; Zareitalabad et al., 2013), landfill leachates (Huset et al., 2011), biosolids used as fertilizer (Lindstrom, Strynar, Delinsky, et al., 2011; Washington et al., 2010), or air emissions (Stoiber et al., 2020).

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is the primary analytical technique used for PFAS analyses (Amin et al., 2020; de Voogt & Saez, 2006; Munoz et al., 2019). Direct injection LC/MS/MS is applicable for a wide range of PFAS at part-per trillion concentrations (USEPA, 2019b; Zintek, 2017). Lower limits of quantitation (LOQ) can be achieved by combining LC/MS/MS with solid phase extraction (SPE). However, reversed-phase sorbents are generally less effective in retaining more acidic short-chain PFAS (Shoemaker et al., 2009; Shoemaker & Tettenhorst, 2018; Shoemaker & Tettenhorst, 2020). On the contrary, weak anion exchange (WAX) sorbents are relatively less effective in retaining less acidic long-chain and nonionic PFAS (Rosenblum & Wendelken, 2019).

EPA Methods 537.1 and 533 are two well-established DW methods using LC/MS/MS combined with reversed-phase and WAX SPE, respectively. These methods are also often used to analyze PFAS in nonpotable waters, including surface water (SW), groundwater (GW), and treated WW. However, there are few studies supporting such applications. Therefore, there is a need for investigating the performance and challenges of these two DW methods for use with various nonpotable water matrices. In this work, four Eurofins laboratories conducted a round-robin study on EPA Methods 537.1 and 533 for PFAS in both potable and nonpotable water matrices. The performance of these two methods was evaluated by measuring the native concentrations of PFAS in the selected water matrices and the matrix spike (MS) recoveries. The results indicate that both methods are generally applicable for pristine SW, GW, and treated WW. However, specific nonpotable water matrices may pose a challenge for meeting the quality control acceptance criteria as described in the methods. This may be successfully resolved or reduced simply by enhancing the postextraction bottle rinsing and elution procedures.

Article Impact Statement
This study indicates that Environmental Protection Agency Methods 537.1 and 533 are applicable for pristine nonpotable water per- and polyfluoroalkyl substance analysis or source water assessment.

2 | MATERIALS AND METHODS

2.1 | Chemicals and standards

The PFASs standards used for the water matrix fortification were purchased from Wellington Laboratories (Guelph, ON, Canada) and Absolute Standards (Hamden, CT). All isotopically labeled PFAS analogues were purchased from Wellington Laboratories. The target PFAS, their associated abbreviations or acronyms, and minimum reporting levels (MRLs) from the four participating laboratories are included in Table 1. Purge-and-trap-grade methanol used for the PFAS standard dilution was purchased from Fisher Scientific (St. Louis, MO).

2.2 | Water matrices and sample collection

The water matrices used in this study included laboratory reagent water (RW), DW, GW, SW, and treated WW. The RW (18.2 MΩ-cm resistance) was collected from a Millipore Milli-Q Academic system (Bedford, MA) of Lab A. 250 mL RW and DW samples were directly collected into 8-oz high-density polyethylene (HDPE) bottles containing the appropriate amounts of preservatives, which were obtained from Environmental Sampling Supply (San Leandro, CA). DW1 was a local city tap water. DW2 was prepared from DW1 by adjusting the hardness with an appropriate amount of magnesium chloride. GW, SW, and WW samples were initially collected in 10-L low-density polyethylene cubitainers or 1-gal HDPE jugs, which contained no detectable PFAS of interest. GW1 was collected from a local GW well. GW2 was prepared from GW1 by adjusting the alkalinity with an appropriate amount of sodium bicarbonate. SW1 was collected from St. Joseph River (South Bend, IN). SW2 was collected from Pleasant Lake (Edwardsburg, MI). Both WW1 and WW2 were collected from two local WW treatment plant effluents, which were chlorinated and then dechlorinated before discharge. No prefiltration was performed for

TABLE 1 A summary of analytes and minimum reporting levels

Analyte	Abbreviation or Acronym	EPA Method 537.1/533 MRL (ng/L)			
		Lab A	Lab B	Lab C	Lab D
Perfluorobutanoic acid	PFBA	NA/2.0	NA/2.0	NA/2.0	NA/5.0
Perfluoropentanoic acid	PFPeA	NA/2.0	NA/2.0	NA/2.0	NA/2.0
Perfluorohexanoic acid	PFHxA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluoroheptanoic acid	PFHpA	2.0/2.0	2.0/2.0	2.0/4.0	2.0/2.0
Perfluorooctanoic acid	PFOA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluorononanoic acid	PFNA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluorodecanoic acid	PFDA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluoroundecanoic acid	PFUnA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluorododecanoic acid	PFDoA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluorotridecanoic acid	PFTrDA	2.0/NA	2.0/NA	2.0/NA	2.0/NA
Perfluorotetradecanoic acid	PFTeDA	2.0/NA	2.0/NA	2.0/NA	2.0/NA
Perfluorobutanesulfonic acid	PFBS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluoropentanesulfonic acid	PFPeS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluorohexanesulfonic acid	PFHxS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluoroheptanesulfonic acid	PFHpS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
Perfluorooctanesulfonic acid	PFOS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
N-ethyl perfluorooctanesulfonamidoacetic acid	NEtFOSAA	2.0/NA	2.0/NA	2.0/NA	2.0/NA
N-methyl perfluorooctanesulfonamidoacetic acid	NMeFOSAA	2.0/NA	2.0/NA	2.0/NA	2.0/NA
1H,1H,2H,2H- Perfluorohexanesulfonic acid	4:2 FTS	NA/2.0	NA/2.0	NA/2.0	NA/2.0
1H,1H,2H,2H- Perfluorooctanesulfonic acid	6:2 FTS	NA/2.0	NA/2.0	NA/2.0	NA/10
1H,1H,2H,2H- Perfluorodecanesulfonic acid	8:2 FTS	NA/2.0	NA/2.0	NA/2.0	NA/2.0
Perfluoro-3-methoxypropanoic acid	PFMPA	NA/2.0	NA/2.0	NA/2.0	NA/2.0
Perfluoro-4-methoxybutanoic acid	PFMBA	NA/2.0	NA/2.0	NA/2.0	NA/2.0
Perfluoro(2-ethoxyethanesulfonic acid	PFEESA	NA/2.0	NA/2.0	NA/2.0	NA/2.0
Nonafluoro-3,6-dioxaheptanoic acid	NFDHA	NA/2.0	NA/2.0	NA/2.0	NA/2.0
Hexafluoropropylene oxide dimer acid	HFPO-DA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
4,8-Dioxa-3H-perfluorononanoic acid	ADONA	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
9-chlorohexadecafluoro-3-oxanonane-1-sulfonic acid	9Cl-PF3ONS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0
11-chloroeicosafluoro-3-oxanonane-1-sulfonic acid	11Cl-PF3OUdS	2.0/2.0	2.0/2.0	2.0/2.0	2.0/2.0

Abbreviation: NA, not applicable.

these nonpotable water samples. 250 mL GW, SW, and WW samples were then poured into the 8-oz HDPE bottles. The unfortified bottles were used to measure the native concentrations of PFAS. The fortified bottles were used to measure the recoveries of PFAS. DW1, DW2, GW1, GW2, SW1, and SW2 were fortified with all PFAS at 10 ng/L. WW1 and WW2 were fortified with all PFAS at 50 ng/L. RW1 fortified with all PFAS at 2 ng/L was used to evaluate the performance of quality control samples close to the LOQ. RW2 fortified with all PFAS at 50 ng/L was used as a reference to compare with the other water matrices. All water samples were shipped, received, and stored as described in EPA Methods 537.1 and 533.

As shown in Table 2, the pH, free chlorine, total organic carbon (TOC), total alkalinity, hardness, nitrate, and heterotrophic plate count (HPC) of these samples were measured by using EPA Method 150.1, SM 4500-Cl G, SM 5310 C, SM 2320 B, SM 2340 B, EPA Method 353.2, and SM 9215 E (SimPlate), respectively. The water hardness was calculated by SM 2340 B based on the concentrations of Ca and Mg cations measured by using EPA Method 200.7. Both chloride and sulfate were measured by using EPA Method 300.0.

TABLE 2 A summary of water quality parameters of studied water matrices

Matrix	DW1	DW2	GW1	GW2	SW1	SW2	WW1	WW2
pH	6.8	7.2	7.3	7.4	7.3	6.7	7.3	7.2
Free chlorine (mg/L)	0.86	0.86	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
TOC (mg/L)	0.516	0.516	0.858	0.427	3.13	5.84	2.44	3.71
Total hardness as $CaCO_3$ (mg/L)	401	563	257	256	278	122	412	324
Total alkalinity as $CaCO_3$ (mg/L)	276	275	208	320	214	109	276	213
Chloride (mg/L)	147	289	37.8	37.4	33.2	12.9	239	182
Sulfate (mg/L)	59.7	59.6	27.6	26.7	39.2	4.6	93.8	53.5
Nitrate as nitrogen (mg/L)	<1.0	<1.0	1.69	2.12	1.84	<1.0	11.0	13.2
HPC (MPN/mL)	NA	NA	311	372	650	1000	440	623

Abbreviations: DW, drinking water; GW, groundwater; HPC, heterotrophic plate count; NA, not available; SW, surface water; TOC, total organic carbon; WW, wastewater.

2.3 | Sample extraction and analysis

EPA Methods 537.1 and 533 were performed for all 29 PFAS without modification. All participating laboratories fully complied with the requirements for calibration standards and curve fits, quality control types and acceptance criteria, SPE procedures, and extraction/analysis batches as specified in the two reference methods. For EPA Method 533, all laboratories used 33 µm Strata X-AW mixed-mode polymeric WAX cartridges (500 mg/6 ml) obtained from Phenomenex (Torrance, CA). For EPA Method 537.1, Labs A, B, and C used 100 µm Strata SDBL polymeric styrenedivinylbenzene cartridges (500 mg/6 ml) obtained from Phenomenex. Lab D used 45 µm Mega Bond Elut Plexa cartridges (500 mg/6 ml) obtained from Agilent Technologies Inc. (Lake Forest, CA). Upon receipt, 250 mL properly preserved samples were fortified with surrogate standards (SS) for EPA Method 537.1 or isotope dilution analogues (IDAs) for EPA Method 533 and then extracted using the SPE cartridges without performing prefiltration. Prior to the LC/MS/MS analysis, the extracts were evaporated to dryness, reconstituted with proper solvents to 1.0 ml, and then fortified with proper internal standards (IS) for EPA Method 537.1 or isotope performance standards (IPS) for EPA Method 533. The PFAS were separated on the C18 columns using a gradient mobile phase of 20 mM ammonium acetate and methanol and then detected by negative electrospray ionization LC/MS/MS in the multiple-reaction monitoring mode.

The PFAS were quantitated using a minimum of five calibration standards for a nonprocedural linear curve or six calibration standards for a nonprocedural quadratic curve. EPA Method 537.1 calibration curves using the IS were in concentration ranges of 2.0–250 ng/L, 2.0–80 ng/L, 1.0–400 ng/L, and 2.0–80 ng/L for Labs A, B, C, and D, respectively. EPA Method 533 calibration curves using the IDAs were in concentration ranges of 2.0–500 ng/L, 2.0–80 ng/L, 1.0–400 ng/L, and 2.0–100 ng/L for Labs A, B, C, and D, respectively. The extracted SS and IDAs were quantitated using multiple calibration points at the same concentration level against the IS and IPS, respectively. The IS were evaluated by comparing with their average peak areas in the initial calibration and the peak areas from the most recent continuing calibration check. The IPS were evaluated by comparing with their average peak areas in the initial calibration. The percent recoveries of the PFAS, SS, and IDAs were calculated by comparing with the true fortified values. The initial method detection limits (MDLs) were determined using the latest 40 CFR Part 136 Appendix B protocol (USEPA, 2017b). The ongoing MDLs were determined following the 2016 TNI Standard (NELAC Institute, 2016). As shown in Table S1 in Appendix S1, the MDLs of less than 0.67 ng/L (1/3 the MRL of 2.0 ng/L) were obtained for all PFAS for EPA Methods 537.1 and 533 with the following exceptions: Lab C had EPA Method 533 MDLs of 0.91, 0.68, 1.0, and 0.74 ng/L for PFBA, PFPeA, PFHpA, and PFOA, respectively. Lab D had nominal EPA Method 533 MDLs of 2.0 ng/L for both PFBA and 6:2 FTS.

3 | RESULTS AND DISCUSSION

3.1 | Native concentrations of PFAS

As shown in Table S2 in Appendix S1, Labs A and B reported that all PFAS in the RW samples were detected at concentrations less than one-third the MRL of 2.0 ng/L. Labs C and D reported all PFAS as not detected, based on their data processing settings. Table 3 indicates the mean native concentrations with standard deviations

TABLE 3 Native concentrations of PFAS resulting from EPA Methods 537.1 and 533

	Mean concentration ± SD (ng/L)							
Analyte	**DW1**	**DW2**	**GW1**	**GW2**	**SW1**	**SW2**	**WW1**	**WW2**
EPA 537.1 results								
PFBS	2.48 ± 0.17	2.53 ± 0.19	5.53 ± 0.25	5.53 ± 0.35	1.4 ± 0.8[a]	0.9 ± 0.5[a]	3.53 ± 0.71	3.99 ± 1.54
PFHxA	1.6 ± 0.9[a]	2.10 ± 0.24	0.7 ± 0.4[a]	0.8 ± 0.5[a]	1.0 ± 0.6[a]	1.1 ± 0.7[a]	14.7 ± 0.8	15.5 ± 1.1
PFHpA	1.5 ± 0.9[a]	1.5 ± 0.9[a]	ND	ND	ND	1.1 ± 0.6[a]	1.98 ± 0.17	1.5 ± 0.3[a]
PFHxS	ND	1.0 ± 0.6[a]	0.8 ± 0.5[a]	0.7 ± 0.5[a]	0.9 ± 0.6[a]	ND	3.19 ± 0.32	5.91 ± 0.66
PFOA	9.70 ± 0.77	10.1 ± 0.6	2.84 ± 0.18	2.85 ± 0.19	1.0 ± 0.6[a]	2.74 ± 0.16	4.55 ± 0.49	3.94 ± 0.33
PFOS	ND	ND	0.7 ± 0.6[a]	0.7 ± 0.6[a]	1.2 ± 0.9[a]	1.1 ± 0.8[a]	3.09 ± 0.86	9.35 ± 1.07
PFNA	ND	ND	ND	ND	ND	ND	0.67 ± 0.30	ND
EPA 533 results								
PFBA	3.2 ± 0.3[b]	3.2 ± 0.3[b]	4.5 ± 0.3[b]	4.6 ± 0.5[b]	2.0 ± 0.3[b]	2.8 ± 0.5[b]	6.89 ± 1.80	7.80 ± 2.92
PFPeA	1.7 ± 0.1[a]	1.7 ± 0.2[a]	0.9 ± 0.1[a]	0.8 ± 0.1[a]	1.4 ± 0.3[a]	1.4 ± 0.3[a]	20.3 ± 1.7	15.4 ± 1.2
PFBS	2.44 ± 0.11	2.41 ± 0.18	5.36 ± 0.35	5.23 ± 0.45	1.3 ± 0.7[a]	0.9 ± 0.5[a]	3.48 ± 0.34	4.04 ± 0.60
PFHxA	2.00 ± 0.12	1.99 ± 0.24	1.0 ± 0.1[a]	0.9 ± 0.1[a]	0.9 ± 0.6[a]	1.0 ± 0.6[a]	14.9 ± 1.7	15.9 ± 1.7
PFHpA	1.8 ± 0.2[a]	1.9 ± 0.2[a]	1.0 ± 0.1[a]	0.8 ± 0.1[a]	ND	1.1 ± 0.2[a]	1.8 ± 0.2[a]	1.5 ± 0.3[a]
PFHxS	1.2 ± 0.1[a]	1.2 ± 0.1[a]	0.8 ± 0.1[a]	0.9 ± 0.1[a]	0.7 ± 0.4[a]	ND	2.88 ± 0.36	5.51 ± 0.64
PFOA	9.63 ± 0.79	9.43 ± 0.71	3.00 ± 0.33	2.79 ± 0.17	0.8 ± 0.5[a]	1.9 ± 1.1[a]	4.03 ± 0.52	3.80 ± 0.60
PFNA	ND	ND	ND	ND	ND	0.7 ± 0.5[a]	ND	ND
PFOS	ND	ND	ND	ND	1.1 ± 0.7[a]	1.0 ± 0.6[a]	2.49 ± 0.29	9.60 ± 0.57

Abbreviations: DW, drinking water; GW, groundwater; ND, not detected; PFBA, perfluorobutanoic acid; PFBS, perfluorobutanesulfonic acid; PFHpA, perfluoroheptanoic acid; PFHpA, perfluoroheptanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeA, perfluoropentanoic acid; SW, surface water; WW, wastewater.

[a]Estimated results of less than the minimum reporting level (MRL) of 2.0 ng/L.

[b]Estimated results of less than Lab D's MRL of 5.0 ng/L.

(SDs) of PFAS measured by the four participating laboratories, which were based on eight replicate samples (i.e., two replicate unfortified samples were run by each laboratory), with an exception of GW1. EPA Method 533 results for GW1 were based on six replicate unfortified samples run by three participating laboratories. The results measured between the MRLs and one-third of the MRLs were included as estimates. Table 3 does not include the analytes, which were not detected by any of the four participating laboratories. As shown in Table 3, the native concentrations resulting from both EPA Methods 537.1 and 533 were generally consistent for the potable and nonpotable water matrices. All four participating laboratories achieved relatively low SDs for all PFAS measured at or greater than the MRLs. In addition, the obtained relative percent differences (RPDs) between the two methods were less than 20% for all PFAS at or greater than the MRLs with a couple of exceptions. At the native concentrations close to the MRL of 2.0 ng/L, the RPDs were 24% for PFHxA in DW1 and 21% for PFOS in WW1, respectively.

3.2 | Accuracy and precision of PFAS analyses

Four MS replicates were extracted and analyzed by each participating laboratory to demonstrate the accuracy and precision. The obtained percent recoveries and trending from all four laboratories were very similar, which can be found in Appendix S1. The overall mean recoveries were calculated from all 16 MS replicates analyzed by the four laboratories. Figures 1 and 2 represent the overall mean MS recoveries of the analytes and SS for EPA Method 537.1, respectively. Figures 3 and 4 represent the overall mean MS recoveries of the analytes and IDAs for EPA Method 533, respectively.

As shown in Figure 1, the obtained mean recoveries of all EPA Method 537.1 analytes were within 80%–120% for the RW, DW, and GW matrices. The mean recoveries for PFAS eluting before NMeFOSAA were within 84%–116% for the SW and treated WW matrices. It is notable that the mean recoveries gradually decreased with the increase in carbon chain lengths or retention time for

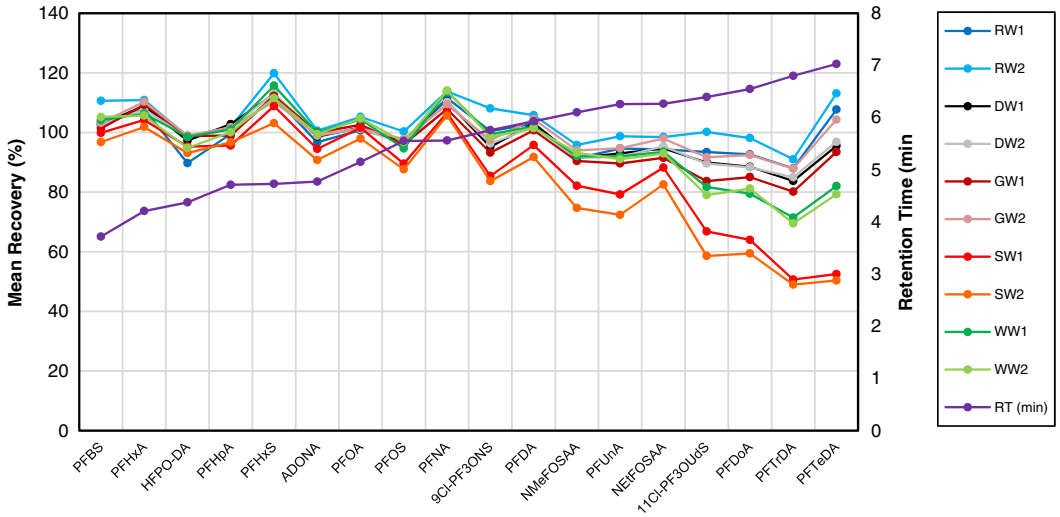

FIGURE 1 Mean recoveries of EPA Method 537.1 analytes resulting from the four participating laboratories, based on 16 matrix spike replicates. The fortification concentrations were 2 ng/L for RW1; 10 ng/L for DW1, DW2, GW1, GW2, SW1, and SW2; and 50 ng/L for RW2, WW1, and WW2, respectively. DW, drinking water; GW, groundwater; RW, reagent water; SW, surface water; WW, wastewater

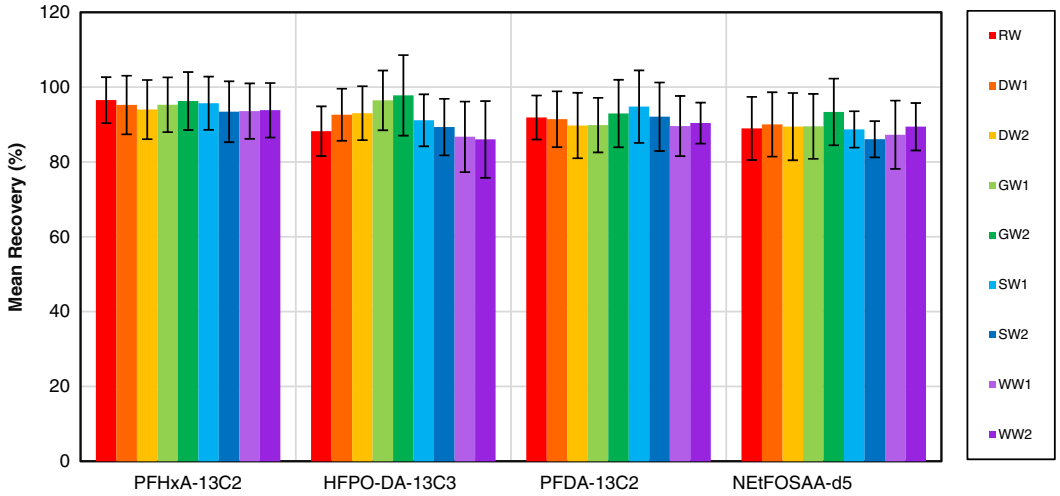

FIGURE 2 Mean recoveries with positive/negative percent relative standard deviations of EPA Method 537.1 surrogate standards resulting from the four participating laboratories, based on 40 reagent water (RW) replicates (32 fortified blanks and 8 unfortified RW samples) and 24 replicates for the other water matrices (16 fortified matrix spikes and 8 unfortified native water samples). The fortification concentrations were 40 ng/L for HFPO-DA-$^{13}C_3$, PFHxA-$^{13}C_2$, and PFDA-$^{13}C_2$; and 160 ng/L for NEtFOSAA-d_5, respectively. DW, drinking water; GW, groundwater; SW, surface water; WW, wastewater

long-chain PFAS. From PFDA to PFTeDA, the mean recoveries varied from 102% to 82% for WW1, from 101% to 79% for WW2, from 96% to 53% for SW1, and from 92% to 50% for SW2, respectively.

