Perfluorinated Alkyl Acids in the Plasma of South African Crocodiles (*Crocodylus niloticus*)

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Abstract

Perfluorinated alkyl acids (PFAAs) are environmental contaminants that have been used in many products for over 50 years. Interest and concern has grown since 2000 on the widespread presence of PFAAs, when it was discovered that PFAAs were present in wildlife samples around the northern hemisphere. Since then, several studies have reported PFAAs in wildlife from many locations, including the remote regions of Antarctica and the Arctic. Although there are a multitude of studies, few have reported PFAA concentrations in reptiles and wildlife in the Southern Hemisphere. This study investigated the presence of PFAAs in the plasma of Nile crocodiles (*Crocodylus niloticus*) from South Africa. Crocodiles were captured from five sites in and around the Kruger National Park, South Africa, and plasma samples examined for PFAAs. Perfluorooctane sulfonate (PFOS) was the most frequent PFAA detected; with median values of 13.5 ng/g wet mass in crocodiles. In addition to PFOS, long chain perfluorinated carboxylic acids were also detected. Correlations between total length and PFAA load were investigated, as were differences in PFAA accumulation between sexes. No correlations were seen between crocodile size, nor were there sex-related differences. Spatial differences were examined and significant differences were observed in samples collected from the different sites ($p < 0.05$). Flag Boshielo Dam had the highest PFOS measurements, with a median concentration of 50.3 ng/g wet mass, when compared to the other sites (median concentrations at other sites below 14.0 ng/g wet mass). This suggests a point source of PFOS in this area.

Keywords: Perfluorinated alkyl acids; Nile crocodiles; South Africa; PFOS; PFNA; Kruger National Park

Introduction

Perfluorinated alkyl acids (PFAAs) are a class of anthropogenic fluorocarbons which have been widely used in a range of commercial products. Manufacturing of PFAAs started in 1948, with the introduction of perfluorooctane sulfonate (PFOS), as the key ingredient in the widely used water repellent (Prevedouros et al., 2006). Since then, PFOS and a wide range of related PFAAs have been manufactured, sold, and distributed throughout the world in various chemical products which include, among others, fire-fighting foams, surfactants, and surface repellents (Prevedouros et al., 2006; Lindstrom et al., 2011). PFAAs are valued within the manufacturing industry due to enhanced stability, surface-tension lowering, water repellent, and stable aqueous foams properties (Haug et al., 2009). However, the chemical and thermal stability of these compounds, which is a result of the aggregate strength of the carbon-fluorine bonds, results in enhanced persistence in the environment and resistance to hydrolysis, photolysis, microbial degradation, and metabolism by vertebrates (Giesy and Kannan, 2002). Furthermore, PFAAs have not only been shown to be ubiquitous in the environment, but it also has been reported that PFAAs bioaccumulate in various food webs, and have been shown to be present in the plasma of arctic mammals far removed from the historical sources of contamination (Giesy and Kannan, 2002; Ostertag et al., 2009; Butt et al., 2010).

Various studies have shown PFAAs persist in the environment, biomagnify up trophic levels (Sedlak and Greig, 2012), and carry over to subsequent generations (Inoue et al., 2004; Hinderliter et al., 2005; Reiner et al., 2011a). PFAAs have been detected in a wide array of fish-eating species, including dolphins, polar bears, mink, eagles, albatross, as well as various species of seals and fish (Giesy and Kannan, 2001; Houde et al., 2011). The common occurrence of PFAAs in aquatic or fish-eating species suggests exposure through diet and/or seepage and runoff, while the appearance of PFAAs in remote locations indicates long-term transportation via

ocean currents and atmospheric processes (Martin et al., 2006). Given the combination of long-term persistence, bioaccumulative potential, widespread occurrence, and the potential toxic influence of PFAAs, a better understanding of the global presence and subsequent effects on the environment is vitally important.

