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Short communication

Human exposure to per- and polyfluoroalkyl substances (PFAS) via the consumption of fish leads to exceedance of safety thresholds

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) receive global attention due to their adverse effects on human health and the environment. Fish consumption is a major source of human PFAS exposure. The aim of this work was to address the lack of harmonization within legislations (in the EU and the USA) and highlight the level of PFAS in fish exposed to pollution from diffuse sources in the context of current safety thresholds. A non-exhaustive literature review was carried out to obtain PFAS concentrations in wild fish from the Norwegian mainland, Svalbard, the Netherlands, the USA, as well as sea regions (North Sea, English Channel, Atlantic Ocean), and farmed fish on the Dutch market. Median sum wet weight concentrations of PFOA, PFNA, PFHxS, and PFOS ranged between 0.1 μg kg^{-1} (farmed fish) and 22 μg kg^{-1} (Netherlands eel). Most concentrations fell below the EU environmental quality standard (EQS_{biota}) for PFOS (9.1 μg kg^{-1}) and would not be defined as polluted in the EU. However, using recent tolerable intake or reference dose values in the EU and the USA revealed that even limited fish consumption would lead to exceedance of these thresholds – possibly posing a challenge for risk communication.

1. Introduction

There is a global regulatory, scientific, and citizen focus on per- and polyfluoroalkyl substances (PFAS), due to their negative effects on human health and the environment (Brennan et al., 2021; Tian et al., 2022). A recent study (Cousins et al., 2022) evaluated environmental PFAS levels in the context of planetary boundaries, defined as the "safe operating space for humanity with respect to the functioning of the Earth System" (Rockström et al., 2009; MacLeod et al., 2014). Cousins et al. (2022) concluded that a planetary boundary has been exceeded based on concentrations detected in rainwater, surface water and soil when comparing these to current guideline values. While some of these guideline values are under debate, this illustrates the problematic nature of extensive PFAS pollution in the environment.

In Europe, the political focus on PFAS is spurred on by the European Commission's (EC) Green Deal (EC, 2020a). The Chemicals Strategy for Sustainability Towards a Toxic-Free Environment details the European Union's (EU) new long-term vision for its chemical policy (EC, 2020c). The accompanying Commission Staff Working Document (SWD) for PFAS (EC, 2020b) outlines why existing regulatory tools are not

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Table 1
Previous and present Tolerable daily Intake (TDI) and Reference dose values in the EU and the USA.

	ng kg^{-1}b.w. per day	Applies for	Year	Reference
Previous EFSA TDI which present day EQS are based on	150	PFOS	2008	(EFSA, 2008)
TDI based on the present day EFSA TWI (4.4 ng kg^{-1}b.w. per week)	0.63	Sum of PFOS, PFHxS, PFNA and PFOA	2020	(Schrenk et al., 2020)
Reference dose values used to set advisories in states in the USA	1.8–77	PFOS	–	(Barbo et al., 2023)
The USA EPA reference dose value for PFOS	0.0079	PFOS	2022	(EPA, 2022)

EFSA = European Food Safety Authority.
TWI = Tolerable weekly intake.
PFOS = Perfluorooctane sulfonate.
PFHxS = Perfluorohexane sulfonate.
PFNA = Perfluorononanoic acid.
PFOA = Perfluorooctanoic acid.
EPA = Environmental Protection Agency.

sufficient to address the concerns of PFAS. In addition, the SWD proposes addressing PFAS as a group whilst highlighting the benefit of applying the concept of "essential use" to this group of substances. However, regulation often has to play catch up – policy developments have primarily been retrospective, reacting to a problem rather than proactive, addressing the problem at its source.

The problem of PFAS is not unique and a similar story can be told for other persistent organic pollutants (POPs) such as dichlorodiphenyltrichloroethane (DDT) (Roberts et al., 2016; Arp et al., 2023) and polychlorinated biphenyls (PCBs), which received considerable public attention (e.g., Carson, 1962; Jensen, 1972; Robertson and Hansen, 2001). These chemicals are detected in almost all media and locations sampled (Turusov et al., 2002; Bhaskar et al., 2019). Similarly, PFAS have been found in rainwater, surface water, drinking water, ice cores, groundwater, biota from varying trophic levels, soils, sediments and the air (Rahman et al., 2014; Ahrens et al., 2015; Hale et al., 2017; Rauert et al., 2018; Langberg et al., 2020; Høisæter et al., 2021; Cousins et al., 2022; Hartz et al., 2023). Many PFAS are relatively water soluble and mobile in water, and despite significant environmental transport via sea spray aerosols, the aquatic environment is the ultimate sink for many PFAS (Johansson et al., 2019). As many PFAS bioaccumulate and biomagnify in aquatic food webs, fish are subject to significant PFAS exposure (Ahrens and Bundschuh, 2014; Langberg et al., 2020). In response to information on the problematic properties of many PFAS, five European countries proposed a broad PFAS restriction to The European Chemicals Agency (ECHA) in 2023, which is currently under consultation (ECHA, 2023). However, as PFAS are extremely persistent, their concentrations will not rapidly decrease even after emissions cease. For example, concentrations of perfluorooctane sulfonate (PFOS) were reported to increase in cod (*Gadus morhua*) liver in the Baltic Sea between 1981 and 2013 (Schultes et al., 2019), and concentrations were reported only to be slowly decreasing in cod livers from the Norwegian coast between 2009 and 2021, despite a reduction in PFOS emissions (Schøyen et al., 2022). It has been stated that there is a lack of comprehensive spatial and temporal environmental monitoring in the EU, and that the present state of information only reflects the top of the iceberg (Sonne et al., 2023). It has been postulated that the main uptake route of PFAS to the general population (i.e., for those whose drinking water is not significantly impacted by PFAS) is via food consumption (Vestergren and Cousins, 2013), especially consumption of fish and other seafood (Schrenk et al., 2020). It has been reported that PFAS intake via fish consumption may pose a risk of exceedance of safety limits for certain groups of the population (Barbo et al., 2023; Schepens et al., 2023). PFAS have been detected in fish from Asia (Lam et al., 2014; Thi et al., 2022), Africa (Abafe et al., 2021), North America (Lescord et al., 2015; Goodrow et al., 2020), South America (Miranda et al., 2021), Arctic (Muir et al., 2019), Antarctica (Gao et al., 2020), Europe (Åkerblom et al., 2017; Valsecchi et al., 2021), and Australia/Oceania (Taylor et al., 2018), confirming ubiquitous contamination.

In this perspective we reflect upon PFAS concentrations in fish polluted by diffuse sources (fish that are not directly affected by a nearby PFAS pollution point source). Previous examples have shown that concentrations of PFAS in the environment are challenging for society: in the Netherlands, building work was temporarily halted in 2019 as PFAS concentrations in soil exceeded the thresholds set for moving soil (0.9 μg kg^{-1} for PFOS and 0.8 μg kg^{-1} for perfluorooctanoic acid (PFOA)) (Wintersen et al., 2019, 2020). PFAS concentrations in rainwater are above drinking water thresholds, calling into question the use of rainwater as a source of drinking water (Cousins et al., 2022). Adding to this, a recent report by the Dutch National Institute for Public Health and the Environment (RIVM; Bilthoven, The Netherlands) concluded that PFAS levels in Dutch surface water must decline to avoid the contribution of drinking water exceeding 20 % of the tolerable daily intake (TDI; Monique et al., 2021). In Denmark, it has been reported that for the 95th percentile of the population PFAS exposure via the consumption of eggs alone exceeds the of the tolerable weekly intake set by The European Food Safety Authority (EFSA) (DTU National Food Institute, 2023). Herein, fish concentrations are compared to current EU environmental quality standards (EQS), as well as current thresholds for tolerable human intake of PFAS in the EU and the USA. We consider whether fish consumption constitutes a risk for exceedance of these thresholds, and challenges related to risk communication.

2. Thresholds and guideline values in the EU and the USA

Thresholds and guideline values vary between areas and have changed over time as detailed for the EU and the USA in the following. Previous and present safety thresholds in the EU and the USA are summarized in Table 1.

As pointed out by Cousins et al. (2022), concentrations of PFOS in surface freshwaters and rainwater exceed the EQS in the EU Water Framework Directive (WFD). The EQS is based on the most critical of the specific quality standards (QS), i.e., the strictest of the QS set to protect top predators ($QS_{biota,\ secpois}$) and the QS for protecting human health ($QS_{biota\ h,h}$). For PFOS, the most critical QS was $QS_{biota\ h,h}$ (EC, 2011b). As shown below, the $QS_{biota\ h,h}$ (that the EQS for water is based on) does not (yet) include new toxicity information.

In 2008, the Scientific Panel on Contaminants in the Food Chain (CONTAM) defined the lowest no-observed-adverse-effect level (NOAEL) for PFOS of 0.03 mg kg^{-1} body weight (b.w.) per day (EFSA, 2008). This value was based on changes in serum levels of high-density lipoprotein cholesterol and thyroid hormones in a single study with Cynomolgus monkeys (*Macaca fasicularis*) (Seacat et al., 2002). By applying an uncertainty factor (UF) of 200 to the NOAEL, a TDI of 150 ng kg^{-1}b.w. per day was established (EFSA, 2008). The TDI was used, together with a factor for the relative source contribution from fish consumption, to calculate $QS_{biota\ h,h}$ for PFOS in biota of 9.1 μg kg^{-1} wet weight (w.w.) (EC, 2011b, 2011a), as shown in Eq. (1).

$$QS_{biota,hh} = \frac{0.1 \times TDI \times b.w.}{Fish_{intake}} \quad (1)$$

The factor 0.1 is from the assumption that PFOS intake via fish consumption contributes 10 % of total PFOS intake; b.w. is body weight (70 kg); and $Fish_{intake}$ is the average fish consumption per day (0.115 kg day^{-1}).

As $QS_{biota\ h,h}$ (9.1 μg kg^{-1}) was the strictest QS, it was used as the EQS for biota (defined as fish) in the WFD. The QS for PFOS in freshwater and saltwater, $QS_{freshwater}$ (0.65 ng/L) and $QS_{saltwater}$ (0.13 ng/L) respectively, were then derived based on $QS_{biota\ h,h}$ (using bioconcentration

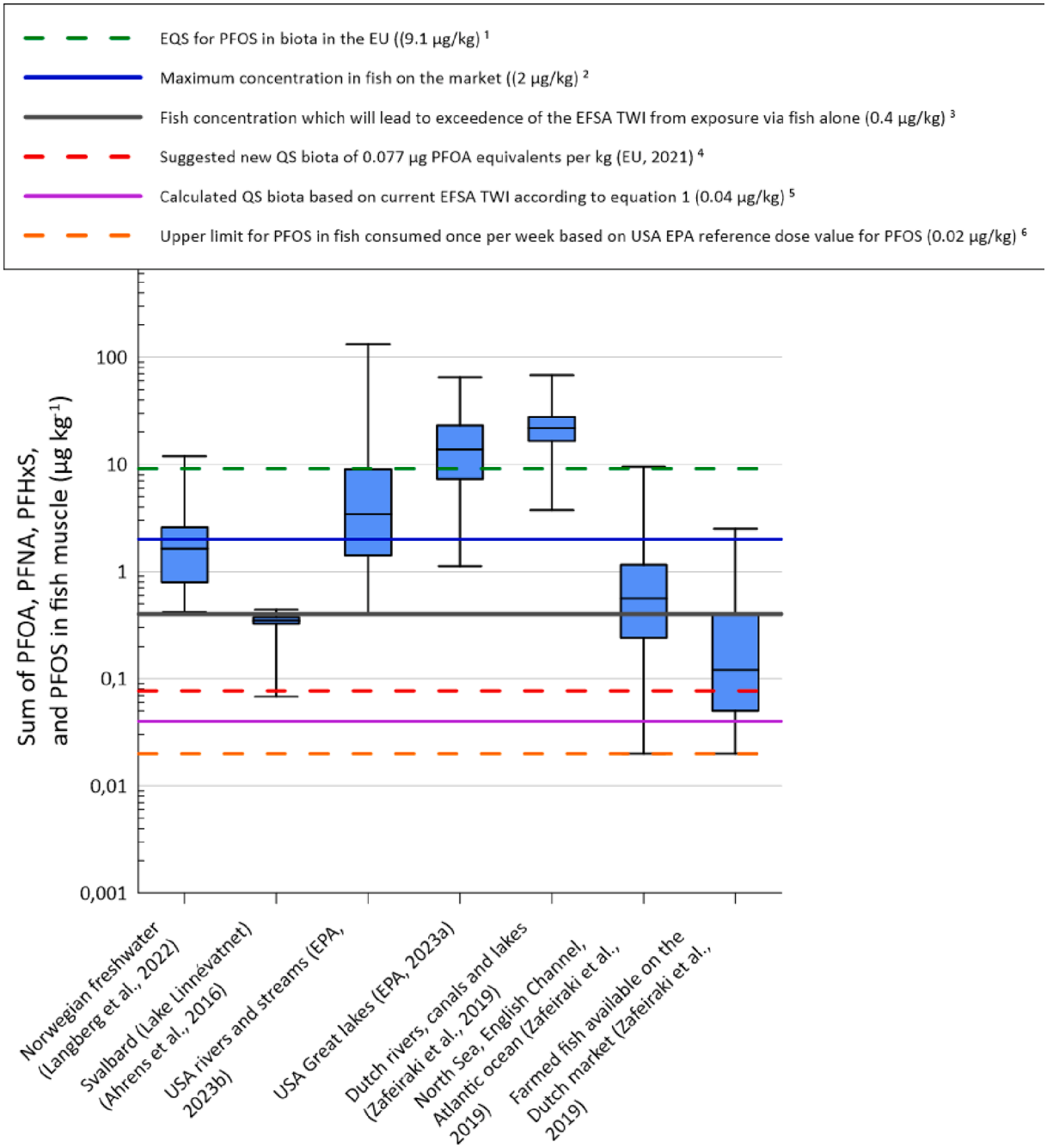

Fig. 1. Sum concentrations of four PFAS (perfluorooctanoic acid [PFOA], perfluorononanoic acid [PFNA], perfluorooctane sulfonate [PFOS], and perfluorohexane sulfonate [PFHxS]) in fish muscle. Boxes indicate the 25th and 75th percentiles, respectively. Centre lines show the medians, and the whiskers indicate the ranges (i.e., max and min). Concentrations below the LOQ were treated as LOQ/2. Selected limit values and thresholds are indicated with lines. Dashed lines show thresholds for other parameters than the sum of the four PFAS (PFOS only or sum of PFOA equivalents). Note: [1] Environmental Quality Standard (EQS) for PFOS in the EU (Directive, 2013/39/EU, 2013). The threshold apply for PFOS only. [2] Maximum concentrations allowed in fish on the European market (EC, 2022). Maximum concentrations were set higher for some specific fish species when not intended for the production of food for infants and young children (EC, 2022). [3] Fish concentration which will lead to exceedance of the Tolerable Weekly Intake (TWI) from the European Food Safety Authority (EFSA) by exposure via fish alone. [4] Suggested new Quality Standard (QS) for biota in the EU (EU, 2021). The red dashed line indicates the threshold for the sum of 24 PFAS expressed as PFOA equivalents. [5] Calculated QS biota based on the current TWI from EFSA according to Eq. (1). [6] The upper limit of the range of maximum levels of PFOS in fish consumed in one meal per week calculated based on the USA Environmental Protection Agency's (EPA) reference dose value and the most widely adopted approaches used by states in the Great Lakes Consortium for Fish Consumption Advisories (Barbo et al., 2023). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Concentrations of linear perfluorooctane sulfonate (L-PFOS) as well as the sum of perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexane sulfonate (PFHxS), and PFOS in fish muscle from the Norwegian mainland, Svalbard, the Netherlands and the USA, as well as wild fish from the sea (North Sea, English Channel, and Atlantic Ocean), and farmed fish available on the Dutch market. Median, mean, maximum and minimum concentrations (μg kg^{-1} w.w.) as well as the percentage of concentrations below the limit of quantification (LOQ) are listed for each dataset. Concentrations below the LOQ were treated as LOQ/2.