Figure 1 indicates that common inorganic water quality parameters (e.g., total hardness, total alkalinity, and common anion concentrations as shown in Table 1) might not significantly interfere with recoveries of PFAS, including late-eluting 11Cl-PF3OUdS, PFDoA, PFTrDA, and PFTeDA. The decreased recoveries of these PFAS for the SW and treated WW matrices might be due to their surface adsorption enhanced by the presence of relatively high HPC and/or TOC concentrations. A hypothesis is that these long-chain PFAS could conjugate with large organic molecules, biological matter, and/or porous debris likely present in the SW matrices. The conjugation might create a co-adsorption effect, which could make the extraction, bottle rinsing, and elution more challenging.

Figure 2 indicates that the mean MS recoveries of all four SS were within 86%–98% with percent relative standard deviations (RSDs) of 4.8%–10.8% for all the studied matrices. However, the results also indicate that the

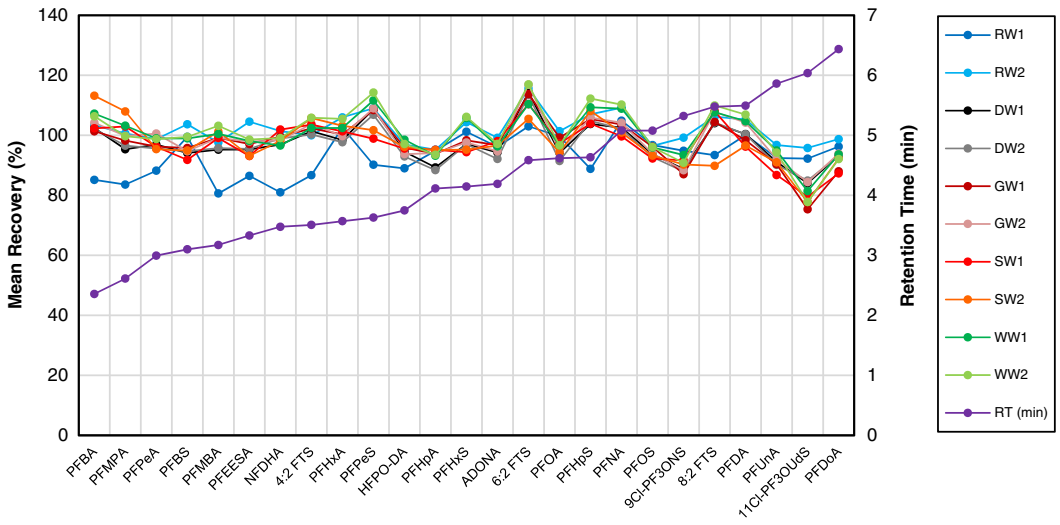

FIGURE 3 Mean recoveries of EPA Method 533 analytes resulting from the four participating laboratories, based on 16 matrix spike replicates. The fortification concentrations were 2 ng/L for RW1; 10 ng/L for DW1, DW2, GW1, GW2, SW1, and SW2; and 50 ng/L for RW2, WW1, and WW2, respectively. DW, drinking water; GW, groundwater; RW, reagent water; SW, surface water; WW, wastewater

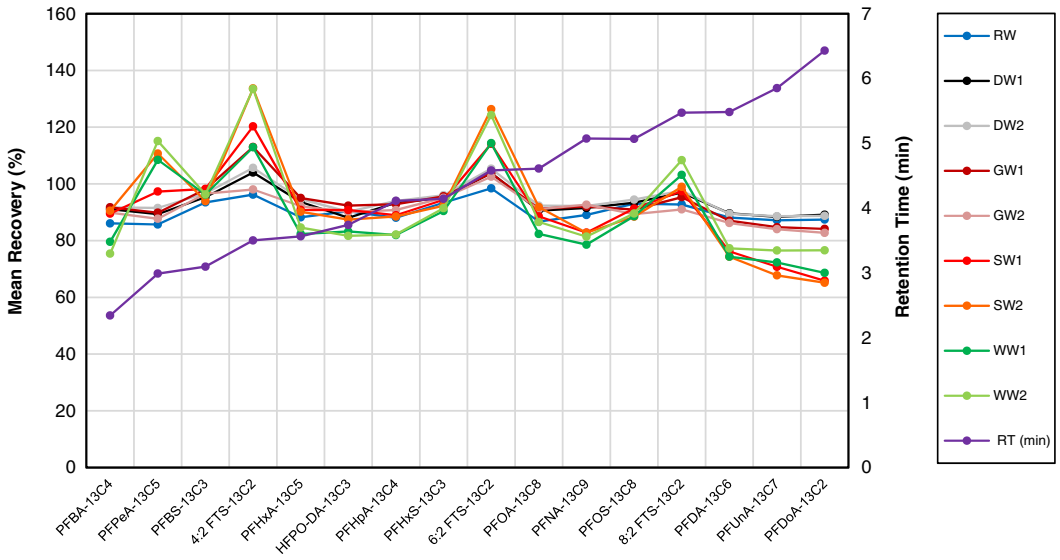

FIGURE 4 Mean recoveries of EPA Method 533 isotope dilution analogues resulting from the four participating laboratories, based on 40 reagent water (RW) replicates (32 fortified blanks and 8 unfortified RW samples) and 24 replicates for the other water matrices (16 fortified matrix spikes and 8 unfortified native water samples). The fortification concentrations were 160 ng/L for 4:2 FTS-$^{13}C_2$, 6:2 FTS-$^{13}C_2$, and 8:2 FTS-$^{13}C_2$; and 40 ng/L for the other isotope dilution analogues, respectively. DW, drinking water; GW, groundwater; SW, surface water; WW, wastewater

latest-eluting surrogate NEtFOSAA-d_5, passing the acceptance criteria of 70%–130% recoveries, does not necessarily predict good extraction efficiency for long-chain PFAS typically eluting after NEtFOSAA or NEtFOSAA-d_5 in challenging nonpotable water matrices like SW1 and SW2. On the other hand, the SS falling below the acceptance criteria will clearly indicate the likelihood of significantly low biased results for the long-chain PFAS.

Figure 3 indicates that the mean MS recoveries of EPA Method 533 analytes did not obviously decrease with the increase in carbon chain lengths for long-chain PFAS. All the analytes were measured with mean recoveries at 75%–117% for all the studied water matrices. It is worth mentioning that EPA Method 533 did not include NMeFOSAA, NEtFOSAA, PFTrDA, and PFTeDA, possibly due to the relatively low extraction recoveries, poor precision, and/or the solubility concern of PFTrDA and

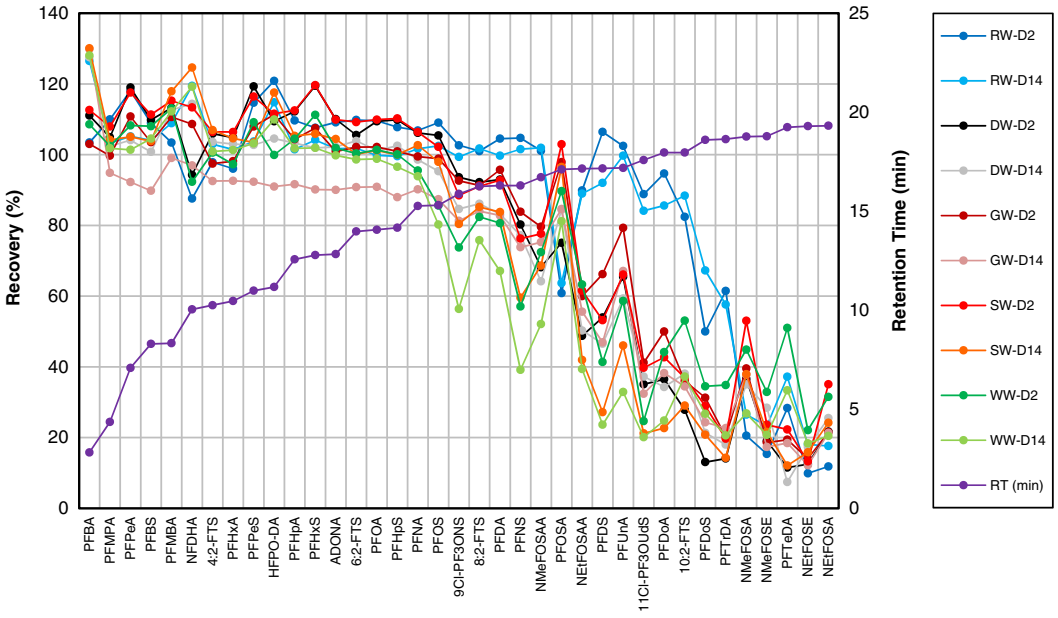

FIGURE 5 Recoveries of per- and polyfluoroalkyl substances fortified into 8-oz high-density polyethylene sample bottles at 400 ng/L and stored at refrigeration temperature (1–6 °C). DW, drinking water; GW, groundwater; RW, reagent water; SW, surface water; WW, wastewater

PFTeDA in the 80:20 volumetric ratio of methanol/water solvent. As shown in Figure 4, all IDAs were measured with the mean recoveries of 65%–134% for all studied water matrices, which were well within the acceptable limits of 50%–200% as described in the method. Similar to Figure 1, the obtained mean recoveries of all EPA Method 533 IDAs were within 83%–113% for the studied RW, DW, and GW matrices. The mean recoveries of 79%–134% were also obtained for the IDAs eluting before PFDA-$^{13}C_6$. It is also notable that the mean recoveries slightly decreased with the increase in carbon chain lengths for long-chain IDAs for the SW and treated WW matrices. From 8:2 FTS-$^{13}C_2$ to PFDoA-$^{13}C_2$, the mean recoveries varied from 103% to 69% for WW1, from 108% to 77% for WW2, from 97% to 66% for SW1, and from 99% to 65% for SW2, respectively. Similarly, the decreased IDA recoveries might be due to their surface adsorption enhanced by the presence of relatively high HPC and/or TOC concentrations in these SW and treated WW matrices. Although the recoveries of some IDAs were slightly low-biased for these challenging matrices, the isotope dilution technique effectively compensated for the matrix interferences, as shown in Figure 3.

It is worth mentioning that it is critical to choose appropriate reference IDAs for those analytes that do not have their own isotopically labeled analogues available. The mean recoveries of early-eluting PFMPA shown in Figure 3 for WW1 and WW2 were quantitated against the IDAs PFPeA-$^{13}C_5$ (Labs A and B) and PFBA-$^{13}C_4$ (Labs C and D), respectively. For Lab A, PFMPA was initially measured with mean recoveries of 143% with RSD of 2.4% for WW1 and 188% with RSD of 1.6% for WW2, corresponding to the mean recoveries of IDA PFBA-$^{13}C_4$ at 65% with RSD of 2.6% and 68% with RSD of 1.9%, respectively. However, PFMPA was measured with mean recoveries of 89% with RSD of 1.6% for WW1 and 90% with RSD of 1.2% for WW2 when the corresponding IDA was switched to PFPeA-$^{13}C_5$. For Lab B, PFMPA was initially measured with mean recoveries of 152% with RSD of 1.6% for WW1 and 161% with RSD of 3.2% for WW2, corresponding to the mean recoveries of IDA PFBA-$^{13}C_4$ at 59% with RSD of 1.2% and 51% with RSD of 7.1%, respectively. However, PFMPA was measured with mean recoveries of 95% with RSD of 0.6% for WW1 and 85% with RSD of 2.8% for WW2 when the corresponding IDA was switched to PFPeA-$^{13}C_5$. As shown in Tables S7–S10 in Appendix S1, compared with the RW, DW, GW, and SW matrices, lower recoveries of IDA PFBA-$^{13}C_4$ were obtained from the treated WW matrices for all four laboratories. The co-extracted polar organic compounds and/or common anions from the WW matrices could co-elute with PFBA-$^{13}C_4$ and cause electrospray ionization suppression.

3.3 | Surface adsorption of PFAS

In a separate surface adsorption study, the same water matrices were collected in 8-oz HDPE bottles, fortified

with PFAS at 400 ng/L, and stored at refrigeration temperature (typically, 1–6 °C). An aliquot of the samples was then transferred into autosampler vials after storage of 2 and 14 days, mixed with an aliquot of ammonium acetate buffer, fortified with isotopically labeled PFAS analogues used as the IS, diluted with methanol in a 60:40 volumetric ratio of water to methanol, and then mixed well prior to the LC/MS/MS analysis. In order to match the adsorption losses in the autosampler vials and analytical system, the calibration standards were also prepared in 40% methanol in RW. This study also included several commonly analyzed PFAS additional to EPA Method 537.1 and EPA Method 533 analyte lists. The obtained results are similar to those resulting from the surface adsorption study on 8-oz high-density polypropylene bottles (Whitaker & Li, 2018). As shown in Figure 5, the recoveries of PFAS were measured as a reciprocal of surface adsorption losses. First of all, the trending variations of mean recoveries were not notably different from 2-day storage to 14-day storage. Secondly, in general, the recoveries of PFAS gradually decreased with the increase in carbon chain lengths or retention time for long-chain PFAS, which means that the surface adsorption of PFAS on the inner walls of the HDPE bottles generally increased with the increase in carbon chain lengths after PFNA. Thirdly, compared with the RW results, the recoveries of PFAS substantially decreased in the DW, GW, SW, and WW, particularly for PFAS between 9Cl-PF3ONS and PFTrDA except neutral PFOSA. This observation indicates that these water matrices could substantially enhance bottle surface adsorption, particularly for those acidic PFAS. However, the RW might result in slightly more bottle surface adsorption of neutral PFOSA than the DW, GW, SW, and WW matrices. Finally, for long-chain PFAS eluting after PFUnA, approximately 50% or more PFAS could be adsorbed on the inner walls of the HDPE bottles.

The obtained relatively low recoveries shown in Figures 1 and 3 of late-eluting EPA Method 537.1 analytes and EPA Method 533 IDAs are basically consistent with the PFAS recoveries shown in Figure 5. These results indicate that hydrolytic, photolytic, and/or biological degradations of environmentally persistent PFAS may be minimal. PFAS adsorption losses are a primary challenge for LC/MS/MS analyses combined with SPE because manual or automated SPE procedures normally involve more surface contact areas in addition to sample bottles. Because adsorption losses of long-chain PFAS are likely enhanced by water matrices (particularly for non-potable water samples containing high concentration of TOC, large organic molecules, biological matter, and porous debris), enhancing postextraction bottle rinsing and elution will become critical. In order to confirm this hypothesis, the participating Lab A repeated once the normal postextraction bottle rinsing and elution steps; i.e., doubled volumes of methanol were used in the postextraction bottle rinsing and elution steps. As shown in Figure 6, the mean recoveries of PFAS were significantly improved, increasing by 6%–30% for SW1 and by 4%–30% for SW2, depending on specific PFAS.

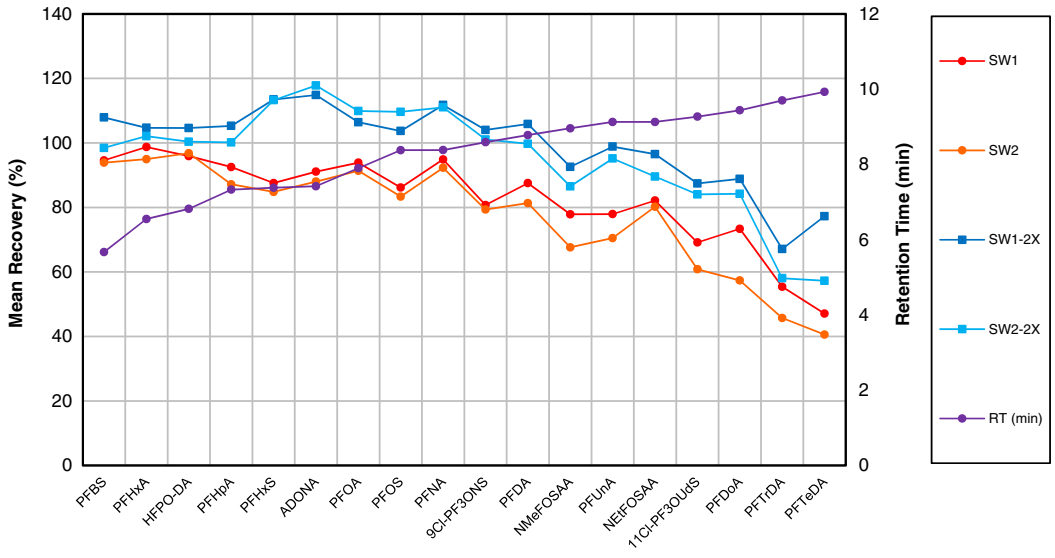

FIGURE 6 Mean recoveries of EPA Method 537.1 analytes, based on four matrix spike replicates fortified at 10 ng/L from Lab a. SW1 and SW2: Normal postextraction bottle rinsing and elution steps. SW1-2X and SW2-2X: Repeated the normal postextraction bottle rinsing and elution steps once. SW, surface water

4 | CONCLUSIONS

The experimental results have demonstrated that both EPA Methods 537.1 and 533 are accurate and precise DW methods. They may be likely applicable for pristine nonpotable water analyses. Bottle surface adsorption losses were demonstrated as a primary challenge for meeting the quality control acceptance criteria as described in the reference methods and for providing accurate analytical results, particularly for nonpotable water matrices such as SW in which high concentrations of TOC, large organic molecules, biological matter, and porous debris may be present. Slightly low biased recoveries (typically, 50%–69%) of late-eluting surrogate NEtFOSAA-d_5 are commonly associated with EPA Method 537.1, which may occur in a small fraction of potable and nonpotable samples. The experimental results indicate that this issue can be significantly reduced by using enhanced postextraction bottle rinsing and elution procedures. Compared with EPA Method 537.1, EPA Method 533 using isotope dilution analysis can tolerate more matrix interference and subsequently provide more accurate results. In addition to bottle surface adsorption losses of late-eluting PFAS, another notable challenge for EPA Method 533 is to choose appropriate IDAs for those analytes that do not have their own isotopically labeled analogues available. Ionization suppression likely resulting from co-extracted polar organic compounds and/or common inorganic anions can significantly affect the response factors between early-eluting analytes and the reference IDAs, which may be dependent on specific SPE procedures. Similar to EPA Method 537.1, enhancing the postextraction bottle rinsing and elution steps can also be expected to a good practice for EPA Method 533, possibly reducing the extraction of common inorganic anions and surface adsorption losses of late-eluting PFAS.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

AUTHOR CONTRIBUTIONS

Yongtao Li: Conceptualization; resources; data curation; formal analysis; supervision; funding acquisition; validation; investigation; visualization; methodology; writing - original draft; project administration; writing-review & editing. **Joshua S. Whitaker:** Conceptualization; data curation; validation; methodology; writing-review & editing. **Robert B. Hrabak:** Data curation; supervision; investigation; methodology; writing-review & editing. **Marnellie Ramos:** Data curation; supervision; investigation; methodology; writing-review & editing. **Charles Neslund:** Conceptualization; data curation; supervision; investigation; methodology; writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Environmental Toxicology

Correlations between Per- and Polyfluoroalkyl Substances and Body Morphometrics in Fledgling Shearwaters Impacted by Plastic Consumption from a Remote Pacific Island

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Abstract: We investigated the concentrations of 45 per- and polyfluoroalkyl substances (PFASs) in fledgling flesh-footed shearwater (*Ardenna carneipes*; $n = 33$) and wedge-tailed shearwater (*A. pacifica*; $n = 9$) livers via liquid chromatography–tandem mass spectrometry and their relationship to body morphometrics and ingested plastic mass recorded in 2019 on Lord Howe Island (NSW, Australia). Sixteen PFASs were detected, of which perfluorooctanesulfonate (PFOS) was the dominant compound, detected in 100% of birds (1.34–13.4 ng/g wet wt). Long-chain perfluorocarboxylic acids, including perfluorodecanoic acid (PFDA; <0.04–0.79 ng/g wet wt) and perfluorotridecanoic acid (PFTrDA; <0.05–1.6 ng/g wet wt) were detected in >50% of birds. There was a positive correlation between PFDA and PFTrDA concentrations and wing chord length ($R_s = 0.36$, $p = 0.0204$; $R_s = 0.44$, $p = 0.0037$, respectively), and between PFDA concentrations and total body mass ($R_s = 0.33$, $p = 0.032$), suggesting that these compounds may impact shearwater fledgling morphometrics. Plastic was present in the intestinal tract of 79% of individuals (<7.6 g), although there was no correlation between PFAS concentrations and plastic mass, indicating that ingested plastic is not the likely primary exposure source. The widespread occurrence of PFASs in fledgling marine birds from a relatively pristine location in the Southern Hemisphere suggests that further studies in adult shearwaters and other marine birds are warranted to investigate whether there are any long-term physiological effects on bird species. *Environ Toxicol Chem* 2021;40:799–810. © 2020 SETAC

Keywords: Per- and polyfluoroalkyl substance (PFAS); Persistent organic pollutants; Avian toxicity; Marine plastics; Contaminants of emerging concern

INTRODUCTION

Similar to the persistent organic pollutants (POPs) of the mid- to late-20th century, per- and polyfluoroalkyl substances (PFASs) are an emerging class of semivolatile and nonvolatile anthropogenic contaminants that may pose a threat to seabirds throughout the world, due to their capacity for long-range transport in the atmosphere and oceans (Armitage et al. 2009). In particular, oceanic transport plays an important role in the fate of nonvolatile compounds, because these compounds can bioaccumulate and biomagnify in biota, potentially leading to detrimental impacts (Butt et al. 2010). Furthermore, as plastics continue to accumulate in the oceans and are mistaken for food by marine birds (Battisti et al. 2019), the role of plastic ingestion as a vector for organic pollutant exposure, including PFASs, is not yet known.