Across the entire continent, very little work has been done measuring PFAA contamination in Africa. Despite the wide spread prevalence of PFAAs there has been an assumption based on literature that high levels in the environment are only likely to be observed in the Northern Hemisphere, due to the greater level of industry in the global north (Giesy and Kannan, 2001, 2002). While the logic behind this argument is reasonable, not enough work has been done to confirm this hypothesis in the form of PFAA examination in wildlife south of the equator. Given the phase out of PFOS in most US manufacturing since 2000 and the increase industrialization south of the equator, an investigation of PFAAs in the Southern Hemisphere should be a valuable study for understanding global PFAA burden.

Additionally, very few studies have been conducted examining PFAA concentrations in reptilian species. Previous studies examined PFAA plasma concentrations of sea turtles (*Caretta caretta*, *Lepidochelys kempii*, *Dermochelys coriacea*, *Chelonia mydas*, and *Eretmochelys imbricata*) off the coast of the Southeastern United States (Keller et al., 2005; O'Connell et al., 2010; Keller et al., 2012), while a study from China examined PFAA levels in captive alligators (*Alligator sinensis*) (Wang et al., 2013a). These studies found detectable levels of PFAAs, with serum concentrations approaching toxic levels in sea turtles (Keller et al., 2005; Keller et al., 2012). Additional laboratory studies have shown PFAAs to possess toxic biological effects in developmental (Lau et al., 2003), immune (Peden-Adams et al., 2008; DeWitt et al., 2009; Guruge et al., 2009), and neurological systems (Zhang et al., 2011).

Crocodilians are an important PFAA sentinel species, due to the fact they are apex predators (hold the potential of accumulating high levels of PFAAs), exhibit high site fidelity, and have inherently long lifespans (70 to 100 years) in aquatic environments vulnerable to anthropogenic runoff. The primary aim of this study was to analyze PFAA concentrations in the plasma of Nile crocodiles (*Crocodylus niloticus*) from South Africa. Since factors such as sex, age, length, and body condition can influence PFAA accumulation, the possibility of sex and length being factors in the concentrations of PFAAs were also examined. Additionally, location specific concentrations were assessed to understand potential site differences in PFAA concentrations in South Africa.

Materials and Methods

Samples and Control Material

In 2012 and 2013, crocodilian blood samples were obtained as a result of an ongoing collaboration with researchers at Kruger National Park (KNP) in South Africa (Supplemental Information Table S1). Crocodile plasma samples (n = 45) were collected opportunistically from five sites in South Africa (Figure 1). These sites included the Flag Boshielo Dam (n = 15, outside and upstream of KNP), along the Olifants River (n = 17, within KNP), Letaba River (n = 5, within KNP), the Foskop Dam (n = 5, a mining dam upstream of KNP), and near the Olifants/Mozambique border (n = 3, outside KNP).

The National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 1958 Organic Contaminants in Fortified Human Serum was used as 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 (www.nist.gov/srm/).

Chemicals

Solutions of the NIST Reference Materials (RMs) 8446 Perfluorinated Carboxylic Acids and Perfluorooctane Sulfonamide in Methanol and RM 8447 Perfluorinated Sulfonic Acids in Methanol were combined to make calibration solutions (Gaithersburg, MD). Combined, the calibration solutions had fifteen different PFAAs: perfluorobutyric acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTA), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), perfluorooctanesulfonic acid (PFOS), and perfluorooctanesulfonamide (PFOSA). All compounds are reported as the total of the linear and branched isomers, using the response of the linear isomer to infer the concentrations of the branched isomers. The internal standards (IS) comprised of $^{13}\text{C}_4$ -PFBA, $^{13}\text{C}_2$ -PFHxA, $^{18}\text{O}_2$ -PFHxS, $^{13}\text{C}_8$ -PFOA, $^{13}\text{C}_9$ -PFNA, $^{13}\text{C}_9$ -PFDA, $^{13}\text{C}_2$ -PFUnA, $^{13}\text{C}_2$ -PFDoA, $^{18}\text{O}_2$ -PFBS, $^{13}\text{C}_4$ -PFOS, and $^{18}\text{O}_2$ -PFOSA were purchased from Cambridge Isotope Laboratories (Andover, MA), RTI International (Research Triangle Park, NC), and Wellington Laboratories (Guelph, Ontario).