Data source	Number of species	L-PFOS (μg kg^{-1} w.w.)	Sum PFOA, PFNA, PFHxS, and PFOS (μg kg^{-1} w.w.)
Freshwater fish (Norwegian freshwater bodies) (n = 315) (Langberg et al., 2022)[1]	4 different species	Median: 0.7	Median: 1.6
		Mean: 1.1	Mean: 2.0
		Max: 8.9	Max: 12
		Min: <LOQ	Min: <LOQ
		Below LOQ: 9.8 %	Below LOQ: 9.8 %
Freshwater fish (Lake Linnévatnet on Svalbard, Norway) (n = 6) (Ahrens et al., 2016)	1 species (Arctic char (*Salvelinus alpinus*))	Median: 0.2	Median: 0.3
		Mean: 0.2	Mean: 0.3
		Max: 0.3	Max: 0.4
		Min: 0.02	Min: 0.1
		Below LOQ: 0 %	Below LOQ: 0 %
Freshwater fish (Rivers and streams in the USA (n = 290) (EPA, 2023b) [2]	37 different species	Median: 3.2	Median: 3.4
		Mean: 7.7	Mean: 8.0
		Max: 131	Max: 131
		Min: <LOQ	Min: <LOQ
		Below LOQ: 8.6 %	Below LOQ: 7.2 %
Freshwater fish (USA Great Lakes) (n = 152) (EPA, 2023a) [2]	17 different species	Median: 12.4	Median: 13.8
		Mean: 16.9	Mean: 18.1
		Max: 64.4	Max: 65.2
		Min: 0.5	Min: 0.7
		Below LOQ: 0 %	Below LOQ: 0 %
Freshwater fish (Dutch Rivers, canals, and lakes) (n = 86) (Zafeiraki et al., 2019)	1 species (European eel (*Anguilla anguilla*))	Median: 20	Median: 22
		Mean: 22	Mean: 23
		Max: 67	Max: 68
		Min: 3.3	Min: 3.8
		Below LOQ: 0 %	Below LOQ: 0 %
Marine fish (North Sea, English Channel, Atlantic ocean) (n = 77) (Zafeiraki et al., 2019)	10 different species	Median: 0.3	Median: 0.6
		Mean: 0.8	Mean: 1.0
		Max: 9.4	Max: 9.4
		Min: <LOQ	Min: <LOQ
		Below LOQ: 38 %	Below LOQ: 27 %
Farmed fish (on the Dutch market) (n = 52) (Zafeiraki et al., 2019)	7 different species	Median: 0.03	Median: 0.1
		Mean: 0.2	Mean: 0.4
		Max: 2.0	Max: 2.5
		Min: <LOQ	Min: <LOQ
		Below LOQ: 69 %	Below LOQ: 58 %

[1] Only data from water bodies reported to not be directly influenced by a PFAS point source were included. [2] Compared to Barbo et al. (2023), the present study reviewed the same dataset for the USA Great Lakes (2015) and a newer dataset from the same USA monitoring program for rivers and streams (2018–2019).

[BCF] and biomagnification [BMF] factors). However, based on decreased immune responses observed in children, in 2020 EFSA set a tolerable weekly intake (TWI) threshold for the sum of PFOA, PFOS, perfluorononanoic acid (PFNA), and perfluorohexane sulfonate (PFHxS) of 4.4 ng kg^{-1}b.w., which corresponds to a TDI of 0.63 ng kg^{-1}b.w. per day (Schrenk et al., 2020). Thus, the 2020 TDI for the $\sum$ PFOA, PFOS, PFNA, and PFHxS is approximately 1/238 of the TDI from 2008 for PFOS (150 ng kg^{-1}b.w. per day) for which present-day EQS are based on.

In 2022, the EC published an amendment to Regulation No 1881/2006 setting maximum levels of PFAS in foodstuffs on the market (EC, 2022). A general maximum level for the sum of PFOS, PFOA, PFNA and PFHxS in fish muscle was set at 2 μg kg^{-1} w.w. (blue line in Fig. 1), while the maximum levels for some fish species were set higher (8 and 45 μg kg^{-1} w.w. respectively, depending on species) when not intended for the production of food for infants and young children (EC, 2022). Assuming a body weight of 70 kg (as in Eq. (1)), a person consuming fish containing the general maximum level of 2.0 μg kg^{-1} w.w. would exceed the EFSA TWI when they consume more than 154 g of fish per week, without other sources of PFAS exposure. Consumption of 154 g of fish per week is low compared to the estimated average fish consumption used in Eq. (1) (115 g per day, or 805 g per week). It is important to notice that this number (154 g per week) does not take into account PFAS intake from other sources than fish exposure (which in Eq. (1) was estimated to contribute to 90 % of the PFAS intake). Consuming even a few grams of fish containing PFAS concentrations corresponding to the new maximum levels for specific fish species of 8 and 45 μg kg^{-1} w.w. (i.e., 39 and 7 g of fish per week, respectively) will lead to exceedance of the present-day TWI, without any other sources of PFAS exposure. Thus, the new maximum levels of PFAS in foodstuff are high considering the new EFSA TWI (4.4 ng kg^{-1}b.w.) and PFAS intake from other sources than fish consumption. Denmark, Germany, the Netherlands, and the Czech Republic have submitted a note to the General Secretariat of the European Council recommending regular reviews, a lowering of existing maximum levels for PFAS in foodstuffs, and setting new maximum levels in additional foodstuffs based on occurrence data in food (General Secretariat of the Council of the European Union, 2023).

Fish advisories in the USA have been under scrutiny and are not coherent between states (Barbo et al., 2023). Reference dose values

(similar to the tolerable intake value in the EU) for PFOS, used to set the advisories in the different states, varied between 1.8 and 77 ng kg^{-1} b.w. per day (Massachusetts and Alabama, respectively) (Barbo et al., 2023). In 2022, the USA Environmental Protection Agency (EPA) published a reference dose value for PFOS of 7.9×10^{-3} ng kg^{-1} b.w. per day (EPA, 2022). Based on the EPA's reference dose value and the most widely adopted approaches used by states in the Great Lakes Consortium for Fish Consumption Advisories, Barbo et al. (2023) calculated a maximum level of 0.008–0.02 μg kg^{-1} for PFOS in fish consumed in one meal per week (the upper limit of this range, 0.02 μg kg^{-1} is indicated as an orange dashed line in Fig. 1).

In conclusion, there is a lack of harmonization within legislations (in the context of tolerable intake) in the EU and the USA. As the consumption of fish and other seafood is reported to be among the most important sources of PFAS exposure to humans, it is relevant to compare concentrations of PFAS in muscle of fish to present-day limit values and tolerable intake estimates. Fish concentrations, relative source contribution from fish consumption, as well as the threshold value for human health used in this comparison will have implications on the amount of fish that can be eaten without exceeding threshold values.

3. Concentrations of PFAS in fish exposed to pollution from diffuse sources

The aim of this study was not to perform a comprehensive review of PFAS concentrations in fish, but to highlight the level of PFAS in fish exposed to pollution from diffuse sources in the context of current safety thresholds. The dataset below presents a non-exhaustive summary of concentrations of PFAS in wild freshwater fish from the countries of the authors' home institutes in Europe (Norwegian mainland, Svalbard, Netherlands) and the USA, as well as wild fish from the sea (North Sea, English Channel, and Atlantic Ocean), and farmed fish available on the Dutch market. Fish concentrations from areas known to be substantially polluted by a particular PFAS point source were excluded from the dataset. Table 2 shows the concentrations of PFOS as well as the sum of PFOA, PFNA, PFHxS, and PFOS (the parameters most relevant for comparison to the thresholds and guideline values listed above) in fish muscle from these areas. Concentrations are shown for the linear isomer and are compared to relevant thresholds in Fig. 1.

4. Comparison of fish data to EQS, and safety thresholds for tolerable intake

There are relatively large differences in PFAS concentrations between the datasets in Table 2. Differences in regional PFAS loads, dilution potential in different water bodies (i.e., freshwater lakes compared to the sea), uncertainties of the analytical methods applied, as well as species are likely some of the explanations for this. Based on the data shown here, it seems that marine fish as well as farmed fish have lower PFAS loads compared to fish from most freshwater sources. A previous study investigating purchased fish in the Netherlands, showed that PFAS concentrations in wild-caught cod and tuna were higher than farmed salmon and pangasius (Schepens et al., 2023).

Except for Dutch eel and fish from the USA Great Lakes, PFAS concentrations in the above reviewed fish are mostly below the present-day EU EQS_{biota} of 9.1 μg kg^{-1} w.w. (dashed green line in Fig. 1) and would therefore not be defined as polluted in the EU. However, given the recent EFSA data (Schrenk et al., 2020), the EQS of 9.1 ug kg^{-1} w.w is now under scrutiny. An assessment by the Norwegian Institute of Public Health (NIPH, 2020) showed that the current EFSA TWI (4.4 ng kg^{-1}b.w. per week) is exceeded for Norwegian children even when PFAS from fish and drinking water consumption are excluded. The data also show that the TWI is exceeded for women when only fish consumption (i.e., without drinking water consumption) is included in the assessment (NIPH, 2020). To ensure that the TWI for adult men and women is not exceeded, the maximum permissible concentrations of PFAS in fish were 0.27 and 0.23 μg kg^{-1}, respectively (NIPH, 2020). Similarly, RIVM calculated the PFAS exposure for the Dutch population and concluded that it exceeds the EFSA TWI, and that fish is an important source of PFAS (Schepens et al., 2023). EFSA has reported that exposure of European children, as well as major parts of the adult population exceeds the present-day TWI (Schrenk et al., 2020). Studies published in the scientific literature have also concluded that human PFAS exposure exceeds health-based guidance values (see e.g., Bil et al. (2023), Uhl et al. (2023) and Brambilla (2024)). Further, a simple calculation shows that a 70 kg person consuming 115 g of fish per day (the values used in eq (1)) would exceed the current EFSA TWI (4.4 ng kg^{-1}b.w. per week) from fish alone if the PFAS concentration in fish muscle exceeded 0.4 μg kg^{-1} w.w. (black line in Fig. 1). However, as the above examples illustrate, fish is not the only source of human exposure to PFAS. If an EQS_{biota} was calculated using the current EFSA TWI according to eq (1), (i.e., assuming 10 % of the TWI could come from fish), the concentration would be 0.04 μg kg^{-1} w.w. (purple line in Fig. 1). As can be seen from Fig. 1, this value is lower than concentrations in most fish. It is also important to note that the value of 0.04 μg kg^{-1} w.w. is lower than most detection limits achieved in routine analysis. For example, the required quantification limit for PFOS, PFOA, PFNA and PFHxS is 0.10 μg kg^{-1} according to European Union Reference Laboratory for halogenated POPs in Feed and Food (2022).

Regardless of whether one concludes that the EQS_{biota} should or should not be calculated using Eq. (1) with the lower EFSA TWI, the current limit values and tolerable intake estimates are not harmonized. In fact, a new $QS_{biota\ h,h}$ of 0.077 μg PFOA-equivalents per kg biota (red dashed line in Fig. 1) has been suggested (EU, 2021). This value is based on the current EFSA TWI (4.4 ng kg^{-1}b.w. per week) and the EC's updated method for calculating $QS_{biota,\ hh}$, i.e., using data on fish intake in the general population in Europe and assuming that 20 % of the total PFAS intake comes from fishery products (EC, 2018; EU, 2021). Comparing the fish data reviewed herein to the maximum level of 0.008–0.02 μg kg^{-1} for PFOS in fish consumed in one meal per week (the level calculated by Barbo et al. (2023), as detailed in section *Thresholds and guideline values in the EU and the USA*) shows that most fish exceed this level. Barbo et al. (2023) concluded that an individual's consumption of freshwater fish is potentially a significant source of exposure to PFAS. That conclusion is in line with indications of the comparisons performed in the present study. These considerations can have serious consequences for the global seafood industry. Overall, the concentrations of PFAS in fish reviewed here are high compared to the present-day EFSA TWI and US EPA reference dose value. Limit values calculated based on present-day EFSA TWI and EPA reference dose value are below the detection limit in most studies (as stated above), which is problematic. Given the current technology, any detection may have to be defined as an exceedance if these values are implemented.

5. How to best communicate risk

It is, in our opinion, important to communicate the potential risk of consuming fish that are contaminated with PFAS from diffuse sources as well as from point sources. However, it is vital to balance and correctly communicate these risks to enable the public to make informed decisions. Fish is an important source of proteins as well as vital micronutrients for human populations around the world (Golden et al., 2016). One possible approach is to provide balanced general advice on the amount (i.e., grams) of fish that can be consumed per week without exceeding thresholds. Such advise should take into account the total amount of PFAS exposure the general population is exposed to, including exposure from sources such as food packaging (Trier et al., 2011; Xu et al., 2013) as well as potential effects of cooking methods (Taylor et al., 2019; Hu et al., 2020). In addition, specific and tailored risk communication could be used for regional areas, freshwater versus seawater fish, or wild caught versus farmed fish. Tailored advice for some locations polluted by nearby PFAS sources are already given. In

2020, the Norwegian public was advised against consuming fish from waters that are polluted by PFAS from a factory producing paper products (Lake Tyrifjorden) and fish from freshwater bodies near airports which are polluted due to the use of aqueous film forming foam (AFFF) for firefighting activities (Mattilsynet, 2024b, 2023). In The Netherlands, RIVM advises reducing consumption to a minimum for fish, oysters, and clams from River Western Scheldt, which is polluted by emissions from the 3M company in Antwerp, Belgium (RIVM, 2022). In the USA, 14 out of 50 states have issued fish consumption advisories for specific water bodies or fish (Barbo et al., 2023). This approach could be extended to region-specific recommended amounts of consumption of fish only exposed to pollution from diffuse sources. In addition, PFAS exposure via consumption of locally caught freshwater fish can disproportionally affect different groups in the population (Barbo et al., 2023). Specific advice for wild freshwater fish is already given in Norway due to the mercury content, where the advice is to not eat large wild caught freshwater fish (Mattilsynet, 2024a). Furthermore, pregnant or breastfeeding women, as well as small children, are advised against eating wild caught freshwater fish at all (Mattilsynet, 2024a). Based on possible pollution with dioxins, PCBs, and PFAS, the Netherlands Nutrition Center advises against the consumption of specific species of freshwater fish from Dutch surface waters (Voedingscentrum, no date).