Per- and polyfluoroalkyl substances are a family of >4700 compounds (Organisation for Economic Co-operation and Development 2018), many of which have been used widely as surfactants since the 1950s in aqueous film-forming foam (AFFF), nonstick cookware, and weather-proof textiles (Buck et al. 2012). Per- and polyfluoroalkyl substances are generally categorized according to their functional group and named by the number of carbon atoms. For example, perfluorocarboxylic acids (PFCAs; $CF_3(CF_2)_xCOOH$) and perfluorosulfonic acids (PFSAs; $CF_3(CF_2)_xSO_3H$) are the most widely studied PFAS groups, because they are the terminal, "forever" products of many PFAS precursors (Wang et al. 2017). Notably, the use of 3 of these compounds has been restricted or banned by the United

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DE09920182

Nations Environment Programme (UNEP) under the Stockholm Convention due to their persistence, bioaccumulation, and toxicity in biota: perfluorooctanesulfonic acid (PFOS; $C_8F_{17}SO_3H$), perfluorooctanoic acid (PFOA; $C_8F_{15}COOH$), and perfluorohexanesulfonic acid (PFHxS; $C_6F_{13}SO_3H$; United Nations Environment Programme 2019).

Per- and polyfluoroalkyl substances include precursor and/or intermediate compounds that contain one or more CH_2 moiety in the aliphatic chain and are increasingly replacing regulated compounds. Consequently, novel PFASs such as fluorotelomer carboxylic acids (FTCAs) and perfluoroalkyl sulfonyl fluorides (PASFs) are being increasingly detected in the environment, where they may pose a potential toxicological risk (Strynar et al. 2015; Barzen-Hanson et al. 2017). A potential source of FTCAs and PASFs may be the biotransformation of semivolatile PFASs, such as n:2 fluorotelomer alcohols (n:2 FTOHs; Butt et al. 2014), which are distributed via long-range atmospheric transport (Armitage et al. 2009).

Flesh-footed shearwaters (*Ardenna carneipes*) and wedge-tailed shearwaters (*A. pacifica*) are marine seabirds from the family Procellariidae. They are distributed in the Pacific and Indian Oceans, with the former also having a range in the Southern Ocean south of Australia (BirdLife International 2018, 2019). Flesh-footed shearwaters are categorized as "near-threatened" by the International Union for Conservation of Nature, whereas wedge-tailed shearwater are listed as species of "least concern" (BirdLife International 2018, 2019). Similar to many seabird species globally, both flesh-footed shearwater and wedge-tailed shearwater have decreasing population trends (Croxall et al. 2012; Dias et al. 2019; Gorta et al. 2019), despite localized areas of variation (Bancroft et al. 2004). Seabirds face many anthropogenic threats such as bycatch, habitat loss, competition for resources, and solid waste pollution (plastic debris; Dias et al. 2019). Because of the large foraging area and high trophic order of seabirds (Mallory et al. 2010), they can be ideal biomonitoring species for understanding the impacts in the world's oceans, due to pollution (Braune et al. 2005), fish stock declines (Frederiksen et al. 2007), and climate change (Thompson and Ollason 2001).

Concentrations of PFASs have mainly been studied in juvenile birds from the Northern Hemisphere, in areas such as the Arctic region, which are exposed due to long-range transport, and near fluorochemical facilities that represent a greater exposure to birds (Table 1). The blood, egg, and liver are the ideal tissues to sample because they tend to have the highest concentration of PFASs relative to the other organs (Custer et al. 2012, 2019; Gebbink and Letcher 2012). However, plasma and eggs are more often sampled due to their nondestructive nature and their consequent usefulness for longitudinal studies (Leat et al. 2013; Route et al. 2014). The most detected and usually the most abundant compound found in juvenile bird livers (Table 1) and plasma is PFOS (Supplemental Data, Table S4). Typically, birds have higher PFAS body burdens adjacent to localized point sources compared with those from remote regions where long-range transport is the primary source (Table 1). Juvenile birds hatched in remote regions have PFOS concentrations up to

TABLE 1: Summary of reported concentration ranges of PFOS, PFNA, PFDA, PFUDA, PFTrDA and ∑PFAS in juvenile bird livers from field biomonitoring studies (in ng/g wet wt, unless stated otherwise)

	Country	Family	Common name	*n*	PFOS	PFNA	PFDA	PFUDA	PFTrDA	Reference
Long-range transport	Australia	Procellariidae	Flesh-footed shearwater	33	0.96–14.1	<0.06–1.02	<0.04–0.79	<0.04–1.06	<0.05–1.58	Present study
	Australia	Procellariidae	Wedge-tailed shearwater	9	1.50–6.0	<0.06–1.08	<0.04–0.71	<0.04–0.94	<0.05–0.46	Present study
Domestic	USA	Laridae	Herring gull	10	16.49–255.9	0.7–2.5	1.14–4.03	0.54–4.97	<1.0–6.4	Robuck et al. (2020)
	USA	Procellariidae	Great shearwater	10	11.14–249.4	0.6–3.5	0.49–5.16	3.31–8.84	<1.0	Robuck et al. (2020)
	South Korea	Laridae	Black-tailed gull	8	34.2–2510	0.73–4.61	1.65–5.04	6.67–10.1	4.46–9.70	Barghi et al. (2018)
Manufacturing	USA	Laridae	Royal tern	6	98.45–242.8	4.7–8.7	14.78–29.46	8.77–14.69	<1.0–2.7	Robuck et al. (2020)
	USA	Laridae	Sandwich tern	2	208.6–279.9	9.9–9.9	32.18–32.98	15.07–18.48	<1.0	Robuck et al. (2020)
	USA	Laridae	Laughing gull	1	114.31	6.00	17.5	4.72	<1.0	Robuck et al. (2020)
	USA	Pelecanidae	Brown pelican	2	46.05–46.71	3–3.3	10.94–11.95	5.95–6.6	<1.0	Robuck et al. (2020)
	USA	Hirundinidae	Tree swallow	24	<9.9–107	NR	NR	NR	NR	Custer et al. (2012)
	USA	Hirundinidae	Tree swallow	10	166–265	<0.64–0.8	n.d.	<0.64–1.40	NR	Custer et al. (2019)
	Belgium	Paridae	Eurasian blue tit	35	69–3323	NR	NR	NR	NR	Hoff et al. (2005)
	Belgium	Paridae	Great tit	48	17–2788	NR	NR	NR	NR	Hoff et al. (2005)

n.d. = nondetectable; NR = not reported. For other abbreviations, see Figure 2 legend.

188 ng/mL in plasma (Sletten et al. 2016). However, near fluorochemical manufacturing facilities, PFOS concentrations that are orders of magnitude higher are reported (35 624 ng/mL in plasma), which decrease as a function of distance from the facilities (Lopez-Antia et al. 2019).

The length of the CF_2 chain is the primary driver in determining the fate of these compounds in the environment. Sediment sorption and bioaccumulation potential can be predicted by the octanol/water partition coefficient (K_{OW}), which increases with CF_2 chain length (Higgins and Luthy 2006). Short-chain PFCAs ($C_n < 8$) and PFSAs ($C_n < 7$) are found less frequently above detection limits in birds (Sturm and Ahrens 2010). However long-chain PFCAs ($C_n > 7$) and PFSAs ($C_n > 6$; Buck et al. 2011) will tend to bioaccumulate in marine and terrestrial organisms and are reported to biomagnify along trophic gradients (Barghi et al. 2018; Ng and Hungerbühler 2014). Odd-chained PFCAs are typically found in greater concentrations compared with their even-chained homologs (e.g., perfluorononanoic acid [PFNA] > PFOA, perfluoroundecanoic acid [PFUDA] > perfluorodecanoic acid [PFDA] and perfluorotridecanoic acid [PFTrDA] > perfluorododecanoic acid [PFDoDA]; Bossi et al. 2015), which may be evidence of the degradation of FTCAs, although this effect has not been observed in avian species (Butt et al. 2014). Recently, replacement compounds and precursor PFASs have frequently been detected in adult bird populations (Letcher et al. 2015; Eriksson et al. 2016). The consequent degradation to terminal perfluoroalkyl acids (PFAAs) and the ecotoxicological impact of replacement compounds could pose a potential risk to vulnerable species and the viability of their offspring.

Concentrations of PFASs in juvenile birds may be due to residual exposure from maternal transfer from adult to egg (Newsted et al. 2007). However, the contribution of PFASs in the egg from the mother varies widely between individuals (Gebbink and Letcher 2012) and will change as a function of the egg-laying order (Lasters et al. 2019), although as Procellariforms invariably lay only one egg, the latter may not be a factor. Overall, diet is the primary pathway of PFAS exposure in juvenile birds after they are hatched (Letcher et al. 2010; Custer et al. 2012, 2019). The contribution of atmospheric exposure of PFASs in birds has been reported (Gewurtz et al. 2016), but few studies have shown concentrations of volatile PFASs and their potential metabolites (Guruge et al. 2011; Eriksson et al. 2016). Previous reports have found that plastic can be found in up to 90% of fledgling shearwater individuals, averaging 2.7 ± 10.6 g plastic/bird ($n = 38$; Lavers et al. 2014). Marine plastic debris may transport and concentrate trace elements and some organic contaminant concentrations from the environment (Bond and Lavers 2011; Tanaka et al. 2019) and can act as a vector for their bioaccumulation into birds (Tanaka et al. 2020). Despite reports that PFASs will also sorb to the surfaces of marine plastic (Llorca et al. 2014), there are no empirical data on the contribution of PFAS to individuals due to plastic exposure in birds, unlike hydrophobic POPs, in which marine plastic is a known vector for body burden contamination (Li et al. 2016).

The estimated median lethal doses (LD50s) of PFOS in juvenile quail and duck are between 61 and 150 mg/kg body weight/d (Newsted et al. 2005), which is orders of magnitude greater than the typical environmental concentrations found around the world (Ahrens 2011; Xiao 2017). In the North Atlantic Ocean, for example, PFOS concentrations range between 8.6 and 36 pg/L (Yamashita et al. 2008), well below the LD50 value. The sublethal effects of chronic exposure to PFASs, such as physiological (Costantini et al. 2019), immunological (Sletten et al. 2016), reproductive (Blévin et al. 2017), biochemical (Lopez-Antia et al. 2017), and genetic (Nakayama et al. 2008) impacts, have been used to assess the risk of the compounds in the environment. Due to the highly proteinophilic nature of some PFASs (Jones et al. 2003), other sublethal effects have been measured in biomonitoring studies such as negative correlation with serum protein (Lopez-Antia et al. 2017), cholesterol (Hoff et al. 2005), alanine aminotransferase, lactate dehydrogenase, and creatinine kinase (Peden-Adams et al. 2009). Furthermore, there is evidence that PFOS will negatively affect the expression of genes involved in carbohydrate transport and metabolism, intracellular trafficking, secretion, and vesicular transport (Nakayama et al. 2008). To assess the risk of PFASs to bird species, the concentrations of each compound must be accurately quantified in individuals from a wide range of species.

The present study reports on the occurrence of 45 PFASs in 2 species of juvenile pelagic seabirds that died as a result of beach-wash and road-kill, from the remote South Pacific Lord Howe Island (NSW, Australia). Correlations between PFAS concentrations, body morphometrics, and plastic ingestion were compared, to determine potential ecotoxicological impacts on juvenile shearwaters. To the authors' knowledge, this is the first study on the occurrence of PFASs in livers of pelagic seabirds from the Southern Hemisphere.

MATERIALS AND METHODS

Sample collection and morphometry

The study was conducted on Lord Howe Island (31°33′15″S, 159°05′06″E) with permissions from the Lord Howe Island Board (no. LHIB07/18), New South Wales Office of Environment and Heritage (no. SL100169), Victorian Department of Environment, Land, Planning and Water (no. 10009019), University of Tasmania Animal Ethics (no. A18480), Department of Primary Industries, Parks, Water and Environment, and the Australian Bird and Bat Banding Scheme. Samples were taken from dead fledglings (80–90 d old) of 2 species of shearwater found beach-washed and as road-kill, collected between 27 April and 9 May 2019 and autopsied within 1 h of recovery. Fledglings tend to take flight just before sunrise, so fresh specimens were sampled each morning within hours of death. Thirty-three flesh-footed shearwaters (*A. carneipes*) and 9 wedge-tailed shearwaters (*A. pacifica*) were sampled in total. The following body morphometric measurements were made: body mass (±10 g), wing chord (±1 mm), culmen and head + bill length (±0.1 mm). The proventriculus and gizzard were examined, and the mass of plastic (±0.0001 g) was recorded for each bird (Supplemental Data, Table S2). The livers of these birds were immediately excised, collected in low-density polyethylene Ziploc bags, and stored at –20 °C until analysis.

Chemicals and reagents

Hypergrade acetonitrile (ACN; CAS 75-05-8), hypergrade methanol (MeOH; CAS 67-56-1), ammonium acetate (>99.99 purity; CAS 631-61-8) were purchased from Merck Millipore. Type I ultrapure water was obtained from reverse osmosis water coupled with a Milli-Q Reference A + system (18.2 Ω; total organic carbon < 5 ppm; Merck). Magnesium sulfate/sodium acetate QuEChERS packets (7.5 g) and C18/primary secondary amine (PSA) dSPE sorbent (100 mg) were obtained from Agilent Technologies. Primary PFAS standards ($n = 45$) and mass-labeled surrogates ($n = 14$) were obtained individually from Wellington Laboratories. Naming conventions for PFASs are according to Buck et al. (2011). A complete list of PFASs, with their long-form names and CAS numbers is available in the Supplemental Data, Table S1. In brief, 11 PFCAs (perfluorobutanoic acid [PFBA], perfluoropentanoic acid [PFPeA], perfluorohexanoic acid [PFHxA], perfluoroheptanoic acid [PFHpA], PFOA, PFNA, PFDA, PFUDA, PFDoDA], PFTrDA, and perfluorotetradecanoic acid [PFTeDA]), 9 PFSAs (perfluorobutanesulfonic acid [PFBS], perfluoropentanesulfonic acid [PFPeS], PFHxS, perfluoroheptanesulfonic acid [PFHpS], PFOS, perfluorononanesulfonic acid [PFNS], perfluorodecanesulfonic acid [PFDS], perfluorododecanesulfonic acid [PFDoDS], and potassium perfluoro-4-ethylcyclohexanesulfonate [PFECHS]), and several novel classes of PFASs including 4 fluorotelomer sulfonic acids (FTSAs: 4:2 FTSA, 6:2 FTSA, 8:2 FTSA, and 10:2 FTSA), 3 FTCAs (3:3 FTCA, 5:3 FTCA, and 7:3 FTCA), 8 PASFs (perfluoro-1-octanesulfoneamide [FOSA], N-ethylperfluoro-1-octanesulfonamide [EtFOSA], N-methylperfluoro-1-octanesulfonamide [MeFOSA], perfluoro-1-octanesulfonamidoacetic acid [FOSAA], N-ethylperfluoro-1-octanesulfonamidoacetic acid [EtFOSAA], N-methylperfluoro-1-octanesulfonamidoacetic acid MeFOSAA, 2N-ethylperfluoro-1-octanesulfonamido-ethanol [EtFOSE], and 2N-methylperfluoro-1-octanesulfonamido-ethanol [MeFOSE]), 3 perfluoroalkyl phosphinic acids (PFPiAs: 6:6 PFPiA, 6:8 PFPiA, and 8:8 PFPiA), 4 fluorotelomer phosphate diesters (diPAPs: 6:2 diPAP, 6:2/8:2 diPAP, 8:2 diPAP, and sodium bis-[2-M-ethylperfluorooctane-1-sulfonamidoethyl] phosphate [diSAmPAP]), and 3 perfluoro ether-based substances (potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate F-53B [6:2 Cl-PFESA], potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate F-53B [8:2 Cl-PFESA], and sodium dodecafluoro-3H-4,8-dioxanonanoate [ADONA]).

PFAS extraction

Whole livers were weighed, transferred to 15-mL polypropylene centrifuge tubes, and homogenized with a rotor–stator dispersal tool for approximately 1 min (PT1200E; Polytron; $n = 42$). The extraction method was adapted from Baduel et al. (2015) and was conducted in batches of no more than 18 samples at a time and included the 2 quality assurance/quality control samples (see *Quality assurance and quality control* section following). Briefly, approximately 25 ng of mass-labeled PFAS internal standard (Supplemental Data, Table S1) was added before the addition of 5 mL ACN, and then the samples were vortexed and sonicated, both for 30 min. Approximately 0.75 g magnesium sulfate and 0.25 g sodium acetate were added before the samples were centrifuged at room temperature (3392 *g*, 10 min). Approximately 1 mL of supernatant was added to a vial containing 50 mg each of C18 and PSA for the removal of large organics such as sugars, lipids, and sterols. The extract was then briefly vortexed and centrifuged (2000 *g*, 5 min), and the resulting supernatant (250 µL) was then transferred to a polypropylene autosampling vial with a polypropylene cap for mass spectrometry analysis.

Liquid chromatography–tandem mass spectrometry analysis

An adapted analytical procedure was performed, as previously detailed (Coggan et al. 2019). In brief, the analysis was performed on an Agilent 1290 Infinity II liquid chromatography system coupled with an Agilent 6495C triple quadrupole mass spectrometer (LC–MS/MS). Chromatographic separation was achieved in a 15-min run with a 50-mm C18 Zorbax Eclipse column using 2 mM ammonium acetate aqueous phase and 100% MeOH organic phase. We used the following gradient of MeOH: $t_0 = 10\%$, $t_{0.5} = 10\%$, $t_{2.5} = 55\%$, $t_9 = 90\%$, $t_{9.1} = 100\%$, $t_{11.5} = 100\%$, and $t_{11.6} = 10\%$. The source conditions were as follows: drying gas temperature and flow = 250 °C at 11 L/min; sheath gas temperature and flow = 375 °C at 11 L/min; nebulizer pressure = 25 psi; capillary and nozzle voltage = 2500 and 1500 V; and iFunnel high- and low-pressure radiofrequency = 90 and 60 V. Transitions and collision energies were optimized for each compound, and a summary can be found in the Supplemental Data, Table S1. A 10-point calibration curve was used to quantify the concentrations of each analyte, ranging between 0.05 and 50 ng/mL ($R^2 > 0.99$) while mass-labeled concentrations remained constant in calibration levels at 5 ng/mL in MeOH.