Extraction and cleanup

Samples were extracted using a method that has previously been described (Reiner et al., 2011b). All samples, quality control materials, calibrants, and blanks were processed identically. Briefly, samples and SRM 1958 aliquots were removed from a freezer and allowed to reach room temperature. The samples, SRMs, calibrants, and water (approximately 1 g, used as a procedural blank) were weighed and gravimetrically spiked with the IS mixture. Samples were vortexed and allowed to equilibrate for 90 min. Samples were extracted using acetonitrile, sonicated, and

centrifuged. The supernatant was removed from all samples and the acetonitrile was solvent exchanged to methanol. Samples were further purified using solid phase extraction, employing Supelco Supelclean ENVI-Carb SPE columns (3 mL, 250 mg 120 – 400 mesh; Bellefonte, PA) with a vacuum manifold (Reiner et al., 2011b).

Extracts were evaporated to a final volume of 1 mL prior to being analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were analyzed using an Agilent 1100 HPLC system (HPLC; Santa Clara, CA) interfaced to an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Samples were injected onto an Agilent Zorbax Eclipse Plus C18 analytical column, (2.1 mm x 150 mm x 5µm). The solvent gradient started at 50% methanol (solvent A) and 50% 20 mmol/L ammonium (solvent B). The gradient was increased to 75% solvent A after 20 min, increased again to 95% solvent A after 5 min, reduced to 50% solvent A at 40.5 min, and then held constant for 9.5 min. Two multiple-reaction monitoring (MRM) transitions for each PFAA were monitored to ensure the validity of results, one MRM for quantitation and one MRM for confirmation (Reiner et al., 2011b).

Levels of each PFAA in the SRM and the samples were calculated using a linear equation of the calibration curve, not forcing the intercept through zero. Compounds were quantified using a relative response ratio to an internal standard compound that most closely matched the compound.

Quality Control

Blanks and NIST SRM 1958 were processed alongside plasma samples for quality control. To assess if methods were in control, the PFAAs measured in SRM 1958 had to agree with the reference values provided on the Certificates of Analysis (Supplemental Information Table S2). A

compound was considered to be significantly above the reporting limit (RL) if the mass of an analyte in the sample was greater than the mean plus three standard deviations of all blanks.

Statistical Methods

All statistical analyses were performed using JMP 11.1.1 (SAS Institute, Cary, NC). Statistical test were performed for PFNA, PFDA, PFUnA, PFHxS, and PFOS concentrations, which were the compounds detected in greater than 90% of the samples. Parametric tests were performed when the concentrations were normally or log-normally distributed. Data analysis for sex based and length based differences in PFAA concentrations included t-test and one-way ANOVA. Compounds less than the RL were set equal to half the RL prior to running the statistical tests.

Results and Discussion

All crocodile samples (n=45) contained at least one quantifiable PFAA, with PFNA, PFUnA, PFHxS, and PFOS being detected in all samples (Table 1). PFDA was frequently detected in the crocodile plasma samples (over 93%). Although PFDoA was only detected in 33% of the samples, it was commonly detected at the Flag Boshielo Dam location (93% of the samples from this site) and in only one other sample from the other four locations. The PFAAs with a carbon chain length less than nine (PFOA, PFHpA, PFHxA, PFPeA, and PFBA) were detected infrequently (< 2% of the samples) and therefore were not included in any statistical analyses.