One obstacle with such an approach is population groups that exceed safety thresholds even without including fish consumption, such as Norwegian children (NIPH, 2020). The large number of PFAS that are, or have been, on the global market (Wang et al., 2017) represent an unknown risk, as present thresholds only account for a few PFAS. Another challenge is the lack of structured concentration data needed for informed decision making. A structured overview of data on PFAS concentrations in fish would support tailored advice specific for regions or population groups. Further, the way in which the above-mentioned existing advisories have affected the dietary choices of the general public is, to the best of our knowledge, unknown.

6. Conclusion

We conclude that the global contamination of fish with PFAS may be of concern in the context of human fish consumption. This adds to the growing body of evidence that global PFAS contamination poses a risk for the world's population, and not only highly exposed individuals. The question remains as to how to tackle this problem.

Several legislations are currently being revised and the hope is a more harmonized policy framework where guideline values are streamlined. Several approaches and strategies are currently being developed and adopted to reduce emissions, such as source control and safe and sustainable by design strategies (Hale et al., 2022). However, it seems inevitable that tolerable intake will be exceeded without advice against eating fish at all. Fortunately, scientists have the tools to close the data gap related to concentrations of known PFAS in fish exposed to diffuse pollution. Mapping and compiling searchable databases of PFAS pollution in fish around the world would help supply data for informed decision making. A similar effort was performed by the Forever Pollution Project, which recently published an overview of more than 17 000 sites where PFAS contamination has been detected in Europe (Le Monde, 2023). With more data, risk communication becomes more informed, and the public can be provided with the correct information to make informed decisions.

CRediT authorship contribution statement

Håkon Austad Langberg: Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Gijsbert D. Breedveld:** Writing – review & editing, Supervision, Project administration. **Roland Kallenborn:** Writing – review & editing. **Aasim M. Ali:** Writing – review & editing. **Sarah Choyke:** Writing – review & editing. **Carrie A. McDonough:** Writing – review & editing. **Christopher P. Higgins:** Writing – review & editing. **Bjørn M. Jenssen:** Writing – review & editing. **Morten Jartun:** Writing – review & editing. **Ian Allan:** Writing – review & editing. **Timo Hamers:** Writing – review & editing. **Sarah E. Hale:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

Christopher P. Higgins is involved in various PFAS litigation activities. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Forever Pesticides: A Growing Source of PFAS Contamination in the Environment

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Background: Environmental contamination by fluorinated chemicals, in particular chemicals from the per- and polyfluoroalkyl substances (PFAS) class, has raised concerns around the globe because of documented adverse impacts on human health, wildlife, and ecosystem quality. Recent studies have indicated that pesticide products may contain a variety of chemicals that meet the PFAS definition, including the active pesticide ingredients themselves. Given that pesticides are some of the most widely distributed pollutants across the world, the legacy impacts of PFAS addition into pesticide products could be widespread and have wide-ranging implications on agriculture and food and water contamination, as well as the presence of PFAS in rural environments.

Objectives: The purpose of this commentary is to explore different ways that PFAS can be introduced into pesticide products, the extent of PFAS contamination of pesticide products, and the implications this could have for human and environmental health.

Methods: We submitted multiple public records requests to state and federal agencies in the United States and Canada and extracted relevant data from those records. We also compiled data from publicly accessible databases for our analyses.

Discussion: We found that the biggest contributor to PFAS in pesticide products was active ingredients and their degradates. Nearly a quarter of all US conventional pesticide active ingredients were organofluorines and 14% were PFAS, and for active ingredients approved in the last 10 y, this had increased to 61% organofluorines and 30% PFAS. Another major contributing source was through PFAS leaching from fluorinated containers into pesticide products. Fluorination of adjuvant products and "inert" ingredients appeared to be limited, although this represents a major knowledge gap. We explored aspects of immunotoxicity, persistence, water contamination, and total fluorine load in the environment and conclude that the recent trend of using fluorinated active ingredients in pesticides may be having effects on chemical toxicity and persistence that are not given adequate oversight in the United States. We recommend a more stringent risk assessment approach for fluorinated pesticides, transparent disclosure of "inert" ingredients on pesticide labels, a complete phase-out of post-mold fluorination of plastic containers, and greater monitoring in the United States. https://doi.org/10.1289/EHP13954

Introduction

Pesticides are commonly used in the United States and around the world to kill or suppress certain organisms on farmland and in areas where people live and work. Although pesticides are often efficacious at killing or preventing the growth of target organisms, they are widely regarded as causing serious unintended harms to both humans and nontarget biota. In the United States alone, roughly 450 million kg of pesticide active ingredients were applied in an estimated 5.3 million cumulative km^2-treatments of farmland throughout the country in 2021.[1]

Therefore, the enormous potential for human exposure and environmental contamination belies the importance of understanding complete product compositions and their environmental fate and transport. Pesticide products generally contain two types of ingredients: active and "inert." Active ingredients are the primary components in pesticide products that kill or suppress the targeted organism.[2] "Inerts" are every other ingredient added to the pesticide product, including emulsifiers, solvents, carriers, aerosol propellants, fragrances, and dyes.[3] However, far from being inert, many of these ingredients have chemical properties that can influence the toxicity or alter the bioavailability of the active ingredient or have unintended off-target effects themselves to people and wildlife.[4,5] Unlike active ingredients, "inerts" are not required to be publicly disclosed on the pesticide label[6] and toxicity testing is limited.[5] This lack of transparency and insufficient toxicity testing—in the pesticide context and many others—accomplishes two things from a public health perspective: It can *a*) hamper the ability of medical professionals to effectively treat patients who fall ill following pesticide exposure and *b*) shield companies from accountability regarding the harms from their products.[5,7,8]

In agriculture, pesticide products are commonly applied with adjuvants, which are separate products that can reduce drift/volatilization, facilitate application, or enhance pesticidal effects of pesticide products.[9] Adjuvant ingredients are widely used in US agriculture, as demonstrated by an analysis of usage data in the state of California.[9]

Fluorination is used to modify chemical attributes, such as stability and lipophilicity, improve stereochemical specificity, and increase residual activity of pesticide ingredients.[10] Pesticide active ingredients are commonly fluorinated, with insecticides and

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N.D.'s employer, the Center for Biological Diversity, currently has active litigation against the US EPA involving some active ingredients that happen to be PFAS for failure to consult under the Endangered Species Act and failure to comply with the Federal Insecticide, Fungicide, and Rodenticide Act. The PFAS classification and extent of fluorination of the active ingredients are not at issue in the litigation. N.D. has provided scientific support for these lawsuits. K.B.'s employer, Public Employees for Environmental Responsibility (PEER), currently has active litigation against Inhance Technologies, LLC, involving formation of PFAS during the fluorination of plastic containers contrary to EPA regulations. K.B. is a Declarant in the lawsuit and has provided scientific support for this lawsuit. K.B., on behalf of PEER, has publicly taken the position that post-mold fluorination of plastic containers is dangerous to human health and the environment and should be discontinued. K.B. and PEER are also representing current and former government scientists on issues relating to PFAS, and PFAS in pesticides. K.B. and PEER have also been involved with Freedom of Information Act (FOIA) litigation against EPA for PFAS related issues. All other authors declare they have nothing to disclose.

Conclusions and opinions are those of the individual authors and do not necessarily reflect the policies or views of EHP Publishing or the National Institute of Environmental Health Sciences.

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acaricides more likely to be highly fluorinated.[11] Fluorination can contribute to the molecular stability of active ingredients—both *in vivo* and in the broader environment—and can influence lipophilicity, which can alter membrane permeability and binding to target proteins.[10] The most common chemotype for fluorinated active ingredients is a trifluoromethyl ($-CF_3$) group followed by a monofluoromethyl group ($-CFH2$).[11]

Numerous patents have demonstrated ways in which fluorinated "inerts" can expedite dispersal of the sprayed pesticide on targeted surfaces such as leaves, aid in surfactancy, and facilitate the penetration of the pesticide into living organisms.[12] The fluorination of inert ingredients can help prevent the formation of foam in the pesticide formulation to ensure efficient spreading of the pesticide after spraying,[12,13] and fluorinated inerts are also used as propellants in aerosol pesticide products.[14] Given that many adjuvant and inert ingredients perform similar functions, it is assumed that at least some adjuvant ingredients are fluorinated.[15]

One subset of fluorinated molecules is per- and polyfluoroalkyl substances (PFAS). PFAS are a serious environmental health concern owing to their highly persistent nature,[16] often potent toxicities,[17] potential to bioaccumulate,[18] and widespread presence in people, animals, and the broader environment.[19,20] Through its PFAS Strategic Roadmap, the US Environmental Protection Agency (EPA) in 2021 committed to not only facilitate the remediation of legacy PFAS contamination but also to intervene to limit the introduction of unnecessary new PFAS into the environment.[21]

A widely used definition of PFAS comes from the Organisation for Economic Cooperation and Development (OECD) and encompasses almost any chemical with at least one perfluorinated methyl group ($-CF_3$) or a perfluorinated methylene group ($-CF_2-$).[22,23] Given the broad nature of this definition, PFAS are often subcategorized by the length of their carbon chain. For the purposes of this commentary, we have further classified PFAS as long-chain, short-chain, or ultrashort-chain, which respectively contain $\geq$6, 4–5, and $\leq$3 fully fluorinated carbon atoms. Although all PFAS are considered extremely persistent owing to the strength of the carbon–fluorine bond, some may differ significantly in other chemical properties, such as mobility, lipophilicity, and potential to bioaccumulate.[24]

Given the diverse array of health impacts that have been linked to PFAS exposure,[25] it is important to understand the extent to which the inclusion of carbon–fluorine bonds within pesticide ingredients is impacting persistence and toxicity. When proposing drinking water limits for six PFAS, the US EPA found that reduced exposure would result in a lower prevalence of kidney cancers, heart attacks, strokes, and developmental effects, as well as a general reduction in harms to the immune, developmental, cardiovascular, hepatic, endocrine, metabolic, reproductive, and musculoskeletal systems of US residents.[26] The majority of studies on PFAS toxicity have focused on just a few compounds, but efforts to catalog the toxicity of other PFAS have indicated shared toxicity end points.[27,28]

The purpose of this commentary is to explore ways that PFAS can be introduced into pesticide products, the extent of PFAS contamination, and the implications this could have for human and environmental health. Here we have identified multiple pathways by which PFAS are introduced into pesticide products—both intentionally and unintentionally—and the regulatory shortcomings that prevent a faithful accounting of the risks posed by this class of chemicals. By focusing on pathways of PFAS introduction, our goal with this commentary is to ultimately identify ways that regulators could reduce PFAS in these products and more fully account for their human and environmental health harms in the pesticide registration process.

Methods

Information Sources Used in This Commentary

Information on the number of currently registered active ingredients, fluorinated inert ingredients, and fluorinated adjuvant ingredients were obtained from public records requests to various state-level government agencies in the United States, US federal agencies, and Canadian agencies and are cited in text in the "Methods" or "Discussion" sections. Multiple publicly accessible databases were also searched for relevant adjuvant ingredient information and water detections of fluorinated active ingredients and are also cited in text in the "Methods" and "Discussion" sections. Data sources used in this commentary can be found in Table 1.

Additional Analyses Conducted for Active Ingredients

As of 31 December 2021, the US EPA had 1,157 pesticidal active ingredients registered with the agency (Excel Table S1).[29] Active ingredients fell into three different categories: biopesticide, antimicrobial, and conventional. Biopesticides[48] are naturally occurring chemicals or living organisms—often used in organic agriculture—that do not contain carbon–fluorine bonds. Antimicrobials[49] are often used indoors in relatively lower amounts. Conventional active ingredients[50] are often thought of as "typical" pesticides—mainly synthetic chemicals used widely in agriculture, around people's homes and in green spaces to kill unwanted insects, plants, rodents, or fungi. These ingredients have a higher potential for broader environmental contamination because they are often used outdoors and in higher quantities than biopesticides or antimicrobials.[51,52] Therefore, we curated the list of active ingredients we received in our public records request down to 471 unique, conventional active ingredients to determine how many were organofluorines or PFAS (Excel Tables S1–S3).

In curating our list of 1,157 pesticidal active ingredients down to 471 unique, conventional active ingredients, we

- Mined US EPA's Pesticide Product and Label System (PPLS) database,[53] the Pesticide Chemical Search tool,[54] and other online materials to identify and exclude any active ingredient

Table 1. Public records, communications, and database sources used in this commentary.

Section	Sources
Active ingredients	US EPA FOIA response[29]
Inert ingredients	US EPA FOIA responses,[30,31] US EPA InertFinder Database,[32] Health Canada PMRA List of Formulants,[33] email communication with Health Canada's Senior Scientific Screening Officer (N. Donley, personal communication)
Adjuvant ingredients	TELUS Label Search,[34] California Department of Pesticide Regulation Public Records Act Request,[35] Washington State Department of Agriculture Spray Adjuvant Ingredients List[36]
Storage container leaching	Analytical testing reports from Eurofins Lancaster Laboratories Env, LLC.[37–42] and Alpha Analytical,[43] US EPA. Analysis of PFAS in selected mosquito control products from the Maryland Department of Agriculture,[44] US EPA. Verification Analysis for PFAS in Pesticide Products[45]
Water contamination	USGS. Dissolved Pesticides in Weekly Water Samples from the NAWQA Regional Stream Quality Assessments (2013–2017)[46]
Pesticide usage	USGS. Preliminary estimated annual agricultural pesticide use for counties of the conterminous United States[47]

Note: EPA, Environmental Protection Agency; FOIA, Freedom of Information Act; NAWQA, National Water-Quality Assessment; PFAS, per- and polyfluoroalkyl substances; PMRA, Canada's Pest Management Regulatory Agency; USGS, US Geological Survey.

that met the definition of an antimicrobial or biopesticide. Antimicrobial pesticides are substances or mixtures of substances used to destroy or suppress the growth of harmful microorganisms, such as bacteria, viruses, or fungi, on inanimate objects and surfaces. Biopesticides are any plant incorporated protectant (PIP), live organism, or naturally occurring extracts from live organisms (e.g., peptides, alcohols, oils, pheromones, extracts). We also excluded any active ingredient whose sole purpose was not for pesticidal use, such as nitrogen stabilization.

- Identified and excluded different precursor forms of the same pesticide because the active pesticide molecule was identical (e.g., dicamba was only represented once in our list even though it had many different registered salt forms). We also identified and excluded different purified isomers or enantiomers that were present in mixtures of a previously registered active ingredient (e.g., alpha-cypermethrin and zeta-cypermethrin were excluded from our list because they were simply two isomers that were present in the previously registered cypermethrin). We also identified and excluded active ingredients that were structurally identical but in a different phase from an active ingredient on our list (e.g., amorphous silica and silicon dioxide were reduced down to a single entry on our list).
- Identified and removed products that only had "technical" or "manufacturing use only" products registered, because we were interested only in active ingredients used in end-use products.