Quality assurance and quality control

The quality of acquired LC–MS/MS data was verified by the addition of a laboratory control sample and method blank, and one of each was included with the corresponding batch of a maximum of 18 samples. The laboratory control sample and method blank were prepared following the same procedure as the samples, except without liver. The laboratory control sample was spiked with 25 ng native PFAS mix to measure the recovery of each compound. The average internal standard response from the samples and quality assurance/quality control were compared with the average internal standard response from the calibration curve. A detailed report on the results can be found in the Supplemental Data, Figure S2. Briefly, internal standard recoveries for each mass-labeled compound fell between 50 and 150%. The internal standard corrected recoveries for laboratory control sample fell between 70 and 130%, and the concentration of PFASs in the method blank fell below the method detection limit for each compound.

The method reporting limit (MRL) was defined by the lowest calibration level for compounds with a signal-to-noise ratio > 3:1. Concentrations of PFASs in samples had to meet the following conditions for quantification: 1) signal-to-noise ratio > 10:1, 2) internal standard recovery response between 50 and 150%, 3) concentration within the calibration range, 4) retention time within 5% of highest calibration result, and 5) qualifying ion ratio (where available) within 20% of highest calibration result. Results that did not meet one or more of these conditions were treated as <MRL.

Statistical analyses

Data were acquired and quantitated using Agilent MassHunter Workstation and Quantitative Analysis Ver 10.1, respectively. Descriptive statistics, statistical analysis, and data visualization were performed with R Ver 4.0.2 (R Core Team 2017) and RStudio (Ver 1.2.5019) with tidyverse Ver 1.3.0.9000 (Wickham et al. 2019), ggplot2 Ver 3.3.2 (Wickham 2016), rstatix Ver 0.6.0 (Kassambara 2020), and psych Ver 2.0.7 (Revelle 2019) packages. Statistical analyses were based on those described by Robuck et al. (2020), and the results of tests were reported according to null hypothesis significance testing guidelines (Dushoff et al. 2019; Erickson and Rattner 2020). Measurements of body morphometry, plastic mass, and concentrations of PFASs were checked for normality using the Shapiro–Wilk test before analysis, where normally distributed data result in $p > 0.05$. All morphometric measurements, plastic mass, and PFOS, PFTrDA, and $\sum_{45}$PFAS concentrations were determined to be non-normal and were $\log_{10}$-transformed, resulting in normal distribution. Geometric mean and 95% confidence intervals (CIs) were calculated for concentrations of each PFAS using MRL/2 for concentrations <MRL and 0 for nondetects when the frequency of detection was >50%. Multivariate analysis of variance (MANOVA) and Cohen's *d* test for effect size was used to test differences in body morphometry and total plastic mass between species. Further statistical analysis of censored data was performed using nonparametric tests. The Mann–Whitney–Wilcoxon *U* test was used to test for differences in concentrations of PFASs. Principal component analysis and Spearman's correlation matrix (R_s) were calculated for morphometric measurements, plastic mass, and PFAS concentrations. The level of significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Flesh-footed shearwater and wedge-tailed shearwater morphology and ingested plastic

The flesh-footed shearwaters had increased body morphometry compared with the wedge-tailed shearwaters (global MANOVA: $p = 0.0000$, $df = 36$; Figure 1). The total body mass of flesh-footed shearwater (mean: 281 g, 95% CI: 210–434 g) was greater than that of wedge-tailed shearwater (mean: 205 g, 95% CI: 170–278 g; $p = 0.0018$, Cohen's $d = 1.07$). The wing chord lengths of flesh-footed shearwater (mean: 255 mm, 95% CI: 231–304 mm) were also greater than those of wedge-tailed shearwater (mean: 231 mm, 95% CI: 212–260 mm; $p = 0.0002$, Cohen's $d = 1.56$). Finally, the head + bill length and culmen lengths in flesh-footed shearwater (mean: 94 mm, 95% CI: 88–99 mm and mean: 42 mm, 95% CI: 39–45 mm, respectively)

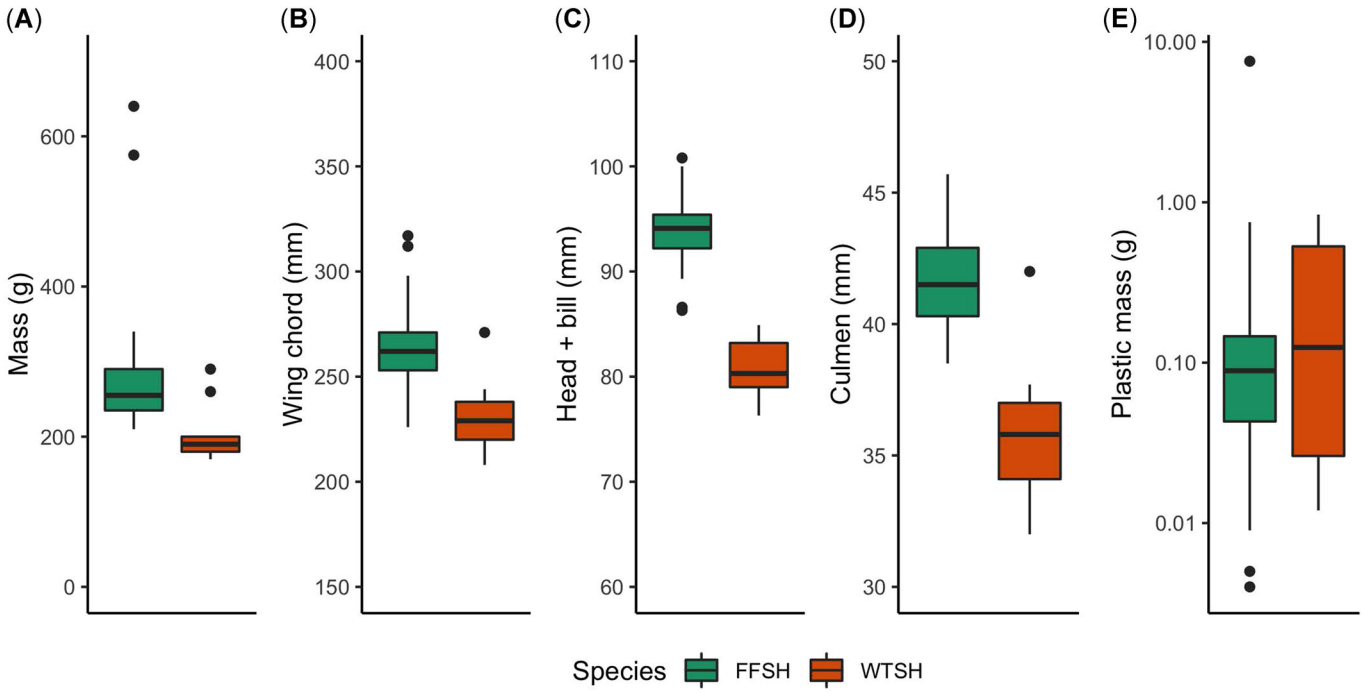

FIGURE 1: Body morphometry results from flesh-footed shearwaters (FFSH; $n = 33$, green boxplots) and wedge-tailed shearwaters (WTSH; $n = 9$; orange boxplots) collected from Lord Howe Island, 2019: (**A**) total mass of individuals ($p = 0.0017$), (**B**) head + bill ($p = 0.0002$) length, (**C**) wing chord ($p = 0.0000$) length, (**D**) culmen ($p = 0.0000$) length, and (**E**) total plastic mass ($p = 0.6585$). Global multivariate analysis of variance (MANOVA): $p = 0.0000$.

DE09920182

were greater than those of wedge-tailed shearwater (mean: 81 mm, 95% CI: 77–84 mm and mean: 36 mm, 95% CI: 32–40 mm, respectively; $p = 0.0000$, Cohen's $d = 4.18$; $p = 0.0000$, Cohen's $d = 2.26$). Each of the morphometric measurements was highly and significantly correlated with one another ($R_s > 0.36$, $p < 0.01$; Supplemental Data, Table S3) The average mass of liver excised from each bird was 1.69 g (95% CI: 1.48–1.70 g). Plastic was found in the intestinal tract of 79% of shearwaters, with no statistically clear differences in the incidence between species ($p > 0.05$; Figure 1). The total masses of plastic found in flesh-footed shearwater and wedge-tailed shearwater intestinal tract were 0.346 g (95% CI: 0–0.58 g) and 0.149 g (95% CI: 0–0.69 g), respectively. The mass of plastic found in shearwaters from 2019 was in the same range as those found previously from these populations on Lord Howe Island by Lavers et al. (2014) in 2011 (range: 0–64.1 g) and Puskic et al. (2019) in 2017 (range: 0–27.4625 g). Full details of the morphometric measurements and plastic masses are presented in the Supplemental Data.

Number and concentration of PFASs

Of the 45 compounds analyzed, 16 PFASs from 5 classes (PFSA, PFCA, FTCA, PASF, and PFPiA) were detected in all juvenile shearwater livers ($n = 42$). The $\sum_{45}$PFASs ranged from 2.08 to 21.51 ng/g wet weight in shearwater livers, and concentrations did not appear to be different between species ($p > 0.05$). In order of decreasing concentration, PFOS, PFNA, PFUDA, PFTrDA, and PFDA were detected in >50% of birds (Figure 2). Liver PFOS concentration (geometric mean: 3.44 ng/g wet wt; 95% CI: 1.5–8.5 ng/g wet wt) was >6-fold greater than the next most abundant compound. In 98% of shearwaters, PFNA was detected, at a mean concentration of 0.58 ng/g wet weight (95% CI: 0.37–0.98 ng/g wet wt). In 95% of shearwater livers, PFUDA was detected, at a mean concentration of 0.55 ng/g wet weight (95% CI: 0.24–0.96 ng/g wet wt). As for PFTrDA, it was detected in 94% of flesh-footed shearwater ($n = 33$) and 78% of wedge-tailed shearwater ($n = 9$), at average concentrations of 0.41 ng/g wet weight (95% CI: 0.19–0.69 ng/g wet wt) and 0.19 ng/g wet weight (95% CI: 0.02–0.42 ng/g wet wt), respectively. In 90% of shearwater livers, PFDA was detected, at a mean concentration of 0.32 ng/g wet weight (95% CI: 0.02–0.72 ng/g wet wt), which was significantly less than the concentration of PFUDA ($p = 0.0000$). Eleven compounds with detection frequencies <50% were not subject to further analysis, particularly from the FTSA, the perfluoroalkyl ether sulfonate/perfluoroalkyl ether carboxylate (PFESA/PFECA), and the diPAP classes, of which none were detected.

There was no statistically clear difference in mean concentrations when we compared PFOS, PFNA, PFDA, and PFUDA levels between flesh-footed shearwater and wedge-tailed shearwater fledglings from Lord Howe Island ($p > 0.05$; Table 2) and thus the 2 species will be discussed together. Furthermore, despite reported differences in foraging areas between the 2 species (Reid et al. 2012; Miller et al. 2018), similar concentrations of PFASs in these species suggests that the environmental concentrations of these compounds surrounding Lord Howe Island are relatively homogenous. By contrast, concentrations of PFTrDA were estimated to be 0.17 ng/g wet weight (95% CI: 0.08–0.29) greater in juvenile flesh-footed shearwater compared with wedge-tailed shearwater (Mann–Whitney–Wilcoxon $U = 259$, $p = 0.0007$, 2-tailed; Table 2). Although there was a real difference in PFTrDA concentrations between species, the difference was relatively small and negligible.

The PFAS PFOS has been the most frequently detected and most abundant compound in marine and terrestrial juvenile

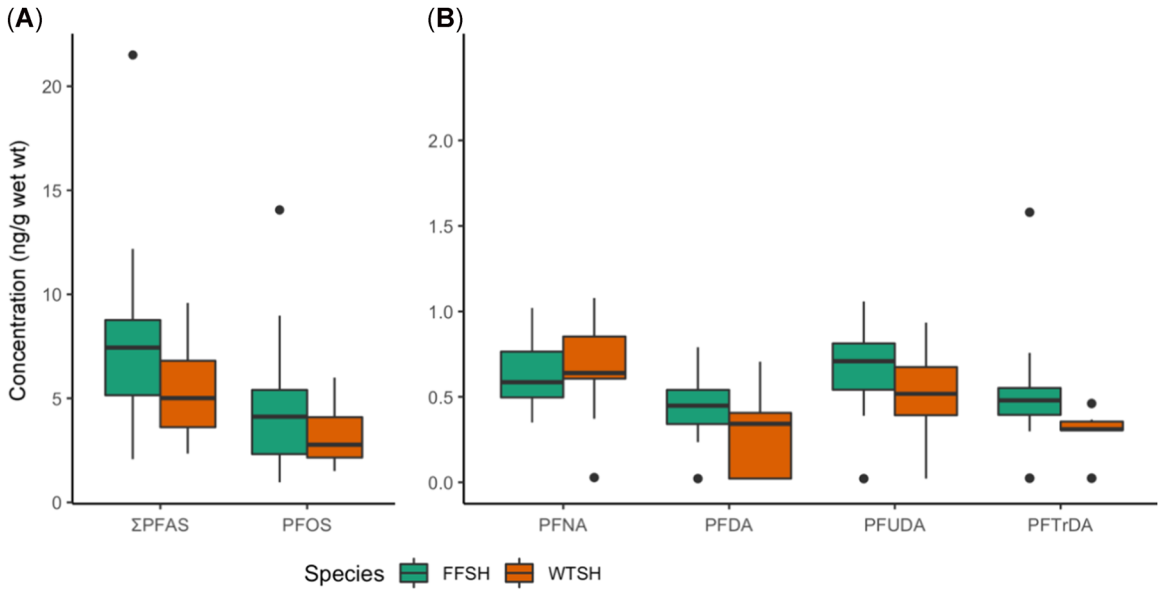

FIGURE 2: Concentration (ng/g wet wt) of (**A**) $\sum_{45}$PFAS and PFOS and (B) PFNA, PFDA, PFUDA and PFTrDA in flesh-footed shearwater (FFSH, $n = 33$, green boxplots) and wedge-tailed shearwater (WTSH; $n = 9$, orange boxplots) liver from Lord Howe Island in April to May 2019. PFAS = per- and polyfluoralkyl substances; PFOS = perfluorooctanesulfonate; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFUDA = perfluoroundecanoic acid; PFTrDA = perfluorotridecanoic acid.

DE09920182

TABLE 2: Summary of detection frequency (%), geometric mean concentration (95% confidence interval, in parentheses), and range (ng/g wet wt, in square brackets) of PFAS found above detection limits in juvenile flesh-footed shearwater and wedge-tailed shearwater from Lord Howe Island in 2019[a]

	Flesh-footed shearwater ($n = 33$)		Wedge-tailed shearwater ($n = 9$)		
Compound	Frequency (%)	Mean/range	Frequency (%)	Mean/range	Mann–Whitney *U* test
PFBS	6	— [<0.05–0.2]	0	n.d.	
PFHxS	30	— [<0.05–0.63]	0	n.d.	
PFHpS	12	— [<0.05–0.22]	0	n.d.	
PFOS	100	3.6 (1.34–8.75) [0.96–14.05]	100	2.93 (1.52–5.43) [1.5–6]	$W = 181$ $p = 0.3266$
PFPeA	3	— [<0.11–0.34]	0	n.d.	
PFHpA	6	— [<0.05–0.17]	0	n.d.	
PFOA	3	— [<0.05–0.07]	0	n.d.	
PFNA	100	0.61 (0.39–0.92) [0.35–1.02]	89	0.48 (0.17–1.04) [<0.06–1.08]	$W = 132$ $p = 0.6238$
PFDA	97	0.40 (0.24–0.74) [<0.04–0.79]	67	0.16 (0.02–0.64) [<0.04–0.71]	$W = 203.5$ $p = 0.0947$
PFUDA	97	0.62 (0.41–0.97) [<0.04–1.06]	89	0.37 (0.11–0.92) [<0.04–0.94]	$W = 203.5$ $p = 0.0948$
PFDoDA	48	— [<0.05–0.45]	0	n.d.	
PFTrDA	94	0.41 (0.19–0.69) [<0.05–1.58]	78	0.19 (0.02–0.42) [<0.05–0.46]	$W = 259$ $\mathbf{p = 0.0007}$
PFTeDA	18	— [<0.05–0.25]	0	n.d.	
EtFOSE	12	— [<0.08–0.4]	11	— [<0.08–0.48]	
7:3 FTCA	6	— [<0.28–4.51]	0	n.d.	
6:6 PFPiA	3	— [<0.06–0.19]	0	n.d.	

[a]MRL/2 was substituted for values below the method reporting limit. The *p* value in bold is statistically significant.
PFAS = per- and polyfluoroalkyl substances; n.d. = nondetectable; PFBS = perfluorobutanesulfonic acid; PFHxS = perfluorohexanesulfonic acid; PFHpS = perfluoroheptanesulfonic acid; PFHpA = perfluoroheptanoic acid; PFOS = perfluorooctanesulfonic acid; PFOA = perfluorooctanoic acid; PFPeA = perfluoropentanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFUDA = perfluoroundecanoic acid; PFDoDA = perfluorododecanoic acid; PFTrDA = perfluorotridecanoic acid; PFTeDA = perfluorotetradecanoic acid; EtFOSE = N-ethyl perfluorooctanesulfonamidoethanol; 7:3 FTCA = 7:3 fluorotelomercarboxylic acid; 6:6 PFPiA = perfluorohexylperfluorooctylphosphinic acid.

birds in biomonitoring studies, and data from the present study are consistent with previous findings (Table 1). Adult shearwaters will travel approximately 400 km on average from the nest to feed their offspring (Alonso et al. 2012). The fact that PFOS has a half-life in birds of 230 d (Tarazona et al. 2015) means that PFOS concentrations measured in the nestling shearwaters on Lord Howe Island are representative of the environmental concentrations within the feeding radius.

Of the PFCAs, the mean concentration of compounds with C > 9 decreased from PFUDA > PFNA > PFTrDA > PFDA (Table 2). Long odd-chain PFCAs have been reported in the literature with greater frequency at high concentrations compared with their next shorter, even-chain moiety (i.e., PFNA > PFOA, PFUDA > PFDA and PFTrDA > PFDoDA; Gebbink and Letcher 2012; Braune and Letcher 2013; Chu et al. 2015; Eriksson et al. 2016; Gewurtz et al. 2016). The 2 compounds PFOA (range: <0.05–0.07 ng/g wet wt) and PFDoDA (range: <0.05–0.45 ng/g wet wt) were detected in 2 and 38% of juvenile shearwater livers from Lord Howe Island, respectively, which is consistent with previous findings (Table 1). In a review of PFAS concentrations in a range of biota, including humans, PFOA was frequently detected in mammals and fish, but not in birds, and thus the absence of PFOA in the present study is supported by the literature (Sturm and Ahrens 2010). The observed PFCA trends in shearwaters may be because C11 and C13 chemistries have greater global emissions compared with those of C10 and C12, respectively (Armitage et al. 2009), and the biomagnification potential for PFNA is greater than

that for PFOA (Tomy et al. 2004; Haukås et al. 2007; Kelly et al. 2009).

Saturated FTCAs, such as 7:3 FTCA, are transformation products of precursor PFAS like FTOHs. Certain FTCAs were previously detected in the liver and eggs of terrestrial bird-of-prey species (Orders: Accipiteriiformes and Falconiiformes) from Japan and Sweden, at amounts ranging between 6.8 and 62 and <0.24 and 2.7 ng/g wet weight, respectively. The compound 7:3 FTCA was also detected in nearshore species, such as swans and mallards to a lesser extent (Guruge et al. 2011; Eriksson et al. 2016), at amounts above the method detection limit (which was 0.28 ng/g wet wt) in 2 particular birds, at 0.64 and 4.5 ng/g wet weight, respectively.

This is the first time 7:3 FTCA has been reported in birds from the Southern Hemisphere. Precursor compounds, such as n:2 FTOHs, are known to degrade in the atmosphere to odd-numbered PFCAs, such as PFNA (Ellis et al. 2004). There is evidence to suggest that odd-chained compounds are the result of preferred biotransformation pathways of precursor compounds that occur in higher trophic organisms by α-oxidation of n:3 FTCAs, an intermediate degradation product of the volatile n:2 FTOHs (Butt et al. 2014). This indicates that atmospheric PFAS emission may play a role in the contamination of these birds.

The compound 6:6 PFPiA was detected in a single flesh-footed shearwater at 0.19 ng/g wet weight (Table 2), the first reported detection of this class of compound found in marine birds; 6:8 PFPiA and 8:8 PFPiA were not detected in any shearwater livers. To the authors' knowledge, PFPiAs have only been detected in one previous study, that of De Silva et al. (2016), who found 6:6 PFPiA in the blood of cormorants from the continental United States averaging in concentration between 0.20 and 1.6 ng/g wet weight; these compounds were used as surfactants in pesticide sprays for agricultural use. The ecotoxicological effects of PFPiAs are not known for birds, but they are suspected endocrine disruptors in zebrafish (Liu et al. 2019), and there is evidence they biotransform to terminal PFAAs after exposure (Lee et al. 2012). The PASF EtFOSE was detected in both flesh-footed shearwater and wedge-tailed shearwater (12 and 11% respectively; Table 2). No other PASFs were detected in shearwater livers in the present study. To the authors' knowledge, this is the first reported detection of EtFOSE in birds.