Finding PFAAs in South African crocodiles is noteworthy, as it has long been assumed that PFAA levels south of the equator were negligible, or at least unknown. PFAA levels have recently garnered attention in South America (Olivero-Verbel et al., 2006; Leonel et al., 2008; Quinete et al., 2009); however, Africa and Australia remain grossly underrepresented in the body

of literature (Giesy and Kannan, 2001, 2002). Largely, this lack of reported data hinges on the argument that the global south is less industrialized thus resulting in fewer point sources of contamination, and also given that atmospheric processes tend to transport northward, thus concerns for PFAAs in wildlife south of the equator has been minimal.

The relationship between sex and PFAA concentrations appears to be a complicated one, with some studies reporting significant differences between sex and PFAA levels while others do not see any significant difference (Reiner and Place, 2015). For all PFAAs examined in this study, there were no significant differences observed between male and female crocodile plasma samples ($p > 0.05$). The relationship between length and PFAA concentrations was also examined in the crocodile plasma samples; however, there were no significant relationships ($p > 0.05$).

The crocodile sample sites included a range of sites both within the KNP and along the Olifants River outside the park, upstream and closer to human settlement (Figure 1). While the Olifants River is known to contain halogenated compounds, few analyses have been performed to examine persistent organic pollutants in this area (Bouwman et al., 2014). The Olifants River runs through KNP and PFAAs seen in the river are likely the result from contaminants upstream and outside of the park (Bouwman et al., 2014). Flag Boshielo Dam is located completely outside of KNP and provides a good profile of upstream sources, as it has a significantly denser surrounding human population (Marble Hall) compared to the other sites, extensive agricultural practices, as well as being upstream of KNP along the Olifants River.

Locational differences of PFAAs were seen among crocodile samples collected at different sites. Among all locations, Flag Boshielo Dam had by far the highest PFOS burden, with a median concentration of 50.3 ng/g wet mass (range 11.5 ng/g to 118 ng/g wet mass) and was significantly higher when compared to the other sites examined in this study (Figure 2a, Table 2;

p < 0.05). PFNA distribution was similar among sites, with significant variation seen between Flag Boshielo Dam and Foskor Dam (Supplemental Information Figure S1). In addition to having the highest PFOS levels, Flag Boshielo Dam also had significantly higher PFDA (Supplemental Information Figure S2) and PFUnA concentrations (median values of 3.40 ng/g wet mass and 2.19 ng/g wet mass, respectively; Figure 2b; p < 0.05). Interestingly, PFDoA was detected frequently at Flag Boshielo Dam, but only once from the four other locations (Table 2; Supplemental Information Figure S3). Since most PFAAs measured in the Nile crocodiles at Flag Boshielo Dam are higher compared to the other locations, this suggests this area could be a source of PFAAs examined in the crocodiles downstream of this location.

The ubiquitous presence of PFOS is a good example of how pervasive PFAAs have become over the past 60 years. While individual PFOS levels greatly varied in Southern Hemisphere Nile crocodiles, as was predicted due to differences in life histories and spatial territories, a comparison of the site median concentrations illustrate a degree of similarity among different groups. It is interesting to note that many samples were collected from within the 19,500 km² KNP, further demonstrating how ubiquitous PFAAs have become worldwide.

Average PFOS levels among all samples in this study of 24.3 ng/g wet mass obtained in this study were slightly higher than those previously reported in captive Chinese alligators (Wang et al., 2013a), thus making these values the highest reported PFOS levels detected in the plasma of crocodilians (Table 3). These levels were also higher than those previously reported for sea turtles (Keller et al., 2005; O'Connell et al., 2010; Keller et al., 2012), though it is important to note that while crocodilians are positioned at a higher trophic level compared to turtles, these levels are lower than those reported in many larger, predatory marine mammals. Since there are very few reptilian studies, this study helps fill a critical gap for assessing the overall/widespread burden of PFAAs.