US Geological Survey Water Data Analysis

Between 2013 and 2017, the US Geological Survey (USGS) analyzed 482 wadable streams for pesticide contaminants in five regions of the United States (Northwest, California, Midwest, Southeast, and Northeast). The methodology used is described in five regional reports,[55–59] and data are available for downloading from the USGS website.[46] We manually identified all analyzed active ingredients that met the OECD PFAS definition, as well as degradates (metabolites) of those active ingredients, in the site's Table 3 text file and extracted the available detection and water concentration data on those chemicals from Data Tables 4–8 on the same site.[46] Data extracted and compiled included the number of positive detections and maximum detected concentrations for 29 analytes (13 PFAS active ingredients plus 16 fluorinated degradates).

Discussion

How PFAS Are Introduced into Pesticides

We sought to document and understand ways in which PFAS were introduced into pesticides and the extent of PFAS contamination in pesticide products. The following sections detail our analyses. There are multiple ways that PFAS can be introduced into pesticide products, which can facilitate their deposition into the environment. We have broadly categorized these PFAS contamination pathways as intentional and unintentional. Below are examples of each.

Intentional addition of PFAS. **Active ingredients.** Of the 471 unique, conventional active ingredients that were currently registered in the United States, 107 (23%) contained at least one carbon–fluorine bond and 66 (14%) met the OECD definition[22] of PFAS (Figure 1 and Table 2; Excel Tables S3–S5) (see the "Methods" section for details). Of the 54 conventional active ingredients that had been approved in the most recent 10 y, the proportion of fluorination increased dramatically with 33 (61%) classified as organofluorines and 16 (30%) as PFAS (Figure 1 and Table 2; Excel Tables S3–S5).

The trend of increasing fluorination of active ingredients in the United States in recent years was consistent with trends in other countries[10] and with the ability of fluorination to impart chemical properties on pesticides that were desirable to manufacturers and users, particularly the addition of a $-CF_3$ moiety.[11] In fact, most of the PFAS active ingredients contained a $-CF_3$ group as the sole criteria for their inclusion as PFAS in this commentary (Table 2 and Figure 2; Excel Tables S4 and S5).

Two active ingredients stood out as having a significantly higher degree of fluorination than the others: broflanilide and pyrifluquinazon (Figure 2; Excel Tables S4 and S5). Both contain a highly fluorinated side chain that is structurally similar to hexafluoropropylene oxide,[62] a component of the highly toxic, known water contaminant GenX. However, despite both having a similar degree of fluorination, the parent molecules differ in their relative persistence as designated by the US EPA. Broflanilide is considered highly persistent, with the parent molecule having soil and aqueous half-lives in the range of 5–6 y.[63] The US EPA has found that the parent broflanilide and its fluorinated degradates have the potential to bioconcentrate and are likely to accumulate in the environment over time.[63] Despite these alarming chemical properties, the US EPA concluded that the pesticide met the registration standard under US pesticide

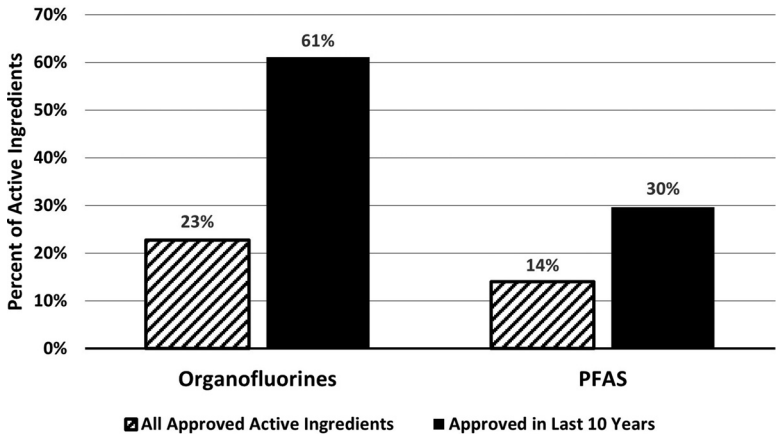

Figure 1. Percentage of conventional US pesticide active ingredients that were organofluorines or PFAS. The striped bars denote the percentage of all US-approved active ingredients ($n = 471$) that were organofluorines (left) or PFAS (right) as of 2021. The solid bars denote the percentage of active ingredients approved between 2012 and 2021 ($n = 54$) that were organofluorines (left) or PFAS (right). For all active ingredients, 107/471 (23%) were organofluorines and 66/471 (14%) were PFAS. For active ingredients approved between 2012 and 2021, 33/54 (61%) were organofluorines and 16/54 (30%) were PFAS. Note: PFAS, per- and polyfluoroalkyl substances.

law.[63] The parent molecule of pyrifluquinazon, on the other hand, is classified by the US EPA as nonpersistent, with soil and aqueous half-lives ranging from 1–16 d.[64] Extractable degradates were similarly short-lived; however, sediment-bound degradates were characterized as very persistent.[64] No studies on the terminal fluorinated degradates of pyrifluquinazon were analyzed by the US EPA, prompting US EPA scientists to convey that "we are concerned that the total accumulation of all PFAS degradates both known and unknown will be a risk issue."[65]

Table 2. PFAS active ingredients approved in the United States and associated registration dates.

CAS No.	Registration date	Active ingredient name[a]
50594-66-6; 62476-59-9	20 August 2018; 20 March 1987	Acifluorfen; sodium acifluorfen
1861-40-1	22 March 1972	Benfluralin
352010-68-5	24 April 2015	Bicyclopyrone
82657-04-3	2 October 1985	Bifenthrin
1207727-04-5	14 January 2021	Broflanilide
63333-35-7	3 October 1985	Bromethalin
122453-73-0	19 January 2001	Chlorfenapyr
180409-60-3	27 June 2012	Cyflufenamid
400882-07-7	9 May 2014	Cyflumetofen
97886-45-8	18 June 1991	Dithiopyr
55283-68-6	2 May 1989	Ethalfluralin
120068-37-3	1 May 1996	Fipronil
104040-78-0	14 May 2007	Flazasulfuron
158062-67-0	26 September 2003	Flonicamid
79241-46-6	25 August 1986	Fluazifop-P butyl
79622-59-6	10 August 2001	Fluazinam
181274-17-9	29 September 2000	Flucarbazone-sodium
131341-86-1	5 October 1995	Fludioxonil
142459-58-3	8 April 1998	Flufenacet
62924-70-3	27 May 1983	Flumetralin
2164-17-2	28 May 1974	Fluometuron
239110-15-7	30 January 2008	Fluopicolide
658066-35-4	2 February 2012	Fluopyram
59756-60-4	31 March 1986	Fluridone
56425-91-3	4 December 1989	Flurprimidol
958647-10-4	13 March 2018	Flutianil
66332-96-5	12 March 1996	Flutolanil
69409-94-5	25 March 1983	Fluvalinate
72178-02-0; 108731-70-0	11 September 1987; 10 April 1987	Fomesafen; sodium salt of fomesafen
76703-62-3; 91465-08-6	31 March 2004; 13 May 1988	gamma-Cyhalothrin; lambda-cyhalothrin
86479-06-3	10 March 1994	Hexaflumuron
67485-29-4	30 September 1982	Hydramethylnon
173584-44-6	30 October 2000	Indoxacarb
141112-29-0	15 September 1998	Isoxaflutole
77501-63-4	1 April 1987	Lactofen
1417782-03-6	26 June 2019	Mefentrifluconazole
139968-49-3	3 August 2007	Metaflumizone
27314-13-2	19 March 1975	Norflurazon
116714-46-6	25 September 2001	Novaluron
121451-02-3	21 September 2001	Noviflumuron
1003318-67-9	31 August 2015	Oxathiapiprolin
42874-03-3	15 June 1981	Oxyfluorfen
219714-96-2	27 September 2004	Penoxsulam
183675-82-3	29 February 2012	Penthiopyrad
117428-22-5	30 November 2012	Picoxystrobin
29091-21-2	7 February 1992	Prodiamine
94125-34-5	3 May 1995	Prosulfuron
365400-11-9	9 August 2007	Pyrasulfotole
179101-81-6	24 April 2008	Pyridalyl
337458-27-2	3 January 2013	Pyrifluquinazon
447399-55-5	15 February 2012	Pyroxasulfone
422556-08-9	27 February 2008	Pyroxsulam
372137-35-4	3 September 2009	Saflufenacil
946578-00-3	6 May 2013	Sulfoxaflor
79538-32-2	17 January 1989	Tefluthrin
335104-84-2	29 November 2007	Tembotrione
112281-77-3	14 April 2005	Tetraconazole
1229654-66-3	10 March 2021	Tetraniliprole
88-30-2	21 August 1964	TFM
1220411-29-9	25 September 2020	Tiafenacil
122454-29-9	2 May 2007	Tralopyril
141517-21-7	20 September 1999	Trifloxystrobin
290332-10-4	29 September 2003	Trifloxysulfuron-sodium
68694-11-1	24 October 1991	Triflumizole
1582-09-8	4 December 1968	Trifluralin
126535-15-7	4 June 1996	Triflusulfuron-methyl

Note: CAS, Chemical Abstracts Service; EPA, Environmental Protection Agency; PFAS, per- and polyfluoroalkyl substances; TFM, 3-trifluoromethyl-4-nitrophenol.
[a]Data in the table were extracted from a public records request to the US EPA.[29] From this list, PFAS pesticides were manually identified and extracted for this table (see the "Methods" section for more detail).

"Inert" ingredients. A public records request to the US EPA, which the agency responded to in December of 2022, indicated that the agency had 24 registered inert ingredients that it had identified as PFAS or that the agency suspected may be PFAS.[30] The provided list appeared to have been compiled of both PFAS inerts and fluorinated inerts that were not PFAS. Since the US EPA produced the list of 24, the agency canceled 12 that were not in any currently registered pesticide products[66] and we identified one as not having any carbon–fluorine bonds, leaving 11 currently registered organoflourine inert ingredients (Table 3). We confirmed this list of 11 by searching for "fluoro" in the ingredient name field on the US EPA's Inert Finder database.[32]

Of the 11 US EPA-registered organofluorine inert ingredients, 8 met the OECD definition of PFAS (Table 3 and Figure 2). Four of these 11 ingredients were approved for both food and nonfood use, whereas the rest were only for nonfood use.[32] All the food-use organofluorine inerts had been exempted from a tolerance,[67,68] meaning that any level of these ingredients was legal on food. Interestingly, 5 of these organofluorine inerts were not in any US-registered pesticide products, whereas 6 were present in 1–67 currently registered products (Table 3).[31] Information on which specific products contained these ingredients was considered "confidential business information" by the US EPA, so it was unclear whether these products were widely used or how they were used.

Canada's Pest Management Regulatory Agency (PMRA) has compiled a list of currently registered inerts (which it calls "formulants") and updates that public list every 6 months.[33] As of 1 October 2022, there were eight organofluorine inert ingredients registered in the country, with seven being PFAS (Table 3). These eight organofluorine inerts were present in anywhere from 1 to 20 Canadian pesticide products (N. Donley, personal communication) (Table 3).

Notably, one inert ingredient approved in both the United States and Canada for both food and nonfood use was the incredibly persistent polytetrafluoroethylene (PTFE), known by the brand name Teflon (Table 3 and Figure 2). Although chemical manufacturers and their consultants consider fluoropolymers like PTFE to be less toxic than their nonpolymeric PFAS counterparts,[69] other researchers have identified serious concerns with their production and use.[70] For instance, PTFE can often be contaminated with nonpolymeric PFAS—at concentrations in the parts-per-million range, well above human toxicity thresholds.[70] This, coupled with its extreme persistence and the inability to recover PTFE once it has been dispersed, makes its use particularly problematic.

During peer review of this manuscript, the US EPA revised the number of products it believes contain PTFE from the 14 it

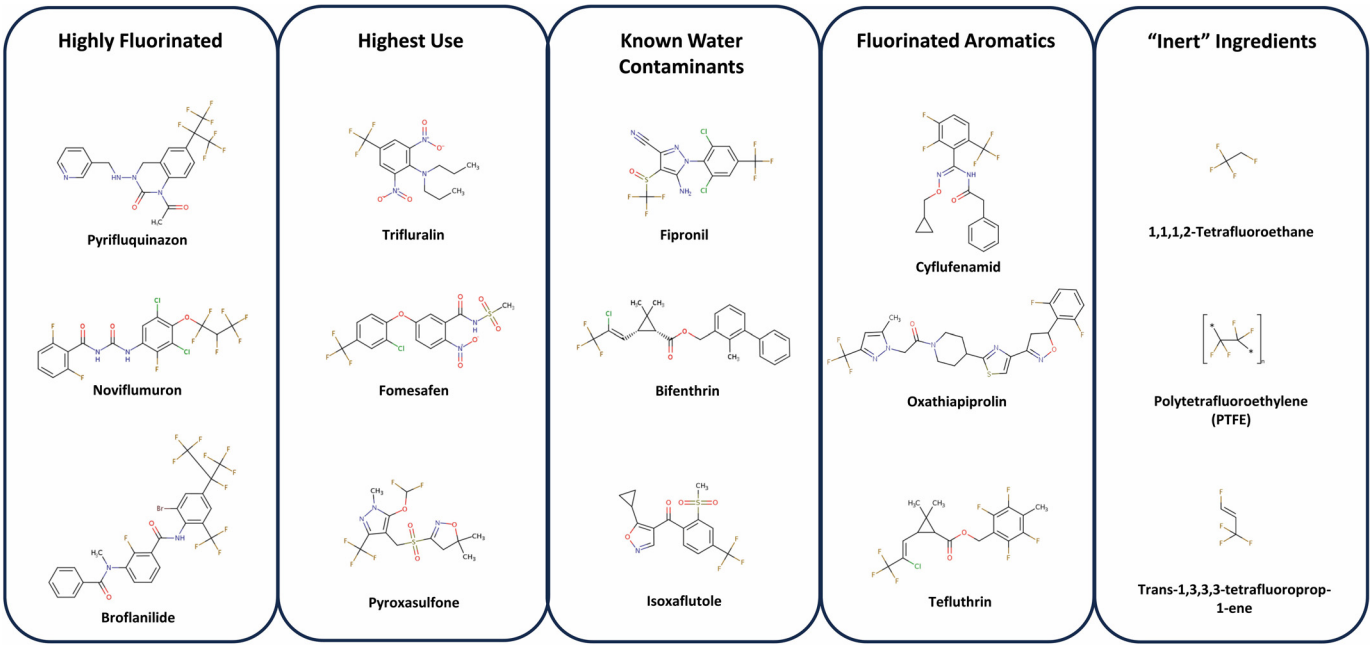

Figure 2. Examples of PFAS chemicals approved for use in US pesticide products. The "highly fluorinated" grouping is the approved PFAS active ingredients with the longest fluorinated chains. The "highest use" grouping is the approved PFAS active ingredients with the highest use by volume, as estimated by the US Geological Survey (Excel Table S6). The "known water contaminants" grouping is the approved PFAS active ingredients that have been widely reported in the literature and identified by government monitoring to be major water contaminants in the United States. The "fluorinated aromatics" grouping displays a few examples of the approved PFAS active ingredients that have fluorinated aromatic structures in addition to a $-CF_3$ moiety. The "'inert' ingredients" grouping displays the US- and Canada-approved inert ingredients that are present in the most pesticide products (Table 3). Structure images were obtained from US EPA's CompTox Chemicals Dashboard.[60,61] Note: EPA, Environmental Protection Agency; PFAS, per- and polyfluoroalkyl substances.

told us in our earlier public records request (Table 3) to zero and proposed to remove PTFE from its list of approved inert pesticide ingredients.[71] We believe this is good news for public health and hope the agency is successful in finalizing that action.