In our study, PFAS concentrations found in juvenile shearwaters were generally lower than those reported for birds impacted by long-range transport and manufacturing plants in the Northern Hemisphere (Table 1), except for golden eagles from Norway, which had a maximum concentration of $\sum_{10}$PFAS = 1.2 ng/mL in plasma (Sonne et al. 2010). In concordance with our study, marine seabirds from a remote island in the Southern Indian Ocean had similarly low concentrations of PFASs compared with Northern Hemisphere counterparts, with average ∑PFAA concentrations ranging between 1.7 and 7.2 ng/g wet weight among 3 species of tern (van der Schyff et al. 2020). Based on the much lower exposure to PFASs in shearwaters from our study, it is likely that the Southern Pacific Ocean surrounding the remote Lord Howe Island is less contaminated than the oceans of the Northern Hemisphere.

Correlation with body morphometrics

The differences in mass and head-to-bill and culmen lengths were clustered along the first and second principal components, representing 69.9% of the data (Supplemental Data, Figure S1). The influence of the body morphometrics and the PFAS was separated along the second principal component. The effects of PFOS, PFNA, PFDA, PFUDA, and PFTrDA were clustered along the first and second principal components (Supplemental Data, Figure S1) and significantly correlated with each other ($R_s > 0.42$, $p < 0.001$; Supplemental Data, Table S3), indicating a possible common source of each of these compounds or similar chemical kinetics that would result in comparable uptake and depuration rates. Furthermore, Puskic et al. (2019) found no relationship between fatty acid composition and body condition of shearwaters previously sampled on Lord Howe Island in 2017.

Concentrations of PFDA and PFTrDA were weakly positively correlated with wing chord length ($R_s > 0.36$, $p < 0.05$; Figure 3). Furthermore, PFDA was weakly correlated with body mass ($R_s = 0.33$, $p = 0.032$; Figure 3). Overall, these findings suggest a possible effect of increased size of body morphometry from PFCA exposure. Tartu et al. (2014) reported negative correlations with PFTrDA and corticosterone concentrations in plasma from adult black-legged kittiwakes (*Rissa tridactyla*) exposed to PFASs by long-range transport, concluding that there is a potential for long-chain PFCAs to have a negative impact on overall body condition. Per- and polyfluoroalkyl substances such as PFTrDA may modulate the action or availability of hormones in birds, due to their ability to disrupt transport proteins in serum (Jones et al. 2003). Decreased concentrations of glucocorticoids (such as cortisol and corticosterone) in birds was found to positively influence the growth of various organs including the muscle, spleen, testis, bursa, and overall body weight (Hull et al. 2007). Conversely, physiological effects of PFASs have not been reported in other juvenile birds (Løseth et al. 2018), nor in birds with significantly greater exposure to PFASs (Custer et al. 2019).

Effect of plastic ingestion

There was no statistically clear correlation between the concentrations of each PFAS or total PFASs and the mass of plastic found in each bird's proventriculus, gizzard, or total intestinal tract (Supplemental Data, Table S3). Determination of the concentration of the sorbed PFAS was not possible because the plastic was characterized using conventional visual analytical techniques (microscope; Fourier transform infrared spectroscopy), which would have resulted in PFAS contamination. It is also difficult to predict the fate of PFASs that have been exposed to the warm acidic conditions of the gastrointestinal tract. Nonetheless, PFASs were found sorbed to plastic pellets and sediment collected from beaches in the

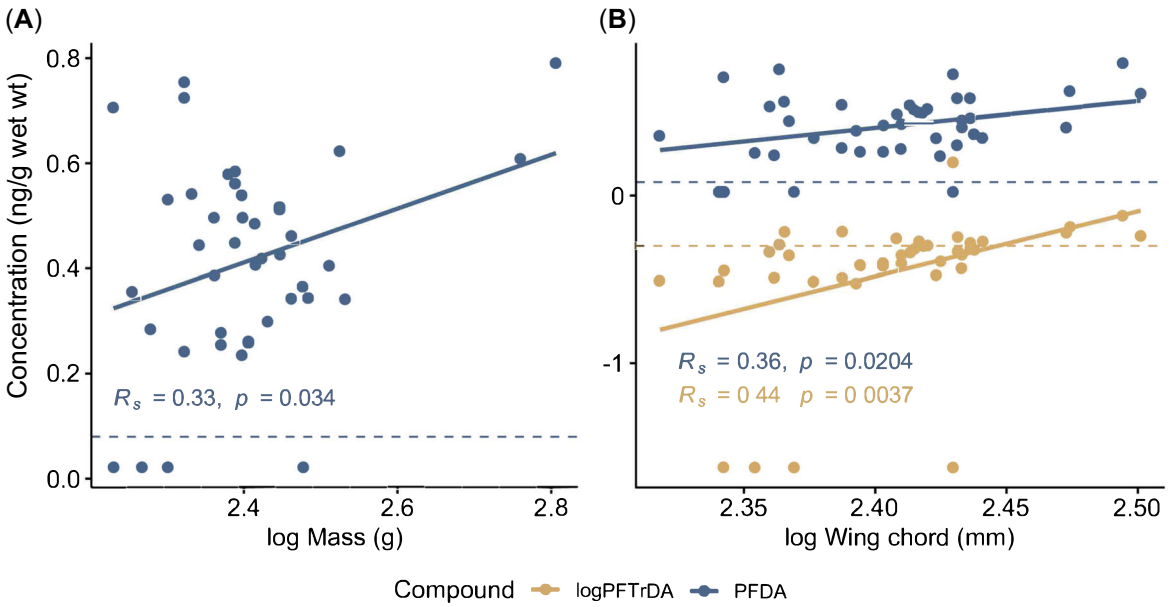

FIGURE 3: Scatterplot of per- and polyfluoralkyl substances (PFAS) concentrations and $\log_{10}$-transformed morphometric measurements in flesh-footed shearwaters and wedge-tailed shearwaters ($n = 42$) from Lord Howe Island in 2019. (**A**) PFDA (blue) and body mass; (**B**) PFDA, $\log_{10}$PFTrDA (yellow) and wing chord length. The dotted line represents the method reporting limit (MRL). The R_s and p values are derived from Spearman's rank correlation. PFTrDA = perfluorotridecanoic acid; PFDA = perfluorodecanoic acid.

Mediterranean Sea at levels up to 115 ng/kg (Llorca et al. 2014). Despite the evidence of PFAS plastics sorption, there is limited information on the potential of plastics to act as vectors for PFAS exposure and the subsequent effects on organisms that ingest plastics. The PFASs detected in the juvenile shearwaters are likely introduced and accumulated from the fish, squid, and other foodstuffs foraged by the adults. Diet is the primary pathway for PFAS exposure to juvenile birds (Gómez-Ramírez et al. 2017), and PFASs such as PFOS and long-chain PFCAs are known to biomagnify in marine seabirds (Barghi et al. 2018).

Strengths and limitations

Our study presents the concentrations of a large suite of PFASs, including novel and emerging classes and products of atmospheric deposition, not well described in the literature. Further studies should be directed toward the potential exposure of semivolatile PFASs such as n:2 FTOHs, to investigate the pathways of biotransformation in birds. As the investigation into exposure and impacts of PFASs continues globally, attention needs to be paid to long-chain PFCAs and n:2 FTCAs, which are being increasingly detected in avian species. Furthermore, the pathways of exposure from semivolatile PFASs such as n:2 FTOHs should be investigated as a source of PFCA and PFSA exposure by way of metabolism and biotransformation.

The ability to detect concentrations of PFASs < 1 ng/g has allowed the authors to understand the potential impacts of long-chain PFCAs without the hindrance of excessive censored data. The trace level detection allowed the examination of correlations between PFASs and body morphometrics, despite the relatively small sample size in our study. Ex situ studies on the sublethal effects of long-chain PFCAs are needed to confirm the apparent trend because there is a lack of data in this field. The developing fields of metabolomics and genomics will also be crucial to understanding the potential sublethal effects of PFASs to birds and other wildlife. The authors also acknowledge the potential impact of cocontaminants we did not report on that may also contribute to the body morphology of the birds.

The absence of many compounds over several classes of PFASs provides information to policymakers for the management of these compounds in the future. Continued study into the fate of PFASs in birds impacted from known sources in the Southern Hemisphere is also warranted, given that AFFF-impacted sites have resulted in highly contaminated avifauna (Sharp et al. 2020). Without studies on PFAS exposure to the adult shearwater population on Lord Howe Island or the contamination present in the food web, it is difficult to speculate as to the precise source. Furthermore, although we did not observe a correlation between plastics ingestion and PFAS, it should be noted that only macroplastics (>1 mm) were recorded and further research should also include smaller micro- (<1 mm) and nanoplastics (<1 µm).

CONCLUSIONS

The present study is the first to quantify a range of PFASs in juvenile marine seabirds ($n = 42$) from the Southern Hemisphere impacted by long-range transport. Of 45 PFAS measured, 17 were detected in the liver of flesh-footed shearwater ($n = 33$), and 6 were detected in livers of wedge-tailed shearwater ($n = 9$) from the relatively pristine location of Lord Howe Island, Australia. In agreement with previous studies, PFOS was detected with the greatest frequency and abundance. There was no statistically clear difference between concentrations of PFASs in flesh-footed shearwater and wedge-tailed shearwater. Long-chain PFCAs,

particularly with carbon lengths between 9 and 13, were also detected in >50% of birds, albeit in lower concentrations compared with PFOS. Concentrations of these compounds were highly and significantly correlated, suggesting a common source of exposure (likely via diet or, to a lesser extent, maternal transfer). Notably, the present study provides evidence of increased body mass and length in individuals with greater concentrations of PFDA and PFTrDA. The significance of increased body morphometry is unclear, and continued monitoring of this population is recommended. The PFAS 7:3 FTCA, EtFOSA, EtFOSE, and 6:6 PFPiA were detected in a few individuals, indicating exposure to novel classes of PFASs that may degrade into stable PFCAs or PFSAs. Finally, there was no clear relationship between the mass of macroplastics found in the digestive tract and concentrations of detectable PFASs. Thus plastic ingestion is not likely contributing to the exposure of juvenile birds to PFASs; instead, they are being exposed by other means, such as diet or maternal transfer.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4924.

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Data Availability Statement—Data, associated metadata, and calculation tools not presented are available from the corresponding author (brad.clarke@unimelb.edu.au).

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PERFLUORINATED ALKYL ACIDS IN PLASMA OF AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*) FROM FLORIDA AND SOUTH CAROLINA

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Abstract: The present study aimed to quantitate 15 perfluoroalkyl acids (PFAAs) in 125 adult American alligators at 12 sites across the southeastern United States. Of those 15 PFAAs, 9 were detected in 65% to 100% of samples: perfluorooctanoic acid, perfluorononanoic acid, perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid, perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid, perfluorohexanesulfonic acid (PFHxS), and perfluorooctane sulfonate (PFOS). Males (across all sites) showed significantly higher concentrations of 4 PFAAs: PFOS ($p = 0.01$), PFDA ($p = 0.0003$), PFUnA ($p = 0.021$), and PFTriA ($p = 0.021$). Concentrations of PFOS, PFHxS, and PFDA in plasma were significantly different among the sites in each sex. Alligators at both Merritt Island National Wildlife Refuge (FL, USA) and Kiawah Nature Conservancy (SC, USA) exhibited some of the highest PFOS concentrations (medians of 99.5 ng/g and 55.8 ng/g, respectively) in plasma measured to date in a crocodilian species. A number of positive correlations between PFAAs and snout–vent length were observed in both sexes, suggesting that PFAA body burdens increase with increasing size. In addition, several significant correlations among PFAAs in alligator plasma may suggest conserved sources of PFAAs at each site throughout the greater study area. The present study is the first to report PFAAs in American alligators, to reveal potential PFAA hot spots in Florida and South Carolina, and to provide a contaminant of concern when assessing anthropogenic impacts on ecosystem health. *Environ Toxicol Chem* 2017;36:917–925. Published 2016 Wiley Periodicals Inc. on behalf of SETAC. This article is a US government work and, as such, is in the public domain in the United States of America.

Keywords: Perfluorooctane sulfonate (PFOS) Perfluorohexanesulfonic acid (PFHxS) Alligator Crocodilian Plasma

INTRODUCTION

Despite being manufactured for more than 50 yr [1], it was not until 2000 that the class of chemicals known as perfluoroalkyl acids (PFAAs) entered the scientific spotlight as a major environmental contaminant of concern [2]. The 2 most commonly known PFAAs, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), were produced by 3M in 1948 and 1947 [1], respectively, the latter of which was subsequently purchased by DuPont in 1951 [3]. A variety of new PFAAs have steadily been introduced to the market since then. Structurally, PFAAs can widely vary; but as a whole, they typically consist of carbon chains of varying length (linear and branched isomers), an acid functional group, and hydrogen atoms substituted with fluorine atoms [4]. The carbon–fluorine bonds are the unique feature of PFAAs and provide chemical and thermal stability [5]. Two well-studied families of PFAAs are carboxylic acids and sulfonic acids [2,6].

The usage of PFAAs has become widespread since the introduction of these chemicals in the 1940s, largely because they exhibit unique surfactant properties that make them attractive components for many consumer-related products, such as nonstick pans, water-repellant surfaces, hair products, plastics, and lubricants [2], as well as firefighting products known as aqueous film-forming foams [7]. Active manufacturing and use of certain PFAAs, such as PFOS and PFOA, have largely ceased as a result of a voluntary phaseout by industry. Current production of fluorinated chemicals includes shorter-chained carboxylic and sulfonic acid substitutes, such as perfluorobutanesulfonic acid (PFBS) and perfluorobutyric acid (PFBA) [8]. In addition, precursor chemicals that have a nonfluorinated structural component attached to a perfluorinated chain may be amenable to microbial or chemical transformation and have the potential to degrade into perfluorinated carboxylic and sulfonic acids over time [9].

The same properties that make PFAAs commercially valuable (e.g., the highly stable carbon–fluorine bonds) also enable them to persist in the environment by resisting chemical, microbial, and photolytic degradation. However, unlike the more lipophilic environmental contaminants, such as organochlorine pesticides, polychlorinated biphenyls (PCBs), and brominated flame retardants (PBDEs) that are sequestered in adipose tissue, PFAAs accumulate in the blood and blood-rich

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organs, such as the liver [10,11]. Conversely, like organochlorine pesticides, PCBs, and PBDEs, PFAAs have also been shown to bioaccumulate and biomagnify in food webs [6]. Increasing PFAA chain length has been shown to correlate with an increasing ability to bioaccumulate [12], and the greatest PFAA concentrations detected in wildlife have been in species occupying high trophic positions [13]. Because PFAAs are bioaccumulative and often observed in higher concentrations in fish-eating marine species [13], humans who consume more fish in their diet may be at higher risk of PFAA exposure than those who consume less fish [14].

Animal studies reveal a wide range of PFAA-related effects that include alterations in liver physiology and serum cholesterol, as well as resulting hepatomegaly, wasting syndromes, neurotoxicity, and immunotoxicity [15–17]. In addition, PFAAs have been mentioned as possible obesogens because of their interaction with peroxisome proliferator–activated receptors [18]. However, although species-specific variations in PFAA excretion rates have been observed [2], the actual mechanism of action of PFAA toxicity is not well understood across species.

Few reports exist on PFAA distribution and body burdens in North American wildlife, and studies of PFAAs in wild reptiles and amphibians have been limited almost exclusively to frogs and sea turtles [19]. Globally, only 3 studies have examined PFAAs in crocodilians [20–22]. Because of their high trophic status, long life span, and high site fidelity, crocodilians are attractive study species for ecotoxicological investigations, particularly those involving exposure and accumulation of persistent environmental contaminants [23–25]. As such, studies examining PFAAs in crocodilians can provide insight into exposure and potential effects in focal species and can identify potential hot spots of PFAA contamination.

In the present study, we examined PFAA concentrations in plasma of wild American alligators (*Alligator mississippiensis*) from 12 sites in Florida and South Carolina, USA (Figure 1). Because factors such as sex, body size, and location may influence PFAA concentrations in alligators, the relationships between PFAA body burdens and these parameters were also examined.

MATERIALS AND METHODS

Study area

American alligator plasma samples ($n = 125$) were collected between 2012 and 2015 as part of multiple ongoing projects examining the biology and ecotoxicology of alligators in Florida and South Carolina [23–25]. In South Carolina, alligator blood samples were collected from the following sites (in order of north to south): Tom Yawkey Wildlife Center (YK; $n = 10$), Kiawah Island (KA; $n = 10$), and Bear Island Wildlife Management Area (BI; $n = 10$) (Figure 1; Supplemental Data, Table S1). In Florida, samples were collected from the following sites (in order from north to south): Lochloosa Lake (LO; $n = 10$), Lake Woodruff (WO; $n = 10$), Lake Apopka (AP; $n = 10$), Merritt Island National Wildlife Refuge (MI; $n = 15$), St. Johns River (JR; $n = 10$), Lake Kissimmee (KS; $n = 10$), Lake Trafford (TR; $n = 10$), Everglades Water Conservation Area 2A (2A; $n = 10$), and Everglades Water Conservation Area 3A (3A; $n = 10$) (Figure 1; Supplemental Data, Table S1).

Sample collection

Immediately following capture of the alligator, a blood sample was collected from the postoccipital sinus of the spinal vein of each animal using a sterile needle and syringe [23–25]. Whole-blood samples were then transferred to 8-mL lithium–heparin Vacutainer blood-collection tubes (BD), stored on ice in the field, and later centrifuged at 2500 rpm at 4 °C for 10 min to obtain plasma, which was stored at –80 °C until analysis. Snout–vent length was measured for each animal as a proxy for size, and sex was determined by cloacal examination of the genitalia [26].

The National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM) 1958 Organic Contaminants in Fortified Human Serum (Freeze-Dried) was used as a control material during PFAA analysis. The freeze-dried human serum SRM 1958 was reconstituted with deionized water according to the instructions on the certificate of analysis [27] and analyzed alongside collected alligator plasma.

Chemicals

Calibration solutions were created by combining 2 solutions produced by the NIST reference materials 8446 Perfluorinated Carboxylic Acids and Perfluorooctane Sulfonamide in Methanol and 8447 Perfluorinated Sulfonic Acids in Methanol. Together, the solution contained 15 PFAAs as follows: PFBA, perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTA), PFBS, perfluorohexanesulfonic acid (PFHxS), PFOS, and perfluorooctanesulfonamide (PFOSA).

Internal standards were purchased from Cambridge Isotope Laboratories, RTI International, and Wellington Laboratories to create an internal standard mixture comprised of 11 isotopically labeled PFAAs, as follows: $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_8$-PFOA, $^{13}C_9$-PFNA, $^{13}C_9$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-PFDoA, $^{18}O_2$-PFBS, $^{18}O_2$-PFHxS, $^{13}C_4$-PFOS, and $^{18}O_2$-PFOSA.

Sample preparation

Samples were extracted using a method described by Reiner et al. [28]. Approximately 1 mL of each alligator plasma sample and SRM 1958 aliquots were thawed and gravimetrically weighed. All samples were then spiked with the internal standard mixture (approximately 600 μL) and gravimetrically weighed. After brief vortex-mixing and 90 min of equilibration, 4 mL of acetonitrile were used to extract the PFAAs from each sample. After sonication and centrifugation, the supernatant was removed from all samples. The collected supernatant was then solvent-exchanged to methanol and further purified using an ENVI-Carb solid-phase extraction cartridge (Supelco). Resulting extracts were evaporated to 1 mL using nitrogen gas prior to being analyzed by liquid chromatography-tandem mass spectrometry.

Samples were analyzed using an Agilent 1100 high-performance liquid chromatographic system coupled to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer with electrospray ionization in negative mode. Samples were examined by liquid chromatography using an Agilent Zorbax Eclipse Plus C18 analytical column (2.1 mm × 150 mm × 5 μm). A ramping liquid chromatography solvent gradient was employed using methanol and deionized water, both containing 20 mmol/L ammonium acetate [28]. Two multiple reaction monitoring transitions for each PFAA were monitored to ensure no interferences with measurements. One was employed for quantitation, and the other was used for confirmation [28].