In addition to the observed PFAA levels in Nile crocodiles from KNP, there are ongoing studies examining the threatened health of the local crocodile population. In 2008, KNP in South Africa experienced numerous mortalities to its resident Nile crocodile population in the Olifants River, with another episode of mortalities following in the spring of 2009. The mortality events of the crocodiles, as well as other aquatic species, were attributed to environmental pansteatitis, a disease hallmarked by inflammation and subsequent hardening of adipose tissue (Osthoff et al., 2010; Huchzermeyer et al., 2013; Lane et al., 2013). To date, no definitive origin of for pansteatitis has yet been determined, though several hypotheses have been noted, which include possible contributions from anthropogenic sources (Larsen et al., 1983; Swanepoel et al., 2000; Ashton, 2010; Masango et al., 2010; Huchzermeyer et al., 2011; Oberholster et al., 2012; Woodborne et al., 2012; Dabrowski et al., 2013; Bouwman et al., 2014; Dabrowski et al., 2014). Of particular interest are those contaminants from anthropogenic sources that have been previously shown to affect lipid pathways and adipose physiology. PFAAs, PFOA in particular, has been demonstrated to be a PPAR- α (peroxisome proliferator activated receptors alpha) agonists, and activation of the PPAR- α heterodimer stimulates β -oxidation of fatty acids, leading to inflammation and adipose tissue atrophy (Grun and Blumberg, 2009). However, PFOA was rarely detected in these samples (< 2% of the samples analyzed). To date no non-lethal mechanism to assess the health/disease status of crocodiles exists. Further work is aimed at determining pansteatitis status to better assess if potential toxicity/health effects are associated with anthropogenic sources.

In addition to expanding research on pansteatitis and the potential health effects associated with historic anthropogenic contaminants, an expansion of measurements for chemicals of emerging concern needs to be addressed. The recent identification of novel fluorinated compounds in aqueous film forming foams and environmental samples can serve as target

compounds to expand PFAA measurements to include possible replacement chemicals (Place and Field, 2012; Ruan et al., 2015; Wang et al. 2013b). Future studies will benefit from the inclusion of these newly identified PFAAs.

To the best of our knowledge, this study provides the first examination of PFAAs in the blood of crocodilians from the Southern Hemisphere in the wild. In general, PFAA levels were comparable to levels exhibited in marine mammals, such as seals, from relatively pristine waters. The levels detected in this study were higher than PFAA levels reported for sea turtles, one of the few other reptile taxa that have been studied. Across the site locations examined, crocodile PFAA levels varied significantly with the highest levels detected at the Flag Boshielo Dam located outside KNP.

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Disclaimer

Certain commercial equipment or instruments are identified in the paper to specify adequately the experimental procedures. Such identification does not imply recommendations or endorsement by the NIST nor does it imply that the equipment or instruments are the best available for the purpose.

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Graphical Abstract

Overhead view of crocodile from South Africa

Table 1. Concentrations of PFAAs (ng/g wet mass) measured in crocodile plasma

	Flag Boshielo Dam (n=15)			Foskor Dam (n=5)			Letaba River (n=5)			Olifants River (n=17)			Olifants River/Mozambique Border (n=3)		
	Range	Median	n > RL	Range	Median	n > RL	Range	Median	n > RL	Range	Median	n > RL	Range	Median	n > RL
PFNA	0.293 - 0.606	0.477	15	0.350 - 0.502	0.366	5	0.392 - 0.611	0.438	5	0.344 - 0.710	0.435	17	0.373 - 0.581	0.394	3
PFDA	1.05 - 6.13	3.40	15	0.199 - 1.87	0.434	5	0.246 - 1.86	0.775	5	<0.118 - 1.98	0.616	14	0.147 - 1.43	0.226	3
PFUnA	0.518 - 3.40	2.19	15	0.266 - 0.740	0.441	5	0.140 - 0.883	0.438	5	0.120 - 0.845	0.309	17	0.092 - 0.618	0.163	3
PFDoA	<0.155 - 0.920	0.550	14	<0.0151	NA	0	<0.148 - 0.198	NA	1	<0.152	NA	0	<0.151	NA	0
PFHxS	0.575 - 1.66	1.06	15	0.851 - 2.26	1.08	5	0.644 - 0.872	0.713	5	0.623 - 2.48	0.748	17	0.676 - 0.805	0.778	3
PFOS	11.5 - 118	50.3	15	10.3 - 28.0	13.5	5	2.11 - 17.9	7.44	5	0.776 - 29.4	7.82	17	1.41 - 17.1	4.31	3