Adjuvants. The US federal government does not regulate adjuvants as pesticides.[9] If an adjuvant product is to be used on food crops, its ingredients may require a tolerance or exemption from a tolerance under the Federal Food, Drug and Cosmetic Act (FFDCA), but there is very little federal oversight.[72]

Some US states regulate adjuvant products. The most robust system is in California, which requires adjuvants to be registered as pesticides, submission of formulation information, and reporting of adjuvant use.[9,73] Adjuvants are widely used in California: Forty-one of the most widely applied 100 pesticide ingredients

Table 3. A list of organofluorine and PFAS inert ingredients approved in the United States and Canada and the number of registered products that contain them.

CAS No.	Ingredient name[a]	PFAS	Food use	Approved in the USA	Approved in Canada	Products in the USA (*n*)	Products in Canada (*n*)
75-37-6	1,1-Difluoroethane	N	Y	Y	Y	67	3
811-97-2	1,1,1,2-Tetrafluoroethane	Y	Y	Y	Y	37	15
9002-84-0	Polytetrafluoroethylene (PTFE; Teflon)	Y	Y	Y	Y	14[b]	2
29118-24-9	*trans*-1,3,3,3-Tetrafluoroprop-1-ene	Y	Y	Y	Y	3	20
188027-78-3	5H-1,3-Dioxolo[4,5-f]benzimidazole, 6-chloro-5-[(3,5-dimethyl-4-isoxazolyl)sulfonyl]-2,2-difluoro	Y	N	Y	N	0	NA
24937-79-9	Ethene, 1,1-difluoro-, homopolymer	N	N	Y	N	0	NA
42557-13-1	Poly(oxy(methyl(3,3,3-trifluoropropyl)silylene)), alpha-(trimethylsilyl)-omega((trimethylsilyl)oxy)-	Y	N	Y	N	0	NA
593-70-4	Fluorochloromethane	N	N	Y	N	3	NA
63148-56-1	Siloxanes and silicones, Me 3,3,3-trifluoropropyl	Y	N	Y	N	1	NA
67786-14-5	2-Naphthalenesulfonic acid, 6-amino-4-hydroxy-5-{{2-(trifluoromethyl)phenyl}azo}-, monosodium salt	Y	N	Y	N	0	NA
88795-12-4	1-Butanol, 4-(ethenyloxy)-, polymer with chlorotrifluoroethene, (ethenyloxy)cyclohexane, and ethoxyethene	Y	N	Y	N	0	NA
98-56-6	Parachlorobenzotrifluoride	Y	INO	N	Y	NA	1
65530-85-0	Alpha-(cyclohexylmethyl)- omega-hydro-poly (difluoromethylene)	Y	INO	N	Y	NA	1
131324-06-6	PTFE, alpha-chloro-omega-(1-chloro-1-fluoroethyl)-	Y	INO	N	Y	NA	1
163440-89-9	PTFE, alpha-hydro-omega-(2,2-dichloro-2-fluoroethyl)-	Y	INO	N	Y	NA	1

Note: CAS, Chemical Abstracts Service; INO, information could not be obtained; Me, methyl; N, no; NA, not applicable; PFAS, per- and polyfluoroalkyl substances; Y, yes.
[a]Data in this table were obtained through database searches, personal communications, and public records requests.[31–33]
[b]After formally responding that 14 products contained PTFE, the US EPA has since publicly stated that zero products contain PTFE and has proposed to remove it from the list of approved inert ingredients in the United States.

are adjuvant ingredients.[74] The high use of these ingredients indicates that they may be a source of PFAS contamination in the environment.

The only sources of information on adjuvant ingredients we found came from the agrochemical industry and the few state-level agencies in the United States that regulate them. The industry views this information as proprietary, so publicly available information is scant. TELUS, a producer of agricultural industry software, maintained a label database[34] that at our date of search encompassed 1,343 adjuvant products. An advanced search for "adjuvant" products containing active ingredients with the term "fluoro" returned zero results. However, it was unclear whether all ingredients were disclosed on this database and whether full chemical names were listed.

We also received public records from California and Washington State. An inquiry to the California Department of Pesticide Regulation (CDPR) asking whether any adjuvants contained fluorinated ingredients elicited the response that "there are no adjuvant products currently registered by DPR which contain fluorinated chemical ingredients."[35] In 2020, the Washington State Department of Agriculture developed a list of spray adjuvant ingredients that identified 313 ingredients in state-registered adjuvant products.[36] The Washington State Department of Agriculture requires only the top three ingredients in adjuvant products to be disclosed to the state,[75] and our search of this partial ingredient list identified no fluorinated ingredients.

Although we found no evidence to indicate that adjuvant products contained fluorinated ingredients or PFAS, our dataset was incomplete and regional, and we concluded that it does not provide strong evidence that no adjuvant ingredients are fluorinated. Rather, the lack of transparency and oversight of adjuvants meant that a robust dataset was not available.

Unintentional addition of PFAS. **Leaching from storage containers.** The practice of fluorinating polyethylene plastic containers to prevent permeability of aromatic chemicals started as early as 1958.[76] Today hundreds of millions of high density polyethylene (HDPE) containers that contain agricultural products, personal care products, household cleaning supplies, home improvement products, and food are fluorinated each year.[77] The most common method of fluorinating hydrocarbon-based plastics is post-mold fluorination,[78] where already molded containers are treated with fluorine gas under high temperature and pressure.

The goal of post-mold fluorination is to swap out the carbon–hydrogen bonds of the HDPE to carbon–fluorine bonds in a thin layer on the surface of the plastic to enhance its barrier properties. If there is any oxygen or water in the fluorination chamber, then the fluorination process will form perfluorinated structures.

In 2011, researchers discovered that a subset of PFAS, perfluorinated carboxylic acids (PFCAs), were formed during the direct post-mold fluorination of HDPE containers when trace amounts of oxygen were present.[77] Eight years later, Public Employees for Environmental Responsibility (PEER) discovered that the insecticide Anvil 10+10 contained perfluorooctanoic acid (PFOA) and hexafluoropropylene oxide dimer acid (HFPO-DA).[79] This finding spurred the US EPA to test the leaching potential of fluorinated HDPE containers that were used to store pesticides, and the agency identified eight PFCAs leaching from various containers—with total concentrations in the 10–60 ppb range.[80] The US EPA's findings that fluorinated HDPE containers leach PFCAs has been reproduced by other groups and is now a well-established contamination pathway for contents stored in these containers.[81] It is estimated that 20%–30% of all hard plastic containers used in the agricultural sector are fluorinated,[82] elevating concerns about widespread PFAS contamination.

Since PEER's initial testing of Anvil 10-10 found PFAS, many other groups have tested and found long- and short-chain PFAS in multiple pesticide products in a manner that is consistent with container leaching (Table 4). It should be noted that the results of this testing by different groups have produced conflicting results that appear to depend on the analytical methodology used and where the testing was conducted, affirming the difficulty of testing complex mixtures such as pesticide products for PFAS.

In late 2023, the US EPA used its authority under the Toxic Substances Control Act (TSCA) to prohibit the production of multiple PFAS in the container fluorination process.[83] Although we believe this strong action would have been an enormous benefit for public health, the US EPA's action was overturned by a federal appellate court, and it is unclear whether the agency will pursue further action under a different legal mechanism.[84]

Other potential sources. Although leaching of PFAS from fluorinated containers appears to be the primary contamination pathway of long- and short-chain PFAS into pesticide products, the testing that has been conducted to date indicates there are other sources of contamination. Multiple groups have found that some pesticides contain perfluorinated sulfonic acids (PFSAs) (Table 4). As mentioned above, container fluorination has only been demonstrated to generate PFCAs that are available for leaching.[80] Therefore, the presence of PFSAs in some products—none of which were approved active or inert ingredients (Table 3; Excel Table S1)—indicates that there are other sources of unintentional contamination.

A recent study on serum levels of long-chain PFAS found that both PFSAs and PFCAs were significantly higher in female Danish greenhouse workers compared with a female Danish urban population measured during the same time period.[85] The authors concluded that this disparity was likely due to differences in exposure to agricultural pesticide formulations and proposed that pesticides may be an important source of long- and short-chain PFAS exposure to agricultural workers.

More research is needed to examine other potential sources for introduction of long- and short-chain PFAS into pesticide products. It is possible that the solvents or other components used in the preparation of some pesticide products could unknowingly be contaminated with PFAS.

Manufacturing by-products and impurities are another potential source of PFAS in pesticides. US EPA regulations allow pesticide products to contain impurities as long as they are $<1{,}000$ ppm and not of "toxicological significance."[6] Toxicological significance is defined with regard to impurities that also happen to be known pesticides[86]; however, its meaning is not formally defined for other impurities. The US EPA views any concentration of an impurity meeting the agency's PFAS definition as toxicologically significant, requiring disclosure.[87] Yet it is unclear whether this reporting requirement is known among the industry or whether companies even know about PFAS impurities in their products, given that many pesticide products contain undisclosed PFAS ingredients (Table 4).[88]

Consequences of PFAS in Pesticides

In addition to documenting sources of PFAS in pesticide products, we sought to understand how PFAS in pesticide products could be impacting human and environmental health in the United States and beyond. Although a lot of knowledge gaps still exist, the available data are cause for concern. It is our view that PFAS in pesticides, particularly PFAS active ingredients, may be having unintended impacts on environmental and public health that must be mitigated or eliminated to prevent irreversible impacts. Below are examples of potential impacts we have identified.

Immunotoxicity. The immune system is highly vulnerable to exposure to chemical toxicants, particularly during development

Table 4. Compilation of studies analyzing long- and short-chain PFAS detections in pesticide products, estimated PFAS concentration found, where the products were tested, and the analytical methodology used.

Date	Product name	US EPA registration no.	PFAS found	Estimated concentration or range	Units	Samples tested (*n*)	Where tested	Analytical method used
27 August 2020[37]	Anvil 10-10	8329-62	Perfluorooctanoic acid (PFOA)	ND–250	ng/L	2	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
			Hexafluoropropylene oxide dimer acid (HFPO-DA)	410–500	ng/L			
15 September 2020[38]	Anvil 10-10	8329-62	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
23 October 2020[39]	Anvil 10-10	8329-62	HFPO-DA	ND–260	ng/L	2	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
19 November 2020[43]	Anvil 10-10	8329-62	Perfluorobutanoic acid (PFBA)	52.8–716	ng/L	9	Alpha Analytical	Modified US EPA method 533
			Perfluoropentanoic acid (PFPeA)	35.2–296	ng/L			
			Perfluorohexanoic acid (PFHxA)	17.6–132	ng/L			
			Perfluoroheptanoic acid (PFHpA)	ND–53.4	ng/L			
			Perfluorohexanesulfonic acid (PFHxS)	ND–59.2	ng/L			
			1H,1H,2H,2HPerfluorooctanesulfonic acid (6:2FTS)	ND–31.6	ng/L			
			PFOA	ND–25.7	ng/L			
			Perfluoroheptanesulfonic acid (PFHpS)	ND–138	ng/L			
			Perfluorooctanesulfonic acid (PFOS)	ND–141	ng/L			
19 March 2021[40]	Permanone 30-30	432-1235	PFOA	3,500	ng/L	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
			HFPO-DA	630	ng/L			
13 May 2021[41]	Frontline Plus for dogs	65331-5	PFHxA	530	ng/L	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
			PFHpA	270	ng/L			
			PFHpS	390	ng/L			
			HFPO-DA	1,200	ng/L			
	Seresto Collar	11556-155	Perfluorododecanesulfonic acid (PFDoS)	0.25	ng/g	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
21 October 2021[44]	Permanone 30-30	432-1235	ND	NA	NA	6	US EPA–Fort Meade	Modified US EPA method 537.1 (oily matrix)
	PermaSease 30-30	53883-459-86291	ND	NA	NA	1	US EPA–Fort Meade	Modified US EPA method 537.1 (oily matrix)
28 March 2023[42]	Malathion 5EC	19713-217	PFOA	510	ng/L	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
			PFHpS	680	ng/L			
	Oberon 2SC	264-719	PFBA	1,500	ng/L	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Intrepid 2F	62719-442	Perfluorobutanesulfonic acid (PFBS)	350	ng/L	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Ultra-Pure Oil	69526-5-499	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Avid 0.15 EC	100-896	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Pedestal	53883-419-59807	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Ortho Home Defense	239-2717	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Triazicide granules	9688-250-8845	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)
	Marathon 1% granular	59807-15	ND	NA	NA	1	Eurofins Lancaster	Modified US EPA method 537 (537 IDA)

Table 4. (*Continued.*)

Date	Product name	US EPA registration no.	PFAS found	Estimated concentration or range	Units	Samples tested (*n*)	Where tested	Analytical method used
18 May 2023[45]	AVID 0.15 EC	NP	ND	NA	NA	2	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Pedestal	NP	ND	NA	NA	2	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Ultra-Pure Oil	NP	ND	NA	NA	1	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Marathon 1%	NP	ND	NA	NA	2	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Oberon	NP	ND	NA	NA	2	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Malathion 5EC	NP	ND	NA	NA	1	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	BotaniGard 22WP	NP	ND	NA	NA	1	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Overture 35WP	NP	ND	NA	NA	1	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	Conserve	NP	ND	NA	NA	1	US EPA–Fort Meade	Two modifications of SW846 test method 8327
	XXpire	NP	ND	NA	NA	1	US EPA–Fort Meade	Two modifications of SW846 test method 8327

Note: EPA, Environmental Protection Agency; IDA, isotope dilution anion (exchange solid phase); NA, not applicable; ND, not detected; NP, not provided; PFAS, per- and polyfluoroalkyl substances.

and in older adults.[89] Long- and short-chain PFAS that have been extensively studied—such as PFOA, PFOS, and perfluorohexanesulfonic acid (PFHxS)—are known to harm the immune system, weaken the antibody response to vaccinations, and increase the risk of infectious disease.[90,91] Studies of impacts on the immune system indicate that it is one of the most sensitive targets of PFAS exposure,[23,92] and both the US EPA and the European Food Safety Authority (EFSA) have identified immunotoxicity as the most potent adverse effect to humans from exposure to certain PFAS.[90] Given the documented sensitivity of the immune system to PFAS exposure, and that immunotoxicity studies are commonly waived during pesticide registration reviews,[93] our analysis focused on this specific health end point. However, we note that with the myriad health effects linked to PFAS exposure, other health end points will likely be of additional interest with regard to fluorinated pesticides.