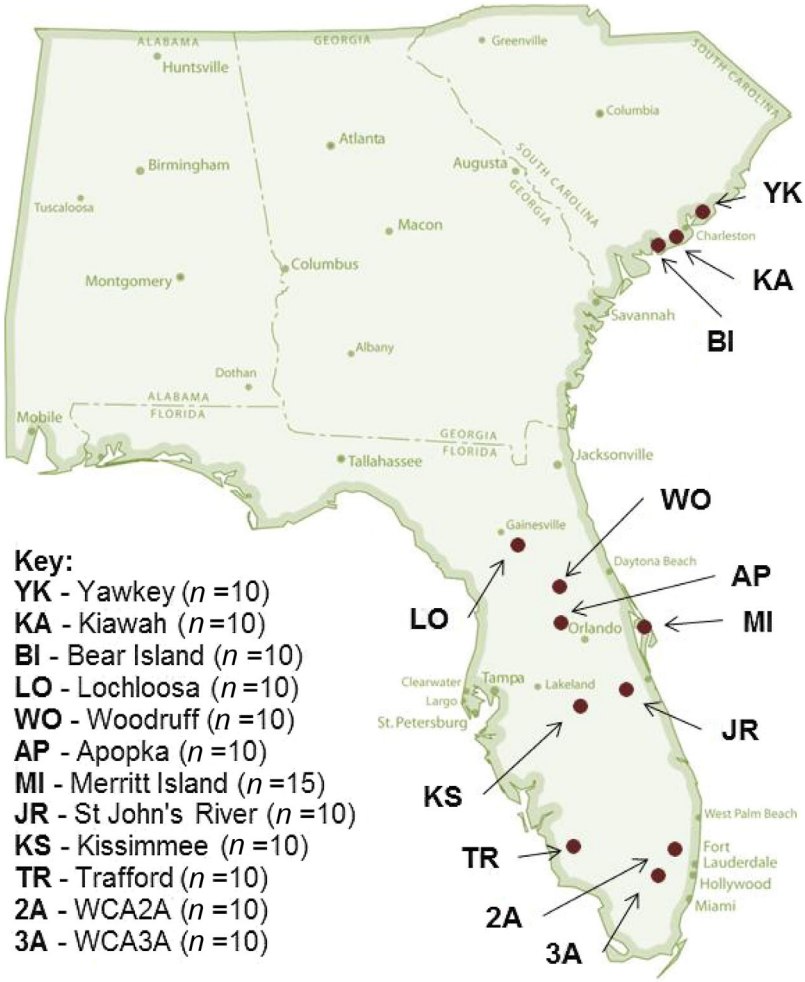

Figure 1. Map showing the 12 sites (SC and FL, USA) from which American alligators (*Alligator mississippiensis*) were sampled in the present study ($n = 125$) during the years 2012 to 2015. Collection sites are listed in decreasing latitude. WCA = Everglades Water Conservation Area.

Quality control

All alligator plasma samples were processed alongside quality control material NIST SRM 1958 to determine the accuracy and precision of the method. The PFAA levels of SRM 1958, processed during our extraction, had to be within the 95% confidence interval as reported on the certificate of analysis. All samples were also processed alongside blanks to assess any background contamination that might be present in the laboratory or a result from the extraction method. Compounds were considered to be above the reporting limit if the mass of an analyte in the sample was greater than the mean plus 3 standard deviations of all blanks.

Statistical methods

All statistical analyses were performed using SPSS statistic 22 (IBM). Statistical tests were performed for the compounds detected in more than 75% of the samples: PFNA, PFDA, PFUnA, PFDoA, PFTriA, PFTA, PFHxS, and PFOS. Unlike previous environmental studies, where PFOA had the second highest concentration measured, PFOA was detected at much lower frequency (detected in only 65% of the samples analyzed). With a full one-third of PFOA measurements falling below the limit of detection, PFOA was excluded from statistical analysis, along with the remaining chemicals (PFHpA, PFHxA, PFPeA, PFBS, and PFBA), which were detected in <2% of the samples. For those PFAAs included in statistical analysis, compounds below the limit of detection were set equal to one-half of the limit of detection prior to running the statistical tests [29].

Sex-based differences of PFAAs in Florida and South Carolina were investigated using univariate analysis of variance with log normally distributed concentration values, and a Friedman's test was used for the PFAAs with non-normally distributed concentration values. Site was set as the nuisance factor, sex as the treatment, and PFAA concentration as the dependent variable. These tests simulated a randomized block design for the collected data. Other parametric tests employed for data analysis of sex-based differences, on a site-by-site basis, and analysis of site differences for PFAA levels included a *t* test and one-way analysis of variance when data were normal or log-normal and Friedman rank test, Mann-Whitney *U* test, and Kruskal-Wallis test when data remained non-normal following log transformation. Pearson correlation and Spearman correlation were used when applicable for correlative measures.

RESULTS AND DISCUSSION

In the present study, we collected a total of 125 plasma samples from alligators at multiple sites in Florida and South Carolina to examine PFAA concentrations in animals from different localities. Of the 15 PFAAs included in the present analysis, all samples contained at least 6 PFAAs. The following 5 PFAAs were detected in every plasma sample analyzed

(in order of highest median concentration to lowest median concentration, among all sites): PFOS (median, 11.2 ng/g; range, 1.36–452 ng/g), PFUnA (median, 1.58 ng/g; range, 0.314–18.4 ng/g), PFDA (median; 1.20 ng/g, range; 0.169–15.1 ng/g), PFNA (median, 0.528 ng/g; range, 0.155–1.40 ng/g), and PFHxS (median, 0.288 ng/g; range, 0.057–23.3 ng/g) (Table 1; Supplemental Data, Table S2). Also, PFDoA, PFTriA, PFTA, and PFOA were detected frequently in alligator plasma samples (in more than 96%, 94%, 75%, and 65%, respectively): PFDoA (median, 0.363 ng/g; range, <0.009–7.27 ng/g), PFTriA (median, 0.416 ng/g; range, <0.026–2.60 ng/g), PFTA (median, 0.050 ng/g; range, <0.008–1.38 ng/g), and PFOA (median, 0.064 ng/g; range, <0.008–0.412 ng/g) (Table 1; Supplemental Data, Table S2). The 9 PFAAs commonly measured over the limit of detection resulted in unique fingerprints for each site (Supplemental Data, Figure S1), which are discussed in the

Table 1. Alligator perfluoroalkyl acid plasma concentrations (ng/g wet mass) at 12 sites from Florida and South Carolina, USA

	Yawkey ($n = 10$)			Kiawah Island ($n = 10$)			Bear Island ($n = 10$)		
	Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]
PFOA	<0.008[b]–0.193	0.050	4	0.028–0.298	0.126	10	<0.008[b]–0.193	<0.100	3
PFNA	0.272–1.32	0.620	10	0.446–1.38	1.19	10	0.155–1.14	0.472	10
PFDA	2.27–15.1	5.88	10	3.72–13.6	6.26	10	0.998–3.21	1.57	10
PFUnA	1.89–18.4	6.25	10	1.87–7.53	3.93	10	1.05–5.02	2.32	10
PFDoA	0.362–3.45	1.01	10	1.32–7.27	3.05	10	0.231–1.88	0.559	10
PFTriA	<0.070[b]–1.85	0.646	8	0.420–2.60	0.919	10	<0.070[b]–1.83	0.674	9
PFTA	<0.082[b]–0.774	0.241	7	0.198–1.38	0.476	10	<0.081[b]–0.733	0.095	7
PFHxS	0.099–0.566	0.353	10	0.313–1.86	0.620	10	0.077–0.824	0.304	10
PFOS	4.50–57.0	20.2	10	38.4–98.2	55.8	10	10.0–44.9	19.5	10
	Lochloosa Lake ($n = 10$)			Lake Woodruff ($n = 10$)			Lake Apopka ($n = 10$)		
	Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]
PFOA	<0.008[b]–0.132	0.071	9	<0.097[b]–0.184	0.062	5	<0.096[b]–0.152	0.126	7
PFNA	0.328–1.19	0.676	10	0.282–1.34	0.578	10	0.251–1.40	0.648	10
PFDA	0.238–1.00	0.615	10	0.350–5.06	2.01	10	0.169–2.44	1.12	10
PFUnA	0.580–1.56	1.03	10	0.633–3.33	1.43	10	0.614–3.39	1.65	10
PFDoA	0.105–0.309	0.182	10	<0.166[b]–0.810	0.317	9	<0.157[b]–0.831	0.315	9
PFTriA	0.181–0.580	0.309	10	<0.070[b]–0.854	0.259	8	0.189–1.00	0.450	10
PFTA	<0.008[b]–0.060	0.018	7	<0.008[b]–0.146	0.029	4	<0.080[b]–0.194	0.049	7
PFHxS	0.069–0.201	0.093	10	0.130–0.623	0.445	10	0.166–0.449	0.332	10
PFOS	2.19–6.16	4.21	10	5.89–41.2	16.0	10	1.98–15.8	11.4	10
	Merrit Island ($n = 15$)			St. Johns River ($n = 10$)			Lake Kissimmee ($n = 10$)		
	Range	Median	$n > RL$[a]	>Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]
PFOA	<0.096[b]–0.412	0.155	7	0.010–0.160	0.080	10	<0.008[b]–0.142	0.104	9
PFNA	0.298–1.10	0.611	15	0.250–1.04	0.471	10	0.275–1.18	0.642	10
PFDA	0.395–3.50	1.02	15	0.492–1.72	1.17	10	0.417–3.15	1.26	10
PFUnA	0.844–5.45	1.82	15	0.655–2.20	1.28	10	0.314–2.47	1.03	10
PFDoA	<0.543[b]–1.07	0.418	14	0.156–0.591	0.362	10	<0.009[b]–0.382	0.147	9
PFTriA	<0.026[b]–1.42	0.654	14	0.173–0.739	0.267	10	0.122–0.677	0.251	10
PFTA	<0.080[b]–0.257	0.076	6	<0.008[b]–0.131	0.022	8	<0.009[b]–0.104	0.025	9
PFHxS	0.684–23.3	3.83	15	0.100–0.308	0.166	10	0.338–1.50	0.505	10
PFOS	38.6–452	99.5	15	3.41–10.2	7.13	10	6.51–25.1	12.2	10
	Lake Trafford ($n = 10$)			WCA-2A ($n = 10$)			WCA-3A ($n = 10$)		
	Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]	Range	Median	$n > RL$[a]
PFOA	0.021–0.117	0.091	10	<0.008[b]–0.077	0.036	2	<0.008[b]–0.042	0.033	6
PFNA	0.239–0.936	0.484	10	0.189–0.382	0.234	10	0.172–0.388	0.301	10
PFDA	0.275–2.05	0.885	10	0.641–2.26	0.900	10	0.406–1.46	0.912	10
PFUnA	0.463–2.19	0.953	10	0.958–3.15	1.43	10	0.719–2.48	1.45	10
PFDoA	0.073–0.737	0.210	10	0.277–0.949	0.392	10	0.172–0.631	0.371	10
PFTriA	0.111–0.528	0.304	10	0.232–0.702	0.370	10	0.162–0.594	0.280	10
PFTA	<0.008[b]–0.096	0.039	9	0.031–0.188	0.109	10	0.011–0.148	0.042	10
PFHxS	0.071–0.320	0.119	10	0.080–0.172	0.112	10	0.057–0.303	0.105	10
PFOS	4.21–14.3	7.82	10	1.36–6.23	2.65	10	1.57–4.71	3.81	10

[a]$n > RL$ indicates the number of samples above the reporting limit (RL).
[b]Values were calculated with one-half the reporting limit substituted for nondetects, as described in *Materials and Methods*; for values shown as "<," however, a specified number describe the actual reporting limit.
PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFUnA = perfluoroundecanoic acid; PFDoA = perfluorododecanoic acid; PFTA = perfluorotetradecanoic acid; PFTriA = perfluorotridecanoic acid; PFHxS = perfluorohexanesulfonic acid; PFOS = perfluorooctane sulfonate; WCA = Water Conservation Area.

section *Site differences*. The shorter-chain PFAAs (PFHpA, PFHxA, PFPeA, PFBS, and PFBA) were detected infrequently (<2% of the samples) and, therefore, not included in any statistical analysis.

Sex differences

As a whole, across all sites, male alligators exhibited significantly higher concentrations of several PFAAs in plasma compared with females as a group: PFOS ($p = 0.01$), PFDA ($p = 0.0003$), PFUnA ($p = 0.021$), and PFTriA ($p = 0.021$) (Supplemental Data, Figure S2). At some individual sites, however, PFAA concentrations were significantly higher in females (e.g., PFOS at AP, PFUnA at KA).

In a population of captive Chinese alligators (*Alligator sinensis*), Wang et al. [21] found the highest PFAA concentration in serum to be that of PFUnA rather than PFOS, the PFAA with the highest concentrations in the present study. However, similar to the present study, male Chinese alligators contained significantly higher concentrations of PFOS and PFUnA compared with females. Wang et al. [21] did not find a sex-based difference for PFDA in Chinese alligators. Christie et al. [22] did not find any sex-based differences in their population of Nile crocodiles (*Crocodylus niloticus*) in South Africa. It is possible that sex-based differences observed for certain PFAAs in alligators are the result of a differential clearance of these contaminants between males and females, as has been observed in rats [30], mice [31], and other mammals [32]. It is also possible that females may offload PFAAs during oviposition, reducing their PFAA body burden compared with males at the same locality. This possibility is supported by studies reporting measurable concentrations of PFAAs in eggs of herring gulls (*Larus argentatus*) [33] and Nile crocodiles [20], confirming maternal transfer of PFAAs in oviparous species. Sex-specific differences in PFAA concentrations may also be the result of differential habitat use by adult males and females, a phenomenon common among crocodilians [34–37]. In such cases, differences in prey availability and contamination between and among habitats within a site could result in different PFAA exposures in males and females.

Because no sex-specific differences in PFOA, PFNA, PFHxS, PFDoA, and PFTA concentrations were observed as a whole (all sites combined), sex-based differences were examined on a site-by-site basis (Supplemental Data, Table S3). Overall, only a few sites exhibited sex-based differences for these 5 PFAAs (Supplemental Data, Figure S3). At LO, male alligators had significantly higher PFNA ($p = 0.016$), PFTA ($p = 0.032$), and PFDoA ($p = 0.032$) plasma concentrations compared with females; and at MI males had significantly higher PFOA ($p = 0.047$) plasma concentrations than females. Interestingly, PFHxS was the only PFAA for which females exhibited significantly higher plasma concentrations (YK, $p = 0.008$; TR, $p = 0.008$) when compared with males (Supplemental Data, Figure S3). It is important to note that our examination of sex-based differences in PFAA concentrations may have been influenced by small sample sizes; in almost all cases, only 5 males and 5 females were sampled per site.

Site differences

Because sex-based differences in PFAA concentrations were observed among alligator plasma samples, site differences were determined separately for males and females. All of the 9 detected PFAAs displayed at least some minor site differences. The PFAAs that displayed the most notable site differences (the highest number of statistically significant groups between the 12 sites) were PFOS, PFDA, and PFHxS. Of those, PFOS exhibited the greatest statistical difference across sites (Figure 2). This is likely because PFOS is generally the most abundant PFAA in the environment. For male alligators only, concentrations of PFOS in plasma ranged from 1.57 ng/g to 452 ng/g. Concentrations of PFOS were highest at MI (median, 106 ng/g) and KA (median, 56.4 ng/g). Males from MI exhibited significantly higher PFOS concentrations compared with males from all other sites, with the exception of KA. In addition, the individual alligator with the highest overall PFOS concentration measured in the present study (452 ng/g plasma) was from MI. After MI, males from South Carolina (KA, YK, and BI) exhibited higher PFOS concentrations than Florida males, with the exception of WO. Some of the lowest PFOS concentrations observed in males in the present study were measured at sites 2A, 3A, LO, and JR.

For female alligators, PFOS concentrations in plasma ranged from 1.36 ng/g to 206 ng/g. Similar to males, females from sites MI (median, 85.5 ng/g) and KA (median, 51.3 ng/g) exhibited significantly higher PFOS concentrations compared with the other sites examined, and the individual female with the highest PFOS concentration was from MI (206 ng/g plasma). After MI and KA, females from the 2 other South Carolina sites (YK and BI) exhibited higher PFOS concentrations than females from

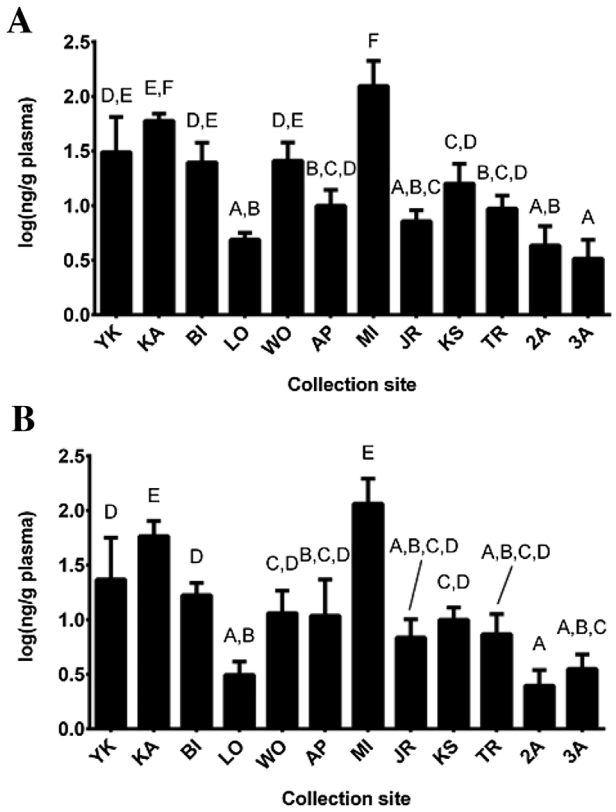

Figure 2. Site comparison of mean (±standard deviation) perfluorooctane sulfonate concentrations (log) in (**A**) male and (**B**) female American alligator (*Alligator mississippiensis*) plasma from multiple sites in Florida and South Carolina. Letters above bars represent statistically significant differences between groups ($p < 0.05$). Samples are listed from left to right in decreasing latitude. 2A/3A = Everglades Water Conservation Areas 2A and 3A; AP = Lake Apopka; BI = Bear Island Wildlife Management Area; JR = St. Johns River; KA = Kiawah Island; KS = Lake Kissimmee; LO = Lochloosa Lake; MI = Merritt Island National Wildlife Refuge; TR = Lake Trafford; WO = Lake Woodruff; YK = Tom Yawkey Wildlife Center.

Florida, with the exception of WO and AP. Some of the lowest PFOS concentrations observed in females in the present study were measured at sites 2A, 3A, LO, JR, and TR.

The concentrations of PFHxS detected in alligator plasma in the present study exhibited a similar trend to PFOS across sites but on a reduced scale (Supplemental Data, Table S4). For males, PFHxS plasma concentrations ranged from 0.057 ng/g to 23.3 ng/g. Males from MI (median, 3.95 ng/g) had significantly higher PFHxS concentrations than those at any other site examined, and the individual male with the highest PFHxS concentration was from Merritt Island National Wildlife Refuge (23.3 ng/g). Males from Kiawah Island and Lake Kissimmee followed closely but were still statistically grouped with other sites (Lake Apopka, Lake Woodruff, and Bear Island). The lowest PFHxS concentrations in males were typically measured at sites 2A, 3A, Lochloosa Lake, and Lake Trafford. For female alligators, PFHxS concentrations in plasma ranged from 0.069 ng/g to 10.0 ng/g. Similar to males, MI females exhibited significantly higher PFHxS concentrations than those at all other sites. Females from KA and KS had the next highest concentrations but were still statistically grouped with those from other sites (AP, WO, and YK). The lowest PFHxS concentrations in females were typically observed at sites 2A, 3A, and LO.

Across the sampling sites, PFDA had a unique signature, one that varied from the patterns observed for plasma PFOS and PFHxS concentrations (Supplemental Data, Table S4). For male alligators, PFDA concentrations ranged from 0.498 ng/g to 15.1 ng/g. KA males had significantly higher PFDA concentrations overall (median, 6.21 ng/g) compared with all sites, with the exception of YK (median, 6.20 ng/g). Males from YK exhibited the next highest PFDA concentrations, but these were not significantly different from those detected in WO males (median, 2.02 ng/g). Males from many of the remaining sites had similarly low concentrations of PFDA. Overall, LO males (median, 0.792 ng/g) had some of the lowest PFDA concentrations of all the sampling sites. For female alligators, PFDA plasma concentrations ranged from 1.69 ng/g to 14.3 ng/g. The 2 sites with the highest (statistically significant) PFDA concentrations in females were also in South Carolina: KA (median, 6.32 ng/g) and YK (median, 5.55 ng/g). Concentrations of PFDA at BI (median, 1.18 ng/g) and WO (median, 1.84 ng/g) followed closely behind but were not significantly different from the other sites sampled. Like males, LO females (median, 0.501 ng/g) had some of the lowest PFDA concentrations across all sites.