n > RL indicates the number of samples above the reporting limit (RL)

Values were calculated with half the RL substituted for nondetects as described in the methods section, but values shown as “<” a

specified number describe the actual RL

NA indicates not available

Table 2. Location differences of PFAs in crocodile plasma samples collected in South Africa.

	PFNA		PFDA	PFUnA	PFHxS		PFOS	
Flag Boshielo Dam (n=15)	A		A	A	A	B	A	
Foskor Dam (n=5)		B		B	A			B
Letaba River (n=5)	A	B		B		C	B	C
Olifants River (n=17)	A	B		B		B	C	C
Olifants River/Mozambique Border (n=3)	A	B		B		B	C	C

Letters above bars represent statistically significant differences between groups ($p < 0.05$)

Table 3. Range of plasma concentrations of PFAAs (ng/g wet mass) determined in reptilian species around the world.

Species (Year; sample size)	Location	PFNA	PFDA	PFUnA	PFDoA	PFHxS	PFOS	Reference
Loggerhead Sea Turtles (2003; n=73)	South Eastern United States	<0.100 - 4.42	<0.100 - 6.35	<0.050 - 2.95	<0.050 - 0.827	<0.050 - 1.08	1.4 - 96.8	Keller et al., 2005
Kemp's Ridley Sea Turtles (2003; n=6)	South Eastern United States	0.258 - 6.50	0.664 - 4.30	0.333 - 2.67	0.241 - 0.586	0.119 - 0.382	13.8 - 60.2	Keller et al., 2005
Loggerhead Sea Turtles (2000-2008; n=59)	South Eastern United States	<0.034 - 14.4	<0.092 - 3.11	<0.063 - 16.0	<0.040 - 1.76	<0.032 - 0.317	0.305 - 36.6	O'Connell et al., 2010
Chinese Alligators (2013; n=48)	Anhui, China	0.2-18.8	4.7-56.0	7.8-63.0	0.8-5.9	0-1.5	6.7-61.8	Wang et al., 2013
Nile Crocodiles (2012-2013; n=45)	South Africa	0.293 - 0.710	<0.118 - 6.13	0.092 - 3.40	<0.148 - 0.920	0.575 - 2.26	0.776 - 118	This study

Values shown as “<” a specified number describe the actual RL

Figure Captions

Fig. 1. Map of crocodile collection sites in South Africa. Sampling sites are (1) Flag Boshielo Dam, (2) Foskop Dam, (3) Letaba River, (4) Olifants River, and (5) Olifants River/Mozambique Border

Fig. 2. (a) Perflourooctane sulfonate (PFOS) and (b) Perfluoroundecanoic acid (PFUnA) concentrations (mean with 95% confidence intervals) in plasma from crocodiles from South Africa. Different letters above bars represent statistically significant differences between groups ($p < 0.05$). Number of samples: Foskop Dam ($n=5$), Letaba Rive ($n=5$), Olifants River ($n=17$), Olifants River (OR)/Mozambique Border ($n=3$), Flag Boshielo Dam ($n=15$)

1

2 **Fig. 1.** Map of crocodile collection sites in South Africa. Sampling sites are (1) Flag Boshielo
3 Dam, (2) Foskor Dam, (3) Letaba River, (4) Olifants River, and (5) Olifants River/Mozambique
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