In 2007, following recommendations from the National Research Council and the US EPA's Science Advisory Panel,[94] the US EPA required all pesticide active ingredients to be subject to T cell–dependent antibody response testing—which the agency uses as a surrogate for immunotoxicity in general.[95] Six years after imposing this requirement, the pesticide industry requested that the US EPA conduct a retrospective analysis of the usefulness of the immunotoxicity assay in pesticide registration decisions.[96] In its 2012 analysis, the US EPA found that, of a representative sample of 155 pesticides that had immunotoxicity testing, the agency only considered 15 (10%) to be immunotoxic.[96] The US EPA's analysis further found that the 15 immunotoxicity findings did not influence the outcome of the pesticides' risk assessment. Following this analysis, the US EPA indicated that it would be receptive to waiving immunotoxicity studies for pesticide active ingredients.[96] Reflecting this position, between 2012 and 2018, the US EPA granted 223 of 229 waiver requests (97%) for immunotoxicity testing of pesticide active ingredients.[93]

However, lost in the US EPA's retrospective analysis, conducted before much of the public or regulatory awareness of the health risks of PFAS, was the fact that 7 of the 15 immunotoxic active ingredients (47%) were organofluorines and 6 of 15 (40%) were PFAS.[96] That compares with 20% and 13% of conventional pesticide active ingredients that were respectively organofluorines or PFAS as of 2012 (Excel Table S3). Immunotoxic effects have also been reported in the peer-reviewed literature for several fluorinated pesticides, including bifenthrin, fipronil, flupyradifurone, and flonicamid.[10]

Troublingly, the number of active ingredients that are fluorinated or that meet the definition of PFAS has increased considerably from 2012 to the present (Figure 1)—the very time period that the US EPA granted 97% of waiver requests for immunotoxicity study requirements.[93] This suggests that fluorinated or PFAS active ingredients may be more likely to be immunotoxic than other types of active ingredients and that any associated immunotoxicity may not be accounted for owing to the lack of requirement for scientific study.

Environmental fate. All PFAS contain perfluoroalkyl moieties that are highly stable in the environment.[16] Even a single $-CF_3$ or a difluoromethylene ($-CF_2$) moiety in a pesticide active ingredient can resist degradation under highly stringent conditions.[97] This all but assures that most PFAS molecules will persist in the environment in perpetuity or break down into a degradate that will similarly persist in perpetuity.[16]

This makes it particularly important to fully understand the metabolic life cycle of fluorinated pesticides *in vivo* and in the environment. For example, highly persistent, fluorinated degradates of the PFAS pesticide fipronil are often found at much higher

concentrations in human serum, plasma, and urine[98–100] and are widespread in the environment.[101,102] These fluorinated degradates are also more persistent[103] and more toxic to a wide range of taxa, including mammals, than the parent pesticide ingredient.[100,104,105] Therefore, a faithful accounting of the pesticide degradates that form within organisms and in the broader environment is essential for proper risk evaluation, particularly for degradates that are highly persistent.

In assessing risk to humans and the environment from the use of a pesticide, the US EPA will estimate exposure to the parent active ingredient and some of its degradates. Which degradates to analyze in the risk assessment is determined via multiple degradation studies—often hydrolysis and photodegradation studies to understand abiotic breakdown and biotic metabolism studies in the terrestrial and aquatic environment.[106] According to US EPA guidelines, the suggested duration of these degradation experiments range from 5 to 30 d for the abiotic degradation studies[107,108] and 100 to 120 d for the biotic metabolism studies.[109,110]

Analyzing the degradation of a chemical over the span of 1–4 months gives the risk assessor an incomplete picture of chemical transformations that happen months or years later. For persistent pesticides and those with persistent degradates, there can be significant uncertainty around what the intermediate and terminal degradates are and how long it takes for terminal degradates to form.[111,112] Current test guidelines were not designed with highly persistent substances in mind, and test duration is specifically cited as one way that limits our understanding of how chemical metabolism proceeds from the parent molecule to its terminal degradates.[113,114]

Even known highly persistent degradates are sometimes omitted from US EPA risk assessments of active and inert pesticide ingredients. The US EPA will often identify which degradates are of toxicological concern either by assessing the acute toxicity of the degradate(s) or conducting a quantitative structure activity relationship to predict toxicity to certain taxa.[115] However, this practice can end up essentially ignoring the release of highly persistent chemicals into the environment. For example, with the PFAS active ingredient sulfoxaflor, the US EPA found that highly persistent fluorinated degradates were expected to contaminate ground and surface water; however, it concluded that the only chemical relevant to assessing ecological risk was the parent molecule because the other degradates were less acutely toxic to aquatic organisms.[116] Similarly, the US EPA conducted a quantitative structure activity relationship for the fluorinated degradates of the PFAS active ingredient bicyclopyrone and determined that the only chemical of ecotoxicological concern was the parent molecule.[117]

The persistence and toxicity of degradates are rarely, if ever, accounted for in the approval of fluorinated "inert" ingredients. A public records request for the degradate/metabolite studies reviewed by the US EPA to support the approval or continued approval of five PFAS inert ingredients [Chemical Abstracts Service (CAS) numbers 42557-13-1, 9002-84-0, 63148-56-1, 67786-14-5, and 188027-78-3] returned no relevant records.[118]

We believe that basing the ecotoxicological relevance of a highly persistent degradate on a limited number of acute toxicity studies or the presence/absence of an active structural site is likely to miss key risks. Pesticide degradates are widespread in the environment[119] and, in many cases, are found in concentrations higher than the parent molecule.[120] There can be serious consequences if the uncertainty involved in a pesticide approval decision ultimately leads to an underestimation of risk coming from pesticide degradates. The generation of fluorinated degradates that have half-lives in the decades or centuries means that any release into the environment will likely be irreversible and will be of ongoing concern if those degradates are found to be more toxic than previously thought. This has led some researchers to propose introducing new regulatory hazard categories that accurately reflect relative persistence of a chemical and its degradates and that high persistence alone should be a basis for regulation irrespective of the toxicities that have thus far been identified.[121,122]

Water contamination. Although most PFAS active ingredients (Table 2) have not been monitored for their presence in the environment across the United States, some older PFAS active ingredients have been actively monitored and found throughout the country. Bifenthrin and fipronil, first approved in 1985 and 1996, respectively, are among the most widely detected pesticides in US streams, lakes, and rivers, and both are often found at levels that exceed aquatic safety thresholds.[101,123–125] In beeswax samples taken from commercial beehives in multiple US states, 98% contained the 1980s-era PFAS pesticide fluvalinate.[126] The older PFAS pesticides isoxaflutole and penoxsulam, and their fluorinated degradates, have been detected in groundwater near sites where they are used.[127,128] Despite making up only 1% of the total applied mass of pesticides that are found in California waters, the PFAS pesticides cyhalothrin and bifenthrin account for 90% of the applied toxicity to aquatic life, indicating they are likely having an outsized impact on aquatic health.[129]

To look more generally at the environmental presence of PFAS active ingredients, we compiled and analyzed USGS data that tested for the presence of a wide variety of pesticides in nearly 500 streams across five regions of the United States between 2013 and 2017 (see the "Methods" section for details).[46] Of the 225 pesticide compounds tested in water samples, 13 were PFAS active ingredients and 16 were their fluorinated degradates (29 total PFAS analytes). Of those tested, 27 PFAS analytes (93%) from 12 PFAS active ingredients were found in US streams (Table 5). Fipronil and isoxaflutole were most prevalent, whereas isoxaflutole and trifloxystrobin were found in the highest concentrations. Only 1 of the 13 tested PFAS active ingredients had >453,000 kg of annual use in US agriculture during the tested time period and many had <45,300 kg of annual use,[130] indicating that these are not highly used active ingredients relative to many others used in agriculture. This suggests that the prevalence of these fluorinated pesticides and degradates in waterways cannot be explained by high agricultural use alone.

Only 13 PFAS active ingredients—of 66 conventional active ingredients that are currently registered (Table 2)—have been actively tracked in surface water across the United States in recent years, and 12 have been found (Table 5). Nearly all of these 13 tested PFAS active ingredients have been registered for >20 y (Excel Table S3), suggesting that the increase in fluorinated pesticide approvals in recent years (Figure 1) is having unknown consequences with regard to water quality. Because of this, we believe that in-depth, targeted monitoring studies of all PFAS pesticides and their fluorinated degradates in the United States is critical.

Total organic fluorine in the environment. Increasing scrutiny of PFAS contamination of drinking water, and sources for drinking water, has led to increasing research on organic fluorine compounds in the environment and biota. Analytical measurements of PFAS have typically been limited to targeted testing for a few dozen PFAS chemicals. Studies that have done targeted PFAS testing in conjunction with total organic fluorine measurements have found that targeted testing is capturing only a small portion of the total organofluorine load in the environment and biota.[131] Not only have many studies found that levels of total organic fluorine are increasing, but the fraction of samples attributed to unknown organofluorine chemicals is often high and has also been increasing in recent years.[131–133]

Table 5. PFAS analytes tested in US surface waters by the USGS between 2013 and 2017, how often they were detected, and the maximum concentration identified.

Active ingredient[a]	Fluorinated analyte	Detections (n)[b]	Max conc (ng/L)
Bifenthrin	Bifenthrin	10	10.7
	cis-Cyhalothric acid[c]	17	961.4
Fipronil	Fipronil	847	61.8
	Desulfinylfipronil	342	10.6
	Fipronil sulfide	441	10.6
	Fipronil sulfone	754	18.1
	Dechlorofipronil	0	—
	Desulfinylfipronil amide	29	14.0
	Fipronil amide	762	84.1
	Fipronil sulfonate	8	72.5
Flubendiamide[d]	Flubendiamide	79	148.9
	Deiodo flubendiamide	2	4.9
Fluometuron	Fluometuron	8	229.5
	Hydroxy mono demethyl fluometuron	2	6.4
	4-Hydroxy-*tert*-fluometuron	1	7.4
	Hydroxyfluometuron	1	3.9
	Demethyl fluometuron	5	5.1
Indoxacarb	Indoxacarb	1	3.4
Isoxaflutole	Isoxaflutole	11	660.1
	Isoxaflutole acid RPA 203328	271	928.4
	Diketonitrile isoxaflutole	496	2,134.90
Lactofen	Lactofen	0	—
Norflurazon	Norflurazon	111	318.6
	Demethyl norflurazon	137	541.8
Novaluron	Novaluron	2	14.5
Oxyfluorfen	Oxyfluorfen	4	70.4
Prosulfuron	Prosulfuron	3	9.5
Tetraconazole	Tetraconazole	56	62.0
Trifloxystrobin	Trifloxystrobin	151	3,670.80

Note: —, not applicable; max conc, maximum concentration detected; PFAS, per- and polyfluoroalkyl substances; USGS, United States Geological Survey.
[a]Data in this table were obtained from the USGS.[46]
[b]The USGS sampled 482 streams between 4 and 12 times each during the 6-to 14-wk study period. Number of detections denotes the number of times the analyte was detected in a sampling event.
[c]Also a metabolic product of lambda-cyhalothrin and tefluthrin, two PFAS active ingredients that were not monitored by the USGS.
[d]Flubendiamide was canceled in the United States in 2016 and is not currently registered.

This indicates that new or unidentified PFAS are increasingly contributing to the overall organofluorine exposure to people and the environment. Increasingly, this unknown total organic fluorine fraction is thought to be coming from short- and ultrashort-chain PFAS,[134–136] which we have defined as respectively containing 4–5 and $\leq$3 fully fluorinated carbon atoms. Short- and ultrashort-chain PFAS are also generally more difficult to remove from contaminated water sources by commonly used filtration methods, making any resulting contamination potentially more difficult to rectify.[137,138] Importantly, the presence of ultrashort-chain PFAS in the environment does not correlate well with the presence of long- and short-chain PFAS, indicating that ultrashort-chain PFAS are coming from different sources.[135,139]

Given that most of the PFAS active pesticide ingredients in the United States contain a $-CF_3$ moiety, it is possible that many of these active ingredients will eventually break down into ultrashort-chain PFAS as their terminal fluorinated degradates. One such degradate is trifluoroacetic acid (TFA), a highly persistent and mobile chemical that is a known water[135,139] and food[140] contaminant and has been detected in several wildlife species.[141,142] A study of Norwegian wildlife found TFA to be a major contributor to total organic fluorine levels in animals.[141] TFA is abundant in human serum and urine samples,[143,144] and exposure to people is thought to occur primarily via contaminated drinking water and indoor household dust.[144]

TFA is a known metabolic by-product of some fluorinated pesticides,[24,97] and TFA levels in waterways and food even correlate strongly with pesticide use.[140,145] Organically grown food has also been found to have lower levels of TFA than food grown with synthetic pesticides.[140] A study by the German Environment Agency found that, when considering the 28 pesticide active ingredients approved in Germany that have a $-CF_3$ group (and could potentially metabolize into TFA), up to 500 metric tons of TFA pollution could be generated annually in the country just from pesticide degradation.[146]

With 66 PFAS active ingredients approved in the United States—and the United States having much higher pesticide use than all countries in the European Union combined[147]—the potential TFA pollution in the United States coming from pesticides is likely significantly greater than that of Germany. The USGS estimates that anywhere from 10.4 to 15.9 million kg of PFAS active ingredients are used across the United States each year (Excel Table S6)[47]—the vast majority of which contain at least one $-CF_3$ group and could potentially metabolize into TFA or other persistent, fluorinated water contaminants. Given the annual volume of use, pesticide active ingredients have the potential to contribute significantly to the presence of ultrashort-chain PFAS and, by extension, the total organic fluorine load in the environment and biota.

Regulatory Recommendations

- Based on ample research and scientific testing, we believe that post-mold fluorination of plastic containers cannot be done without producing harmful PFAS that are available for leaching. This practice should be discontinued and substituted with other options, such as barrier methods for plastic that do not use fluorine, and possibly in-mold fluorination if it is found not to produce PFAS.
- The United States and other countries must require that all pesticide ingredients, including inerts, and their relative proportions be disclosed on pesticide labels and material safety data sheets. The American Medical Association made this same suggestion nearly 30 y ago in an effort to protect the public, to no avail.[148] It is our view that the pesticide industry should not be allowed to hide behind spurious claims of confidentiality at the expense of the public's knowledge of the potentially harmful chemicals in widely available products.
- Immunotoxicity studies should no longer be waived for fluorinated active ingredients or inerts, and the US EPA should issue a data call-in for any pesticide ingredients that do not have the necessary testing in place.
- All PFAS pesticides, and all intermediate and terminal degradates, must be fully evaluated for environmental persistence, and the most persistent ones, such as broflanilide, should be mitigated heavily and targeted for replacement with nonchemical or less persistent alternatives. This can be modeled after a P-sufficient framework[121] to prevent potential devastating consequences of releasing highly persistent chemicals with no means for recovery.
- The US federal government must expand environmental monitoring and biomonitoring programs to include all PFAS pesticides to gather timely data on their bioaccumulation and their potential impact on human and ecosystem health.
- Once it identifies all terminal and intermediate degradates from PFAS pesticides, the US EPA must assess the cumulative impacts from fluorinated degradates that are common to multiple active ingredients, such as TFA. The US EPA must also assess how the cumulative use of all fluorinated pesticides can impact the total organic fluorine load in the environment and food.