Overall, male and female alligators from both MI and KA exhibited some of the highest PFOS concentrations measured to date in a crocodilian species (median PFOS concentrations in plasma: MI males = 106 ng/g; MI females = 85.5 ng/g; KA males = 56.4 ng/g; KA females = 51.3 ng/g). In comparison, the mean PFOS concentration in serum from captive Chinese alligators was 28.7 ng/mL (28.0 ng/g) [21], whereas the median concentrations in wild Nile crocodiles at several sites in South Africa ranged from 4.31 ng/g to 50.3 ng/g [22]. In a study of other reptiles, loggerhead sea turtles (*Caretta caretta*) along the coast of South Carolina and Florida exhibited median PFOS plasma concentrations of 2.87 ng/g and 3.80 ng/g, respectively [38]. In another study, hawksbill sea turtles (*Eretmochelys imbricata*) off of Juno Beach (FL, USA), south of MI, showed higher than expected PFOS levels at 11.9 ng/g [39] when compared with several other species of turtles along the east coast. The authors discuss geographic differences as possible reasons for the higher levels of PFOS in the hawksbill. Comparing the results Keller et al. [39] found for hawksbill with the present results would suggest that there might be a potential source of PFOS off the east coast of Florida. It is possible that, for the present study, the high concentrations of PFOS and PFHxS detected in male and female alligators at MI may be related to the aeronautic facilities located in and around MI, which comprise a large part of Florida's Kennedy Space Center. The past use of aqueous film-forming foams at Kennedy Space Center may have played a role in PFAAs in the surrounding environment and wildlife. Historically, aqueous film-forming foams have been shown to contain PFAAs, such as PFOS and PFHxS, as well as a number of other proprietary PFAA mixtures [7], and can be resistant to remediation [9]. Perfluoroalkyl acids have been measured in firefighters [40], in wildlife [6], and downstream of their use [41]. Potential sources of PFOS and PFHxS at KA are more speculative. In addition, it should be noted that, with the exclusion of MI, alligators from the South Carolina sites (BI, YK, and KA) had some of the highest PFOS concentrations compared with the Florida sites. In Florida, WO exhibited mid to high concentrations of PFOS, PFHxS, and PFDA compared with other sites sampled. For many years, WO has been used as a reference site for multiple studies on alligator ecotoxicology because of its relatively low concentrations of organochlorine contaminants, such as DDT, its metabolites, and other organochlorine pesticides [42]. The results of the present study indicate that WO would not be a suitable reference site for future studies involving PFAAs. In contrast to WO, sites 2A and 3A, which are located in the Everglades, exhibited some of the lowest concentrations of PFOS and PFHxS measured in Florida. Surprisingly similar levels of PFOS were found in loggerhead sea turtles (3.67 ng/g) from Florida Bay close to the 2A and 3A alligator sampling sites [38]. Interestingly, whereas PFAA concentrations appear to be relatively low in 2A and 3A alligators, the same adult alligators at these sites have been reported to contain some of the highest mercury concentrations in Florida and throughout the range of the species [25,43,44].

Correlations

For all alligators included in the present study, snout–vent length was uniform across sites for males and nearly uniform across sites for females (Supplemental Data, Figure S4). Thus, data from all sites were combined within each sex to investigate relationships between PFAA concentration and alligator snout–vent length. Because MI had a very different pattern of PFAAs compared with the other sites, correlations were run with and without this site included. The significance did not change based on the inclusion or exclusion of this site, and thus it was included in the analysis. Correlations comparing both male snout–vent length and female snout–vent length to PFAAs resulted in a number of significant positive correlations (Table 2). Overall, females exhibited higher correlation coefficients between PFAA concentration and snout–vent length when compared with males. The highest correlation coefficients for females were with PFTriA, which explained 57.0% of the variation, followed closely by PFOS, which explained 55.1% of the variation. In contrast, the highest correlation coefficients for male snout–vent length and PFAA concentration were for PFUnA, which explained 35.5% of the variation, followed closely by PFOS, which explained 33.1% of the variation. This would seem to refute the hypothesis that female alligators may have a lower PFAA burden than male alligators as a result of maternal transfer of PFAAs during oviposition. Collectively, these data suggest that concentrations of some PFAAs in adult

Table 2. Correlation coefficients between plasma perfluoroalkyl acid concentrations and snout–vent length for American alligators (*Alligator mississippiensis*) sampled in Florida and South Carolina, USA[a]

Snout–vent length	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTA	PFTriA	PFHxS	PFOS
Male ($n = 65$)	0.072	0.252[b,c]*	0.206	0.355[b]**	0.279[b]*	0.209	0.273[b]*	0.273[b]*	0.331[b,c]**
Female ($n = 60$)	0.133	0.261[b,c]*	0.443 [b,c]**	0.489[b]**	0.469[b]**	0.468[b,c]**	0.570[b]**	0.412[b]**	0.551[b,c]**

[a]All significant results were positively correlated. Values were calculated using log normal concentrations.
[b]Indicates significant correlation coefficients.
[c]Indicates a correlation coefficient determined using the Pearson correlation. All other correlation coefficients were determined using the Spearman correlation.
*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFUnA = perfluoroundecanoic acid; PFDoA = perfluorododecanoic acid; PFTA = perfluorotetradecanoic acid; PFTriA = perfluorotridecanoic acid; PFHxS = perfluorohexanesulfonic acid; PFOS = perfluorooctane sulfonate.

Table 3. Correlation coefficients between concentrations of various perfluoroalkyl acids in plasma of American alligators (*Alligator mississippiensis*) sampled in Florida and South Carolina ($n_{male} = 65$, $n_{female} = 60$)[a]

	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTriA	PFTA	PFHxS	PFOS
Male									
PFOA	—	0.615[b]**	0.226	0.092	0.036	0.152	0.181	0.260	0.386[b]**
PFNA		—	0.550[b]**	0.322[b]*	0.144	0.313[b]*	0.273[b]**	0.339[b]**	0.541[b]**
PFDA			—	0.840[b]**	0.743[b]**	0.439[b]**	0.654[b]**	0.307[b]*	0.550[b]**
PFUnA				—	0.920[b]**	0.783[b]**	0.826[b]**	0.445[b]**	0.654[b]**
PFDoA					—	0.751[b]**	0.846[b]**	0.316[b]*	0.528[b]**
PFTriA						—	0.770[b]**	0.395[b]**	0.489[b]**
PFTA							—	0.238	0.399[b]**
PFHxS								—	0.827[b]**
PFOS									—
Female									
PFOA	—	0.648[b]**	0.332[b]*	0.186	0.098	0.064	−0.003	0.441[b]**	0.440[b]**
PFNA		—	0.585[b]**	0.444[b]**	0.365[b]**	0.339[b]**	0.196	0.387[b]**	0.538[b]**
PFDA			—	0.890[b]**	0.827[b]**	0.529[b]**	0.560[b]**	0.337[b]**	0.595[b]**
PFUnA				—	0.938[b]**	0.684[b]**	0.578[b]**	0.331[b]*	0.691[b]**
PFDoA					—	0.763[b]**	0.708[b]**	0.226	0.635[b]**
PFTriA						—	0.713[b]**	0.190	0.598[b]**
PFTA							—	0.130	0.454[b]**
PFHxS								—	0.654[b]**
PFOS									—

[a]Values were calculated using log normal concentrations. All significant correlations resulted in positive correlation coefficients.
[b]Indicates significant correlation coefficients.
*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFUnA = perfluoroundecanoic acid; PFDoA = perfluorododecanoic acid; PFTriA = perfluorotridecanoic acid; PFTA = perfluorotetradecanoic acid; PFHxS = perfluorohexanesulfonic acid; PFOS = perfluorooctane sulfonate.

American alligators increase with increasing body size in both males and females. Conversely, Wang et al. [21] found that PFAA (specifically PFUnA, PFDA, and PFNA) concentrations decreased with increasing body size (total length). These observed differences between American and Chinese alligators may be the result of many factors, including the combination of including animals from all sites in the present study, interspecific differences in food consumption, growth rate differences, and differences in body size [45], as well as differences in toxicodynamics and toxicokinetics of PFAAs. In addition, differences in diet and numerous environmental variables between wild (present study) and captive [22] alligators may influence growth and body burdens of PFAAs.

With all sites combined for each sex, significant correlations were observed between different PFAAs measured in plasma, suggesting somewhat similar sources of PFAA contamination across the sampling localities. The varying levels of PFAA contamination from site to site are likely the result of varying distances from these potential PFAA sources. Some correlative relationships between the PFAAs were stronger than others (Table 3). Of all the PFAAs, correlations between PFUnA and PFDoA for male ($p < 0.01$, $r = 0.920$) and female ($p < 0.01$, $r = 0.938$) alligators across the sites were the most highly significant relationships observed in the present study.

CONCLUSIONS

The present study is the first to quantitate PFAA concentrations in American alligators and one of the few studies to quantitate PFAAs in crocodilians [20–22]. All alligator samples ($n = 125$) contained the 5 following PFAAs: PFOS (median, 11.2 ng/g; range, 1.36–452 ng/g), PFUnA (median, 1.58 ng/g; range, 0.314–18.4 ng/g), PFDA (median, 1.20 ng/g; range, 0.169–15.1 ng/g), PFNA (median, 0.528 ng/g; range, 0.155–1.40 ng/g), and PFHxS (median, 0.288 ng/g; range, 0.057–23.3 ng/g). The present findings support sex-based differences in PFOS and PFUnA concentrations previously observed in captive Chinese alligators [21], while demonstrating opposite relationships between PFAA concentration and body size for American (wild) and Chinese (captive) alligators.

The high number of significant PFAA-to-PFAA correlations suggests common point sources throughout the sampling sites in Florida and South Carolina. The present study also reveals potential hot spots for various PFAAs (e.g., PFOS at KA and MI) that warrant further investigation and provides another contaminant of concern to be combined with organochlorines, metals, and others when assessing overall anthropogenic impacts on ecosystem health.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3600.

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Disclaimer—Certain commercial equipment or instruments are identified to specify adequately the experimental procedures. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the equipment or instruments are the best available for the purpose.

Data availability—Data are available upon request from the author (jessica.reiner@nist.gov). Additional sample information and data are provided in Supplemental Data.

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Environmental Toxicology

Determinants of Legacy Persistent Organic Pollutant Levels in the European Pond Turtle (*Emys orbicularis*) in the Camargue Wetland, France

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Abstract: Many banned persistent organic pollutants (POPs) remain for decades in the aquatic environment and can have harmful effects on long-lived predators because of their high bioaccumulation and biomagnification potentials. We investigated the occurrence and levels of 18 polychlorinated biphenyls (PCBs) and 16 organochlorine pesticides in European pond turtles ($n = 174$) from April to July 2018 in the Camargue wetland, France. Although the Camargue was highly contaminated in previous decades, plasma occurrence and levels of POPs were very low: we were able to quantify only 3 of the 34 compounds we analyzed in >10% of the turtles. The burdens from POPs did not differ between males and females and were uncorrelated with sampling date and body mass. We observed differences in POP burdens between turtles from the 2 sampling sites. One possible explanation is that the sampling sites were in different agricultural hydraulic systems: plasma occurrence and levels were higher for PCB-52 and hexachlorobenzene in turtles captured in drainage channels and for PCB-153 at the site that receives irrigation. Finally, the occurrence and levels of PCB-153 in turtles increased with age, likely because of bioaccumulation and much higher exposure 20 to 30 yr ago than now. *Environ Toxicol Chem* 2021;40:2261–2268. © 2021 SETAC

Keywords: Reptiles; Polychlorinated biphenyl; Organochlorine pesticides; Delta wetlands

INTRODUCTION

In Europe, the production and use of persistent organic pollutants (POPs) have been prohibited or severely restricted since 2004 through the Stockholm Convention, but POPs still cause several environmental concerns today. Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) remain in the environment for decades because of their slow biodegradation and because of improper handling of contaminated wastes. Levels of POPs in animals increase with age (Vives et al. 2005; Binnington and Wania 2014) and trophic level (Goutte et al. 2020). Exposure to POPs causes a wide range of adverse effects, including neurotoxicity, endocrine disruption, immune dysfunction, reproductive impairment, and developmental abnormalities, which may ultimately compromise survival and reproductive output and lead to population declines of wild vertebrates (Goutte et al. 2014, 2015; Salice et al. 2014).

Pollution, especially from industrial and agricultural discharges, is one of the major threats to freshwater ecosystems (Holt 2000; Dudgeon et al. 2006). Wetlands support an extremely rich biodiversity but are among the most transformed and threatened ecosystems of the world (Revenga et al. 2005), experiencing rates of population decline and species extinction far higher than those in forests, grasslands, and coastal ecosystems (Dudgeon et al. 2006). Deltas are often exposed to high water pollution from intensive agriculture (Kuenzer and Renaud 2012) and from discharge of contaminants in upstream waters. The Camargue, in the Rhône River delta, is the largest wetland in France and is of international importance under the Ramsar Convention (Conference of the Contracting

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Parties 1971); but it is heavily impacted by human activities (Cheiron et al. 2018). The contamination of water bodies by agricultural, industrial, and urban discharges has been well studied over the past decades (Comoretto et al. 2007). In particular, levels of PCBs in sediments of the Rhône River increase from upstream to downstream, reaching 417 µg/kg dry weight (for the sum of 7 PCB congeners: 28, 52, 101, 118, 138, 153, and 180 [Mourier et al. 2014]). Mean flux over the 2011 to 2016 period was 14 kg/yr for PCB-180 in suspended particulate matter at the outlet of the Rhône River (Poulier et al. 2019). Previous studies in the Camargue confirmed that both birds (Berny et al. 2002) and fish (Roche et al. 2002, 2003, 2009b) are exposed to OCPs and PCBs.

Freshwater turtles remain poorly studied in ecotoxicology, although they can provide useful information on local contamination because of their longevity (Campbell and Campbell 2002; El Hassani et al. 2019; Gaus et al. 2019), high trophic level, and low dispersal capacity (Châteauvert et al. 2015; Ming-ch'eng Adams et al. 2016). Moreover, as ectothermic vertebrates, turtles have a lower ability to metabolize pollutants than endothermic mammals and birds (de Solla 2015). The European pond turtle, *Emys orbicularis*, a long-lived (>40–80 yr) opportunistic predator (feeding on fish, amphibians, aquatic insect, gastropods, and crayfish) and scavenger (Ottonello et al. 2005; Ficetola and De Bernardi 2006; Ziane et al. 2020), is facing significant population declines as a result of multiple environmental alterations, including water pollution (Cheylan 1998). The European pond turtle is listed as "near threatened" on the International Union for Conservation of Nature's Red List of threatened species (Tortoise & Freshwater Turtle Specialist Group 1996). Pollution by trace metal elements has been studied in *E. orbicularis* (Namroodi et al. 2017; Guillot et al. 2018; Beau et al. 2019), but data are lacking on burdens of POPs.

We studied contamination levels in 2 populations of *E. orbicularis* in the Natural Reserve of the Tour du Valat in the Camargue, France. Several individuals were of known age owing to a capture–mark–recapture program initiated more than 20 yr ago (Olivier et al. 2010; Ficheux et al. 2014; Arsovski et al. 2018). The hydraulic system consists of irrigation canals originating from the Rhône River and of drainage canals, which receive various environmental contaminants from the Rhône River and agricultural plots, especially rice fields (Chauvelon 1996). In the present study, we assessed recent (2018) levels of OCPs and PCBs in plasma of the European pond turtle ($n = 174$). We also tested the effects of individual traits (mass, sex, and age) on POP burdens. We expected that older and larger individuals should have higher levels of POPs because of bioaccumulation and length of exposure.

Moreover, we compared POP burdens of turtles from 2 populations in different locations in the agricultural hydraulic system (irrigation vs drainage). We expected high levels of OCPs in turtles from the drainage site, as a possible consequence of the remobilization of pesticides from agricultural soils, and high levels of PCBs in turtles from the irrigation channel, as a possible consequence of the historical contamination. We acknowledge the limitations to our inferences about the possible causes of site differences in POP burdens because we had a single site in irrigation canals and a single site in drainage canals. Therefore, we are unable to disentangle site effects from hydrologic effects. Consequently, we only cautiously offer some plausible explanations for site differences.

MATERIAL AND METHODS

Sampling sites and capture

We conducted the study in the Natural Reserve of the Tour du Valat (43°30′N, 4°40′E; Figure 1) in France. We captured European pond turtles ($n = 174$) in canals and marshes from 24 April to 26 July 2018 by hand or with funnel traps (Olivier et al. 2010; Ficheux et al. 2014). We captured turtles at 2 sites: 1) irrigation canals and their associated marshland (site of Esquineau, $n = 126$), and 2) drainage canals of the Fumemorte basin and marshes filled with water by these canals (site of Faïsses, $n = 48$). The sex ratio was the same between the 2 sites (58.3% females at Faïsses and 60.3% females at Esquineau, Pearson's chi-squared test, $p = 0.95$).

Individual turtles were identified as part of a long-term capture–mark–recapture program with unique combinations of shallow notches on the marginal and nuchal scales (Olivier 2002; Olivier et al. 2010). Individuals were weighed and sexed by visual observation of the secondary sexual characteristics. The year of birth can be determined if the first capture occurred within the first 5 yr of life by counting the number of growth streaks (Castanet 1988). The long-term capture–mark–recapture program started in 1997, but a few dozens individuals were marked as adults in 1976. In our data set, 66 of the 174 individuals were of known age (5–26 yr old, corresponding to a first capture between 1997 and 2018), and some were first captured as adults in 1976, thus being considered more than 44 yr old. When turtles were first captured after their first 5 yr of life ($n = 108$), we determined stages of growth based on growth streaks on the plastron (Olivier 2002). By combining known ages and stages of growth, we assigned an age class to each turtle: 4 to 8 yr ($n = 20$), 9 to 13 yr ($n = 46$), 14 to 22 yr ($n = 54$), and 23 to 44+ yr ($n = 54$). Turtles were released on the same day at their capture site.

Blood sampling

Plasma is a good matrix to determine individual burdens of POPs because blood sampling is minimally invasive and because circulating levels of POPs in blood are significantly correlated to concentrations of POPs stored in fat (Keller et al. 2004; Dabrowska et al. 2006) and in liver, kidney, and muscle tissues (Van de Merwe et al. 2010). We collected blood samples (2 mL, always <1% of the turtle body mass) from the dorsal coccygeal vein (Keller et al. 2004; Innis and Knotek 2020) with a Terumo® syringe preimpregnated with heparin to prevent blood clotting during collection and equipped with a 25G needle. We then centrifuged the samples to separate the plasma from the red blood cells. Samples were stored at −18 °C until analysis in the UMR 7619 METIS of Sorbonne Université.

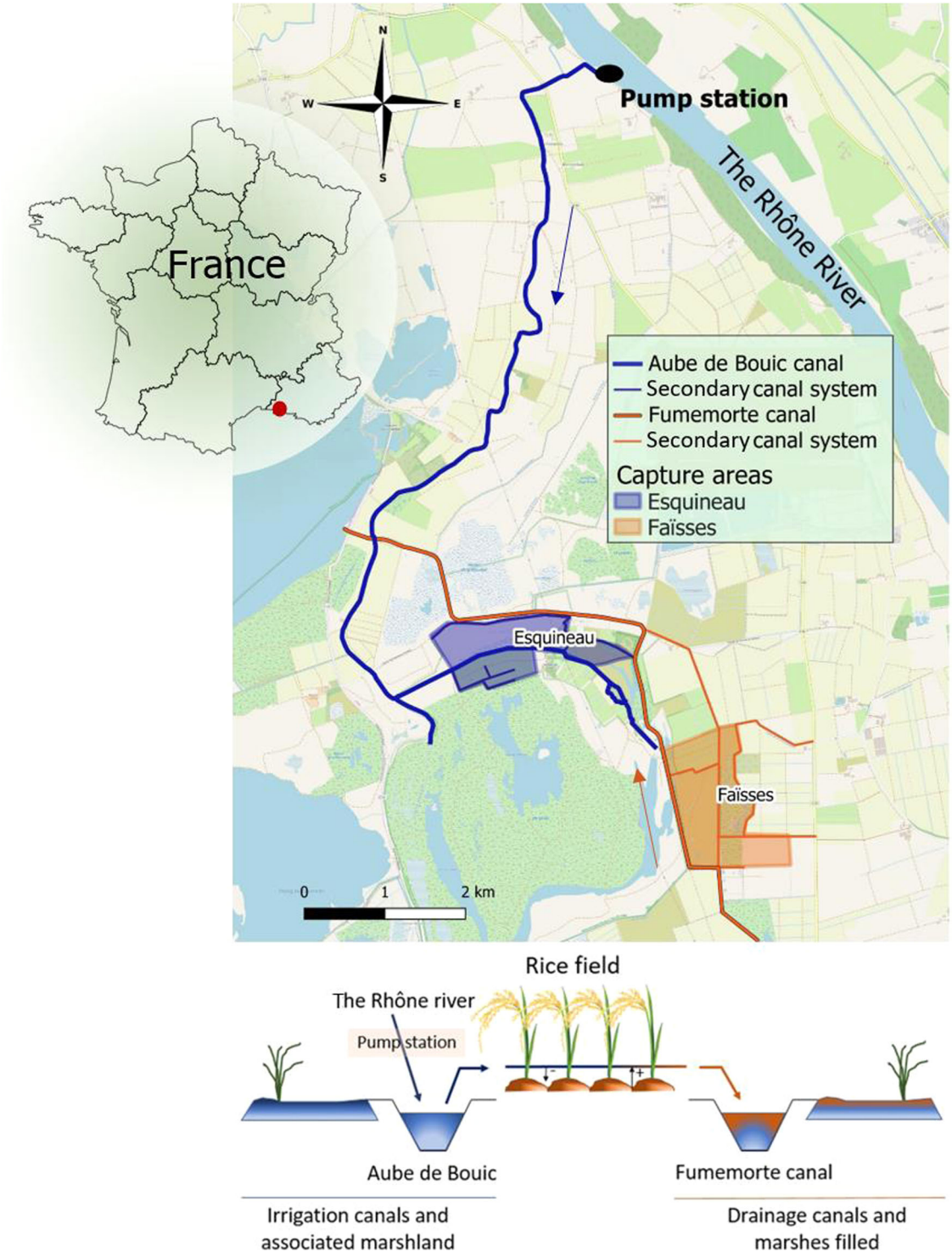

FIGURE 1: Sampling locations for European pond turtles in the Regional Nature Reserve of Tour du Valat, Camargue, France. The 2 capture locations are Esquineau (in blue), irrigated with water pumped from the Rhône River, and Faïsses (in orange), consisting of drainage canals of agricultural parcels.