Conclusions

Pesticide products increasingly contain fluorinated ingredients, and this is happening via multiple pathways. A major contributor of long- and short-chain PFAS (>3 fully fluorinated carbon atoms) into pesticide products was through leaching of PFAS from fluorinated containers (Table 4). The polymer PTFE is also an approved inert ingredient in the United States and Canada, but its use currently appears to be limited to about a dozen products (Table 3). The available data also pointed to unknown sources of long- and short-chain PFAS contamination in pesticide products, which have yet to be identified (Table 4).

The biggest contributor of ultrashort-chain PFAS (≤3 fully fluorinated carbon atoms) in pesticide products was active ingredients and their degradates (Table 2). Although 23% of US conventional pesticide active ingredients were organofluorines and 14% were PFAS, those percentages jumped to 61% organofluorines and 30% PFAS when looking just at active ingredients approved in the past 10 y (Figure 1). In our review of US EPA risk assessment documents, these PFAS active ingredients are either extremely persistent themselves or break down into intermediate or terminal degradates that are extremely persistent. The majority of PFAS active ingredients contained a single $-CF_3$ moiety and the few that had been monitored are known to pollute waterways across the United States (Table 5; Excel Tables S4 and S5).

We believe these data indicate that some pesticide products contain complex mixtures of ultrashort-chain to long-chain PFAS that are present in parts-per-billion concentrations for some of the long- and short-chain PFAS and up to parts-per-hundred concentrations for some of the ultrashort-chain PFAS active ingredients. The long-term impacts of using mixtures of extremely persistent chemicals on potentially hundreds of millions of acres of US land every year is, to us, a cause for concern. Most, if not all, PFAS in pesticide products or their degradates are going to be chronic persistent pollutants[16] for the foreseeable future of humanity, and their ultimate impact on human and environmental health are largely unknown. Here we have identified steps the US government can take to mitigate potential impacts of fluorinated components in pesticides with the ultimate goal of eliminating or reducing their use altogether.

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Targeted analysis and Total Oxidizable Precursor assay of several insecticides for PFAS

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ABSTRACT

Targeted analysis for 24 Per- and Polyfluoroalkyl Substances (PFAS) was conducted on 10 insecticide formulations used on a United States Department of Agriculture crop research field. Perfluorooctane sulfonic acid (PFOS) was found in 6 of the 10 formulations with concentrations ranging from 3.92 to 19.2 mg/kg. Further analysis of soil and plant samples collected at the site found several additional PFAS, with PFOS being the most prominent. Suspect screening was then conducted on the formulations and provided several suspected PFAS in addition to the 24 targeted analyzed PFAS in 7 of the 10 samples, one of which showed no PFAS during targeted analysis. PFAS-precursor oxidation was then conducted on the two insecticide formulations with the greatest lists of suspected PFAS as validation of potential unknown PFAS in the formulations. This study revealed a previously unknown potential PFAS contamination source for rural and agricultural environments.

1. Introduction

The chemical class per- and poly-fluoroalkyl substances (PFAS) have drawn regulatory focus due to their potential toxicity (Bach et al., 2016; Barry et al., 2013; Gallo et al., 2012; Halldorsson et al., 2012; Jantzen et al., 2016; Johansson et al., 2009; Melzer et al., 2010; Midgett et al., 2015; Savitz et al., 2012; Steenland et al., 2013; Wielsøe et al., 2015), tendency to trophic transport (Awad et al., 2011; Giesy and Kannan, 2001; Hagenaars et al., 2008; Kwadijk et al., 2010; Vestergren et al., 2013), and their environmental mobility and persistence (United States Environmental Protection Agency, 2019). Within the PFAS chemical group, perfluoroalkyl acids (PFAAs) have been the primary focus of research and legislation due to a strong display of the previously mentioned traits and relatively high environmental occurrence.

In February 2019, the United States' Environmental Protection Agency (EPA) published an action plan concerning PFAS exposure and contamination in the United States (United States Environmental Protection Agency, 2019). One of the research areas identified by the action plan as needing additional input was "What are the sources, fate and transport pathways, and exposures to humans and ecosystems?" (United States Environmental Protection Agency, 2019). The most common characterized sources of environmental PFAS contamination are associated with wastewater and biosolids, aqueous firefighting foam (AFFF), and products containing PFAS and PFAS precursor manufacturing and use (Key et al., 1997; Prevedouros et al., 2006). This list is not comprehensive, especially for agricultural or rural communities. To promote advancement in this area, the United States' EPA allocated $5 million on August 20th, 2020 for new research on managing PFAS in agricultural and rural communities.

In a trial run of a prior study on plant uptake of PFAS (Lasee et al., 2019, 2020), it was discovered that there was detectable PFAS contamination in control plant samples grown in a United States Department of Agriculture (USDA) cropping systems research laboratory greenhouse. Targeted Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis was performed to find the source of the PFAS contamination; identified PFAS in the soil on site, other research plants grown on site, and various insecticides used on the site, while site water, potting soil, and fertilizers were all non-detect for PFAS. The objective of this study was to characterize the PFAS found in the tested insecticide formulations and to attempt to connect that PFAS to PFAS found in the soil. Suspect screening was conducted on the insecticide products in an effort to identify possible "unknown" PFAS in the products. Then we

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conducted the Total Oxidizable Precursor assay to quantify how much "unknown" PFAS were observed in two of the insecticide samples.

2. Materials and methods

2.1. Materials

All calibration (4:2 FTS, 6:2 FTS, 8:2 FTS, N-MEFOSAA, N-EtFOSAA, PFBA, PFPeA, PFBS, PFHxA, PFPeS, PFHpA, PFHxS, PFOA, PFOS, PFHpS, PFNA, PFOSA, PFDA, PFNS, PFUdA, PFDS, PFDoA, PFTrDA, and PFTeDA) and stable isotope (^{13}C4-PFBA, ^{13}C5-PFPeA, ^{13}C3-PFBS, ^{13}C5-PFHxA, ^{13}C2–4:2FTS, ^{13}C4-PFHpA, ^{13}C3-PFHxS, ^{13}C8-PFOA, ^{13}C2–6:2FTS, ^{13}C9-PFNA, ^{13}C8-PFOSA, ^{13}C8-PFOS, ^{13}C6-PFDA, ^{13}C2–8:2FTS, ^{13}C7-PFUdA, d3-MeFOSAA, d5-EtFOSAA, ^{13}C2-PFDoA, ^{13}C2-PFTeDA) standards were obtained from Wellington Laboratories (Guelph, Ontario). The 24 PFAS selected were those included in the EPA SW-846 Test Method 8327. Tested insecticides formulations were collected from the test site (a USDA crop research laboratory).

It is important to note that we have observed some 50- and 15-mL test tubes and analysis grade solvents have shown trace PFAS residuals that can lead to contamination of a sample. We recommend the careful use of solvent blanks and prior analysis of materials and products to remove the risk of sample contamination from these sources. LC-MS/MS-grade methanol, water, and acetonitrile used in this study were purchased from Honeywell (Charlotte, North Carolina). 50- and 15-mL test tubes used in this study were VWR® High-Performance Conical-Bottom Centrifuge Tubes with Flat Cap, Polypropylene (Radnor, Pennsylvania). Prior analysis of these solvents and test tubes did not show concentrations of the 24 PFAS targeted in this study. Scoopulas used in this study were disposable polypropylene scoopulas from VWR® (Radnor, Pennsylvania).

2.2. Insecticide collection and analysis

Ten different insecticide formulations were collected from the crop research site after the analysis of soil from the site found concentrations of a variety of PFAS species. The selected insecticides were only those recorded as used on the site in 2017. In 2020, the insecticides were confirmed to still be in use at the site. Insecticide formulates sampled were collected from a cabinet designated for storage of all pesticides in use on site. All pesticides stored in the cabinet were kept, if possible, in their original resealable packaging. If the original packaging did not allow for sealing or the seal was damaged, the pesticide, still in its original packaging, was place inside a secondary sealable plastic container. None of this studies sampled insecticides were stored in secondary containers.

Formulations samples were collected with disposable scoopulas and were placed into 15 mL centrifuge tubes for storage. Samples were stored in a hood at 20 °C. Formulations were diluted as 10–100 mg in 10 mL LC-MS/MS-grade methanol and were allowed to dissolve over 24 h in 15 mL centrifuge tubes in triplicates. Formulations were then sonicated in a 20 °C water bath for one hour. Each formulation solution was then diluted to 10 μg formulation/1 mL (10 ppm) with LC-MS/MS-grade methanol in a new 15 mL centrifuge tube. No extraction or filtration steps were used due to concerns that these steps could remove fractions of non-targeted PFAS. To prepare for targeted analysis, 537 μL of formulation/methanol dilution, 3 μL of a 120 ng/mL internal standard (in methanol), and 1260 μL of LC-MS/MS-grade water were added to an auto injector vial (recovery of internal standards presented in S1). To prepare samples for suspect screening, 540 μL of each 10 μg/1 mL formulation/methanol dilution and 1260 μL of LC-MS/MS-grade water were added to an auto injector vial. Samples were stored at 5 °C until analysis. For both targeted and non-target analysis, results were calculated between triplicates.

PFAS suspect screening was conducted on all tested insecticides. The list produced by the suspect screening was only partially validated and is therefore incomplete. Accordingly, the current work and discussion is presented in the Supplemental information. Library matches did validate the existence of PFOS in samples. Further identification of suspected PFAS was outside the scope of the current study. Additional information on the suspect screening is presented in the Supplemental information, with the results of the suspect screening presented in Table S2.

2.3. Total Oxidizable Precursor assay

The Total Oxidizable Precursor (TOP) assay developed by Houtz and Sedlak (2012) was used to convert suspected PFAS to PFAAs for which standards were available (ie. PFBA, PFBS, PFPeA, PFPeS, PFHxA, PFHxS, PFHpA, PFOA, PFOS, PFNA, etc.). Insecticide 6 was chosen for this technique because suspect screening (Table S2) showed that insecticide 6 was the only insecticide with a targeted analysis hit (PFOS in insecticides 1–6) with a suspected PFAS with an area of the same order of magnitude as its known PFAS (109,500 vs. 324,100). All other PFAS with a targeted analysis hit did not have suspected PFAS with an area of the magnitude as their known PFAS indicating that they may not have a large "unknown" PFAS fraction. Additionally, insecticide 6′s is one of the most commonly used organophosphate. Insecticide 10 was selected for TOP analysis due to being the only tested insecticide that did not show PFAS concentrations during targeted analysis, but showed activity during suspect screening (Table S2). Many of insecticide 10′s suspected PFAS had large areas indicating that TOP analysis may reveal a large "unknown" PFAS fraction.

2.4. Soil and vegetation sample collection and preparation

The study site was a USDA crop research laboratory that uses the 5 fields on site to said crops. Soil and vegetation samples were collected from these fields. Soil and vegetation sample were collected by a nitrile gloved hand and placed in 50-mL test tubes. Prior to ownership by the USDA, the site was owned by Texas Tech University and was kept as native rangeland. Wastewater, biosolids, or municipal sludge (known PFAS contamination sources) have not been applied to the site. Nearby fields (within 2 miles) also had PFAS concentrations in the soil. Accordingly, none of them were used as controls. This is not surprising as most agricultural fields in the area grew cotton and likely used the same or similar pesticides.

At the time of sampling, Fields 1 and 4 were planted with cotton, Fields 2 and 3 were planted with sorghum, and Field 5 as planted with corn, cotton, sorghum, peanuts, and beans. Approximate sampling locations are presented in Fig. 1. Soil samples were collected as a composite of 5–6 surface grab samples taken from a single field. It rained 0.4 in. the morning before samples were collected. Corn, bean, and peanut grab samples were collected from Field 5; corn samples were collected as kernels only from immature cobs, bean samples were collected as both seed and pod, and peanut samples were collected as seed and pod from the soil. Each sample was washed in DI water to remove clinging soil. Samples were then dried at 70 °C for 24 h. Dried soil and plant samples were then homogenized. Approximately 2 g of dried soil and 0.5 g of dried vegetation sample were placed in 50-mL polypropylene centrifuge tubes and stored at room temp (20 °C) to await extraction.

2.5. Soil and vegetation extraction

Soil and vegetation samples were extracted as published in Zhao et al. (2013) with the exception of filtering the final extract with a nylon filter. Prior work conducted in the laboratory showed that nylon filters may remove significant fractions of some longer chained PFAS and PFAS precursors. Extractions were reconstituted in 30 % methanol/ 70 % water and stored in 2-mL auto-sampler vials at 5 °C until analysis. Average recoveries for the 19 internal standards (IS) are presented in Table S3 for plant tissue samples. Recoveries using this technique were

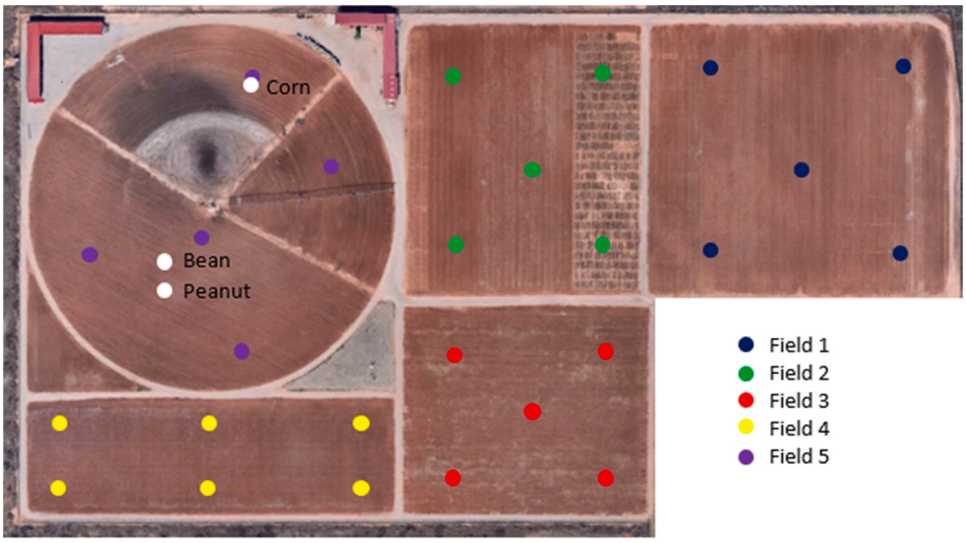

Fig. 1. Soil and plant sampling locations on the study site. All soil samples taken from the same field were combined as a composite sample for analysis.

low for several PFAS IS in soil samples, so soil samples were extracted again using a basic methanol extraction technique modified from Higgins et al. (2005) (IS recoveries presented in Table S4).