Chemical analyses

We determined the levels of 17 OCPs (p,p′-dichlorodiphenyltrichloroethane [DDT] and metabolites, p,p′-dichlorodiphenyldichloroethylene [DDE] and p,p′-dichlorodiphenyldichlorethane [DDD]; pentachlorobenzene [PeCB]; hexachlorobenzene [HCB]; pentachloronitrobenzene [quintozene]; 4 isomers of hexachlorocyclohexane [α-, β-, γ- (lindane), δ-HCH]; aldrin; endrin; isodrin; telodrin [isobenzan]; heptachlor; heptachlor epoxide; heptachlor endo-epoxide), 7 marker PCBs (International Union of Pure and Applied Chemistry [IUPAC] nos. 28, 52, 101, 118, 138, 153, and 180), and 12 dioxin-like PCBs (DL-PCBs; IUPAC nos. 77, 81,

105, 114, 118, 123, 126, 156, 157, 167, 169, and 189) in the 174 plasma samples.

Samples were processed by solid phase extraction, using a hexane to dichloromethane ratio of 9:1, with a validated protocol (Tapie et al. 2011) that was adapted for plasma samples (see Supplemental Data for a detailed description of the procedure and method validations). PCBs and OCPs were analyzed using an Agilent 7890 A gas chromatograph coupled to a 7000 B triple quadrupole mass spectrometer system (Agilent Technologies). Recovery rates of compounds were assessed on replicate plasma samples of European pond turtles ($n = 4$) with spiked solutions (100 ng of each compound). The repeatability of the method was assessed in terms of relative standard deviation of the recovery (Supplemental Data, Table S2). Recovery rates were not satisfactory (<75 or >125%) for isodrin, endrin, heptachlor epoxid, heptachlor-endo-epoxide, p,p′-DDD, PCB-77, and PCB-189 (Supplemental Data, Table S2); and these chemicals were thus not considered further.

Statistical analyses

We performed statistical analyses with R software, Ver 3.3.2 (R Development Core Team 2016). Only quantifiable POPs (i.e., values higher than the limit of quantification [LOQ]) in at least 10% of the samples were included in the statistical analyses. Because age and mass were highly positively correlated ($t = 3.65$, $df = 64$, $p = 0.0005$) and females were significantly heavier than males ($W = 474$, $p < 0.0001$), effects of body mass, sex, and age were evaluated in separate analyses.

We used generalized linear models with a binomial distribution and a logit link function to test for the effects of sex, site, the interaction sex × site, sampling date, age, and body mass on contaminant occurrence. For each model, we used a backward elimination to progressively remove nonsignificant terms ($p > 0.05$). For all analyses, model specification and validation were based on residual analysis.

The effects of sex, sampling site, date, age, and body mass on pollutant concentrations were tested by using all data and applying statistical methods for left-censored data to handle values below the LOQ (Helsel 2005). To do so, group comparisons and linear regressions were performed using Peto-Prentice tests and tobit models, respectively, with the function cendiff of the NADA package and the function tobit of the AER R-package (Shoari and Dubé 2018).

RESULTS

POP levels and occurrence

In 37 of the 174 samples, which represented 10.4% of turtles sampled in Faïsses and 24.4% of turtles sampled in Esquineau, all contaminant levels were below the LOQ. Eleven compounds were never detected at levels above the LOQ in the plasma samples: PeCB, lindane, quintozene, heptachlor, and the DL-PCBs: 118, 105, 114, 126, 156, 157, and 167. The following 7 compounds were detected at levels above the LOQ at least in one sample from Esquineau but not in samples from Faïsses: alpha-HCH, 44′-DDT, and PCBs 28, 101, 123, and 169 (Table 1). Overall, the contaminant concentrations in the plasma samples were very low (Table 1).

Influence of individual traits, sampling site, and date on POP levels and occurrence

Only 3 POPs (HCB, PCB-52, and PCB-153) occurred commonly, being quantified in >10% of individuals (Table 1).

TABLE 1: Concentrations of organochlorine compounds (ng/mL wet wt) in plasma samples of European pond turtles from the 2 sites, Esquineau and Faïsses[a]

		Esquineau ($n = 126$)			Faïsses ($n = 48$)		
Variable	LOQ	Mean ± SD	Max.	*df* (%)	Mean ± SD	Max.	*df* (%)
Organochlorine pesticides							
α-HCH	1.9	0.04 ± 0.29	2.39	2	0.00 ± 0.00	0.00	0
β-HCH	0.9	0.08 ± 0.39	3.22	5	0.02 ± 0.14	0.98	2
δ-HCH	1.3	0.03 ± 0.23	2.19	2	0.08 ± 0.37	2.22	4
HCB	0.2	0.02 ± 0.10	0.77	7	0.07 ± 0.18	1.00	19
Aldrin	1	0.01 ± 0.09	1.03	1	0.02 ± 0.17	1.16	2
Isobenzan	0.6	0.02 ± 0.11	0.79	2	0.17 ± 0.50	2.02	13
4,4′-DDE	1.1	0.04 ± 0.29	2.33	2	0.05 ± 0.34	2.30	2
4,4′-DDT	2.4	0.05 ± 0.41	3.62	2	0.00 ± 0.00	0.00	0
PCBs							
PCB-28	1	0.14 ± 0.53	3.16	8	0.00 ± 0.00	0.00	0
PCB-52	0.2	0.54 ± 1.30	10.18	36	0.73 ± 0.91	3.80	69
PCB-81	0.9	0.02 ± 0.21	2.10	2	0.02 ± 0.17	1.16	2
PCB-101	2.3	0.06 ± 0.48	4.82	2	0.00 ± 0.00	0.00	0
PCB-123	2.6	0.02 ± 0.23	2.61	1	0.00 ± 0.00	0.00	0
PCB-138	1.8	0.23 ± 0.94	7.28	8	0.07 ± 0.27	1.27	6
PCB-153	0.2	0.22 ± 0.34	1.57	46	0.13 ± 0.25	1.03	29
PCB-169	1.4	0.01 ± 0.17	1.86	1	0.00 ± 0.00	0.00	0
PCB-180	0.3	0.02 ± 0.10	0.72	2	0.03 ± 0.14	0.83	4

[a]Means and standard deviation are calculated considering all the data (i.e., levels > limit of quantification [LOQ] and data <LOQ by assigning them zero for value).
HCH = hexachlorocyclohexane; HCB = hexachlorobenzene; 4,4′-DDE = 4,4′-dichlorodiphenyldichloroethylene; 4,4′-DDT = 4,4′-dichlorodiphenyldichlorethane; PCB = polychlorinated biphenyl; SD = standard deviation.

TABLE 2: Results from a generalized linear model with binomial distribution and a logit link function model with persistent organic pollutant levels having an occurrence >10% as dependent variables, as a function of sex, sampling site, and their interaction, sampling date, age, and mass

	PCB-52		PCB-153		HCB	
	χ^2	*p*	χ^2	*p*	χ^2	*p*
Sex	1.699	0.192	0.162	0.688	0.433	0.511
Site	14.347	<0.001	4.488	0.034	4.052	0.044
Date	1.289	0.256	0.951	0.329	3.828	0.050
Sex × site	2.659	0.103	0.285	0.594	0.306	0.580
Age class[a]	4.870	0.182	10.603	0.014	0.840	0.840
Mass[a]	2.119	0.146	0.264	0.608	0.760	0.383

[a]Age class and mass were not tested in the same model because these 2 variables were highly correlated (see *Material and Methods*).
HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.

TABLE 3: Effects of sex, sampling site, age, date, and mass on pollutant levels were tested, using Peto-Prentice tests (group comparisons) and tobit models (linear regressions)

	HCB		PCB-52		PCB-153	
Peto-Prentice tests						
	χ^2	*p*	χ^2	*p*	χ^2	*p*
Sex	0.7	0.403	1.1	0.300	0.6	0.444
Site	5.2	0.022	12.3	<0.001	3.7	0.054
Age class	1.0	0.800	6.7	0.082	9.5	0.023
Tobit models						
	Wald	*p*	Wald	*p*	Wald	*p*
Mass	0.498	0.481	0.086	0.769	1.265	0.261
Date	2.814	0.093	0.191	0.662	0.607	0.436

HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.

Plasma occurrences of PCB-52 and HCB were significantly higher in turtles captured at Faïsses compared with Esquineau, whereas the occurrence of PCB-153 was higher in turtles captured at Esquineau (Tables 1 and 2). The occurrence of HCB slightly increased with sampling date (Table 2). Sex, mass, and age class did not explain POP occurrence in turtles, except for PCB-153 whose detection frequency was higher in older individuals (Table 2).

Levels of HCB and PCB-52 were significantly higher in turtles from Faïsses, whereas PCB-153 levels tended to be higher in turtles from Esquineau (Tables 1 and 3). Moreover, levels of PCB-153 were lower in the youngest turtles (Table 3 and Figure 2). Sex, mass, and date did not explain variation in levels of POPs among turtles (Table 3).

DISCUSSION

The aim of the present study was to characterize legacy levels of POPs (OCPs and PCBs) in the plasma of European pond turtles in the Camargue, France, and to determine whether these levels were a function of individual traits and habitat type. Plasma levels of POPs were low and often below LOQ (0.2–3.1 ng/mL). We found significant differences in burdens of POPs between turtles from the 2 sampling sites, and these differences could be attributed to the hydraulic system (drainage/irrigation), keeping in mind that we lack site replication to conclude firmly an effect of the hydraulic system. The occurrence and levels of PCB-153 were higher in older turtles.

The Rhône River has been historically contaminated, leading to an accumulation of POPs in sediments downstream (Mourier et al. 2014). Previous studies conducted on several animal taxa in the Camargue confirmed high exposure during the last decades, with high concentrations of PCBs in muscles of eels (*Anguilla anguilla*) fished between 1997 and 2000 (Roche et al. 2004) and in eggs of little egrets (*Egretta garzetta*) collected in 1996 (Berny et al. 2002). Levels of POPs were low in the pond turtle in Camargue, which could indicate a decline in exposure to legacy POPs for wild species in the Camargue. Although a longitudinal study of the same species at the same locations would be required to test this hypothesis, decreasing PCB concentrations have been observed in Rhône River sediments in previous decades (Liber et al. 2019), and water analyses conducted by the National Nature Protection Society have not detected PCBs and OCPs in the Fumemorte canal since 2011 (Cheiron 2019). Moreover, organochlorine contamination across food webs tended to diminish through time in the Vaccarès lagoon (Roche et al. 2009b), into which the Fumemorte canal flows. Exotic red swamp crayfish (*Procambarus clarkii*), the main prey of European pond turtles in the Camargue (Ottonello et al. 2005), were not contaminated by POPs in 2019 (i.e., <LOQ, with LOQ ranging 0.9–1.98 ng/g dry wt; A. Goutte, EPHE, PSL Research University, unpublished data).

One of the first studies looking at the plasma concentration of POPs in turtles documented high PCB and OCP levels in snapping turtles (*Chelydra serpentina*) in Ontario, Canada, in 2001 to 2004 (Letcher et al. 2015): plasma concentrations of OCPs (sum of 17 contaminants) ranged from 0.2 to 236 ng/g wet weight, and the most abundant pesticide was p,p′-DDE (mean ± standard error 27 ± 6 ng/g wet wt). In our study,

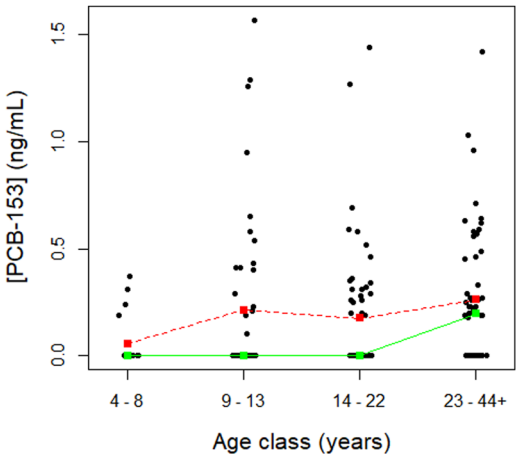

FIGURE 2: Plasma polychlorinated biphenyl 153 levels in European pond turtles increase with age class. Each point represents an individual. Red and green squares correspond to mean and median levels for age class, respectively. Age class was determined based on growth streaks on the plastron (see *Material and Methods*). PCB = polychlorinated biphenyl.

plasma concentrations of POPs (sum of 16 contaminants) were much lower, ranging from 0 to 2 ng/g wet weight, and p,p′-DDE levels did not exceed 2.3 ng/g wet weight. Snapping turtles are freshwater turtles with similar feeding habits to those of European pond turtles; snapping turtles also consume plant and animal matter, including aquatic invertebrates, fish, frogs, and reptiles (Ernst et al. 1994). Other studies of turtles documented plasma concentrations of POPs comparable to those in our study; for instance, for PCB-153 and HCB in another freshwater species, the western pond turtle (*Actinemys marmorata*), in Sequoia National Park, USA, in 2012 (Meyer et al. 2016), as well as in marine turtles such as loggerhead sea turtles (*Caretta caretta*) in the eastern Atlantic Ocean in 2011 and 2012 (Bucchia et al. 2015) and in green turtles (*Chelonia mydas*) and hawksbill turtles (*Eretmochelys imbricata*) in Cape Verde in 2009 to 2011 (Camacho et al. 2014).

In terms of occurrence of POPs, our results in European pond turtles were low compared to other studies (Bucchia et al. 2015; Meyer et al. 2016). The occurrences of DL-PCBs in 8% of European pond turtles, as well as p,p′-DDE in 2% of individuals, were much lower than those found in plasma of loggerhead sea turtles, which ranged from 63% in the Atlantic Ocean to 100% in the Adriatic Sea for DL-PCBs and 100% for p,p′-DDE (Bucchia et al. 2015).

Despite low detection frequencies and levels, we found differences in POP burdens between turtles from the 2 sampling sites. Turtles living in the drainage waters of Faïsses exhibited significantly higher occurrence and levels of HCB compared to turtles from Esquineau, likely because of the remobilization of trapped OCPs in soils. On the other hand, the occurrence and levels of PCB-153 were higher in turtles from Esquineau, a site receiving water from irrigation channels. A previous study has documented higher concentrations of PCBs in the aquatic fauna in irrigation canals compared to the drainage canals downstream of rice fields (Roche et al. 2009a). In contrast to the situation for PCB-153, concentrations of PCB-52 were higher in turtles in Faïsses, which may be due to the latter PCB's lower sedimentation rate associated with its lower molecular weight and greater solubility as a result of a lower level of chlorination (Gong et al. 1998; Alkhatib and Weigand 2002). The processes of deposition and release of PCBs from the irrigation part to the drainage part of the canals need to be further studied, and other populations of pond turtles in the 2 types of hydraulic systems should be studied to confirm this pattern.

The occurrence and levels of PCB-153 were lower in the youngest turtles (4–8 yr old), which could be attributed to a shorter exposure period. This is a recalcitrant chemical with a high hydrophobicity (high octanol–water partition coefficient), high resistance to metabolic transformation, and slow respiratory elimination through air exhalation (high octanol–air partition coefficient). It is also more prone to bioaccumulation than is the case for many other PCB congeners (Kelly et al. 2007), especially in long-lived air-breathing predatory species (Rowe 2008). It is also possible that there is a dietary shift during growth in pond turtles, leading to the consumption of higher–trophic level prey with age; and this diet would be more PCB-contaminated as a result of biomagnification. This hypothesis, however, is not consistent with dietary studies of *E. orbicularis* in Camargue, which instead documented a shift to a more herbivorous diet with age based on prey identification in fecal samples (Ottonello et al. 2005) or no difference in the proportion of plants, invertebrates, and vertebrates in the diet based on metabarcoding (Ducotterd et al. 2020).

We did not detect differences in POP levels between male and female pond turtles, despite males often being more contaminated than females in other turtle species (Guirlet et al. 2010; Bangma et al. 2019; Lambiase et al. 2021) because of a transfer of pollutants from the mother to the eggs through vitellogenesis (Moss et al. 2009; de Solla 2010).

CONCLUSION

The concentrations of POPs were very low in European pond turtles, probably much lower than they were several decades ago in wild vertebrates in Camargue, France. The occurrence and concentrations of POPs may have been influenced by the hydraulic system where the turtles were captured and by turtle age. Further studies are needed to assess the impact of current contaminants on biodiversity, especially for aquatic top predators such as turtles.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5077.

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Ethics Statement—The present study was performed in accordance with laws relative to the capture, transport, and experiments on *E. orbicularis* (DREAL permit CERFA_13616-01), and all procedures were approved by an independent ethical committee (APAFIS 17899-201812022345423 v2).

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Author Contributions Statement—A. Olivier, L. Burkart, C. Le Gac, and N. Martin conducted the fieldwork and collected the blood samples; L. Burkart and F. Alliot conducted the laboratory work to prepare plasma samples for analysis with gas chromatography and tandem mass spectrometry; L. Burkart, O. Lourdais, A. Goutte, A. Olivier, G. Blouin-Demers, and M. Vittecoq conceived and coordinated the study and participated in data analysis and in writing the

manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (aurelie.goutte@ephe.psl.eu).

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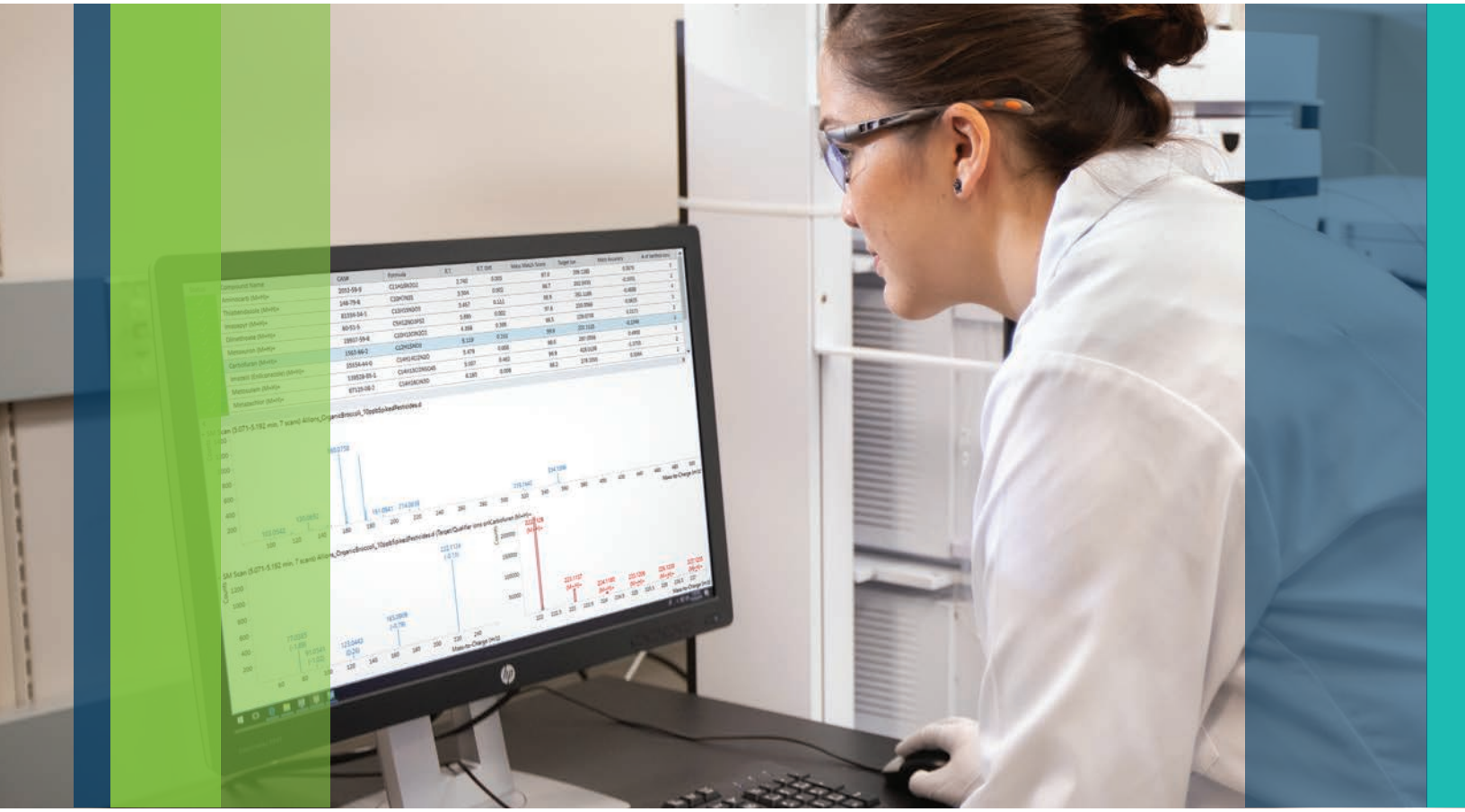

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