2.6. Quality assurance

All samples (insecticide formulations, soil, and plant tissue extractions) were injected in triplicate. Every 9 injections (3 samples) alternating 10 ng/L and 500 ng/L standards were injected for quality control. Extraction blanks were utilized for the plant tissue and soil extractions, and a solvent blank was used for the insecticide formulations as no extraction was done with these samples. Significant 6:2 FTS contamination was observed in the plant and soil extraction samples and as a result, 6:2 FTS concentrations in these samples were not reported due to concerns in their authenticity. SW-846 Test Method 8327 was used for acceptable recovery range (70–130 %). Limits of quantification (LOQs) were determined by injection of 1, 2, 5, and 10 ng/L standards and are presented in the Supplemental information.

2.7. Instrument conditions

Chromatographic separation was carried out using a SCIEX ExionLC™ equipped with a Phenomenex Gemini® C18 column (100 × 3 mm; 3 μm particle size) with a Phenomenex SecurityGuard™ Gemini® C18 (4 × 2 mm) guard column. The column oven temperature was set to 40 °C. The following conditions were used: elution solvents were 20 mM ammonium acetate in water (A), methanol (B) mobile phase composition (A:B; v/v) was 95:5 at 0 min, increasing to 35:65 at 1.6 min, increasing 0:100 at 8 min, and switching to 5:95 at 12.8 min which is maintained until 16 min. The flow rate was 700 μL/min and the injection volume was 500 μL. The LC was coupled to a X500R Quadrupole Time-of-Flight mass spectrometer (SCIEX). These settings were used for both the targeted analysis and suspect screening. Suspect screening was conducted using Electrospray Ionization in negative mode.

3. Results

3.1. Targeted analysis of formulations

The results of PFAS targeted analysis of the insecticide formulations are presented in Table 1. PFAS concentrations were above the LOQ for only one of the 24 species (PFOS) in the 10 analyzed formulations. PFOS

Table 1
Average concentration of PFOS in the analyzed insecticide formulations (mg PFAS/kg formulation or ppm, ± standard deviation). The concentrations reported were calculated from the dilution described previously in the "Insecticide Analysis section". PFAS with no concentrations above LOQ were not included in this table.

Sample ID	Formulation type	Active ingredient	PFOS (mg/kg)
1	Liquid concentrate	Abamectin	3.92 ± 0.51
2	Emulsified suspension	Novaluron	9.18 ± 0.34
3	Liquid concentrate	Mineral Oil (Petroleum oil)	8.64 ± 0.67
4	Emulsified suspension	Imidacloprid	13.3 ± 1.4
5	Emulsified suspension	Spiromesifen	19.2 ± 1.2
6	Liquid concentrate	Malathion	17.8 ± 0.7
7	Wettable powder	*Beauveria Bassiana*	0
8	Wettable powder	Pyridalyl	0
9	Emulsified suspension	Spinosad	0
10	Wettable powder	Spinetoram, Sulfoxaflor	0
BLANK			0

was found in 6 of 10 formulations (3.92–19.17 mg/kg). Peaks for a variety of other PFAS were observed in the samples, primarily PFHxS and PFBS, although none of these peaks surpassed the instrument LOQ (1–10 pg/g in dilutions). This is not surprising as PFAS tend to exist as complex mixtures. Additionally, if the source of the PFOS found in the samples were PFAS precursors, PFAS precursors often degrade into several different PFAAs (Gebbink et al., 2015; Mejia Avendaño and Liu, 2015; Vestergren et al., 2008). The sample injection was a 1:100,000 dilution in methanol, therefore the < LOQ concentrations of PFHxS and PFBS could be detectable in a lower dilution and may still accumulate in soils overtime.

While the PFAS concentrations found in this study are a cause for concern, these insecticides are a highly concentrated product. The dilution and application directions for most of the collected insecticide formulations were approximately 4–8 fluid ounces diluted in 100 gallons of water. At 8 fluid ounces, that is a 1600-fold dilution by volume.

3.2. Targeted analysis of soils

Results of the targeted analysis of surface soil of the 5 tested fields are presented in Table 2. PFOS was the PFAS species with the highest

Table 2
Average soil concentrations (ng PFAS/kg dry soil, ± standard deviation) of PFAS from the targeted analysis of soil samples from five fields. All samples were aggregates of 5–6 surface soil grab samples that were homogenized. Standard deviations are presented in parentheses.

	Field sampled					
PFAS	1	2	3	4	5	BLANK
4:2 FTS	51 ± 7.0	36 ± 7.3	32 ± 5.3	23 ± 3.5	30 ± 5.0	< LOQ
PFOA	42 ± 9.2	72 ± 12	173 ± 38	46 ± 5.1	47 ± 6.5	< LOQ
PFNA	18 ± 2.5	33 ± 6.7	43 ± 7.5	12 ± 1.8	14 ± 1.5	< LOQ
PFOS	698 ± 120	1150 ± 165	1720 ± 299	156 ± 26	247 ± 14	0.0
8:2 FTS	31 ± 7.5	23 ± 4.6	19 ± 2.6	12 ± 0.8	11 ± 2.9	0.0
PFUdA	52 ± 13	58 ± 14	69 ± 8.8	30 ± 1.8	40 ± 8.9	0.0

concentration found in the soil followed by PFOA and 4:2 FTS, 8:2 FTS, PFNA, PFOA, and PFUdA (which all had similar concentrations). Many of the other 24 PFAS species in the targeted analysis were below the LOQ. The full results are reported in Table S5. The targeted analysis placed Field 3 as the field with the highest PFAS concentrations followed by Field 2, Field 1, Field 5, and Field 4. The goal of this sampling technique was to create a single sample that could be a qualitative representative of both known (targeted analysis) and unknown (non-target analysis) PFAS in a field. Additionally, PFAS are known to distribute heterogeneously in soils (Rankin et al., 2016). The soil sampling was only of the surface; different PFAS are known to have a variety of soil distribution patterns (Guelfo and Higgins, 2013). Given those three points, we would not consider concentrations presented in Table 2 to be accurate representatives of a quantitative distribution of PFAS in the tested fields.

The water used to irrigate the research center was also analyzed by mixing 1.4 mL of water with 0.6 mL methanol and directly injecting it. No quantifiable concentrations of target PFAS were found in the water, although, solid phase extraction of a greater volume of water could produce quantifiable concentrations of PFAS.

3.3. Targeted analysis of plant tissues

The results of PFAS targeted analysis of corn kernel, string bean, and peanut are presented in Table 3. In the analyzed insecticides, PFOS was the primary component observed, followed by PFHxS and PFBS (both were below the LOQ). The corn and bean samples, which were collected from the above ground portions of the plants, had PFAS concentrations an order of magnitude higher for PFBA, PFHxA, PFHxS, and PFOS than the peanut sample, which was collected as a below ground portion. For PFHpA, the concentration in the peanuts was an order of magnitude higher than those in the corn and bean tissues. These plant tissues were collected as single, opportunistic grab samples. Replicate sampling

Table 3
Average tissue concentrations (ng PFAS/kg dry plant tissue or ppt) of PFAS from the targeted analysis of corn kernel, string bean pod, and peanuts. All samples are were collected from the commonly consumed tissue of these plants. Standard deviations are presented in parentheses.

	PFBA	PFHpA	PFHxA	PFHxS	PFOA	PFOS
CORN	1120 ± 143	38 ± 2.2	1020 ± 130	4900 ± 147	349 ± 138	3230 ± 316
BEAN	3300 ± 48	37 ± 0.8	138 ± 76	1150 ± 104	176 ± 72	4260 ± 154
PEANUT	580 ± 31	313 ± 39	0	200 ± 59	162 ± 35	407 ± 13
BLANK	0	0	0	0	0	0

throughout the field was not done. Thus, concentrations found in these samples should not be considered representative of the harvested crop.

3.4. Total Oxidizable Precursor assay

The TOP assay was done on insecticides 6 (active ingredient Malathion) and 10 (active ingredients Spinetoram and Sulfoxaflor). The results comparing the before assay to after assay concentrations are found in Fig. 2. The TOP assay technique converts PFAA precursors to PFAAs, although it is not a perfect or complete process. Both insecticides saw an increase in moles of PFAS after the TOP assay was conducted. Suggesting that both insecticides had significant "unknown" PFAS concentrations. Insecticide 6's total PFAS moles nearly tripled (pre – 0.24 μmoles/L vs. post – 0.64 μmoles/L) and insecticide 10 was revealed to have nearly as much PFAS in it as insecticide 6 (0.61 μmoles/L vs. 0.64 μmoles/L) despite not showing any PFAS concentrations in targeted analysis.

4. Discussion

4.1. Targeted analysis

All insecticides tested in this study are still in production under the same brand names, though the formulations tested should not be assumed to be the same as the ones currently in production, as the sampled product was not new. However, PFAS are known to be incredibly environmentally stable, consequently, historic use of insecticides containing PFAS or PFAS precursors can translate into persistent soil contamination. Soil PFAS have been shown to be absorbed and translocated into plant tissues (Lasee et al., 2019; Bizkarguenaga et al., 2016; Blaine et al., 2014; Lechner and Knapp, 2011; Shobhna et al., 2020; Stahl et al., 2009; Wen et al., 2014). Manufacturing of PFAS began in 1949 (3M, 1999). Historical PFAS containing pesticide use could translate into high concentrations of several different PFAS in agricultural soils that can persist in the soil for many years.

Targeted analysis of PFAS concentrations in the tested insecticides (Table 1) showed PFOS to be the primary PFAS found in the formulations. This was reflected in the aggregate soil samples. Inspection of the chromatographs (Fig. 3, Figs. S1–S10) showed a split peak that is indicative of two isomers (a branched and linear) of PFOS being present. Although similar, the chromatographs are not identical in shape. Soil samples showed a smaller peak for the branched isomer than the formulations. An explanation for this phenomenon could be that the soil samples collected were of surface soil and branched PFOS isomers have shown greater environmental mobility than linear PFAS (Chen et al., 2015), leading to a disproportionately greater decrease of branched PFOS surface soil concentrations over time compared to its linear counterpart. In addition, these soil samples are environmental, so multiple PFAS input sources are likely. It is not uncommon to find a variety

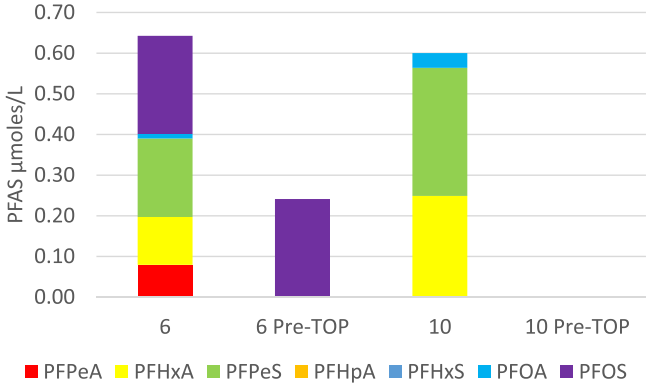

Fig. 2. Average pre- vs. post-TOP PFAS concentrations (μmoles/L) in insecticides 1 and 6.

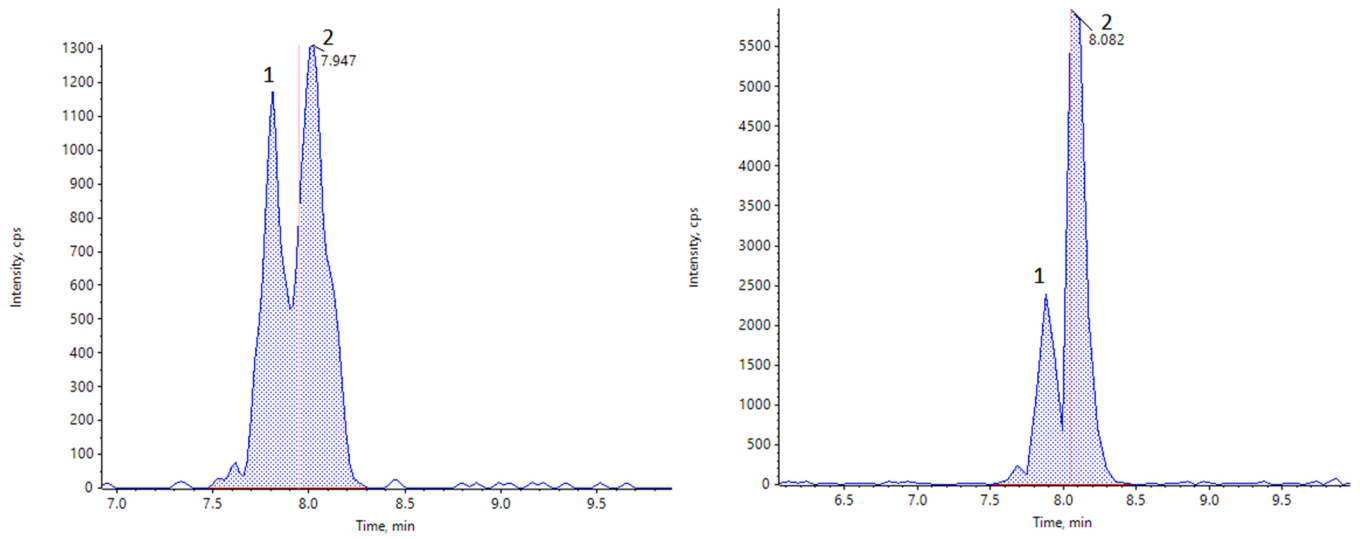

Fig. 3. Chromatographs of PFOS in insecticide 5 (right) and field 3 (left). The branched isomer of PFOS is labeled with 1 and the linear isomer of PFOS is labeled with 2.

of different PFAS in any soil grab sample. PFAS are solely made anthropogenically and many have been known to undergo long-range transport in the environment. Rankin et al. (2016) found dry weight concentrations ranging between 29 and 14,300 ng/kg for total perfluoroalkyl carboxylates and < LOQ-3270 ng/kg for total perfluoroalkyl sulfonates from surface soil samples collected from all continents, including areas judged to have no evident human impact.

Electrochemical fluorination (ECF) and telomerization are the two primary processes used in the production of PFAS and PFAS-related products. Production of PFAS by ECF was mostly phased out in the US in 2002. The existence of branched isomers of PFAS and homologs (like PFHxS for PFOS) are indicative of the ECF production process for PFAS (Benskin et al., 2010). The PFOS chromatograms of the sampled soil and insecticides (that contained PFOS) showed branched isomer peaks (Figs. S1–12). Another hallmark of PFOS produced by ECF is the significant presence of PFHxS also being found in the sample. In the present study's plant tissue grab samples, significant PFHxS concentrations were observed alongside significant PFOS concentrations.

4.2. Plant samples

Blaine et al. (2013) found that negligible amounts of soil PFAS were taken up and deposited in corn grains from corn plants grown in PFAS contaminated biosolid-amended soils. Scher et al. (2018) found negligible concentration of PFBA (the PFAS they found to have the highest bioconcentration potential) in corn kernels and low PFAS concentration in bean pods watered with PFAS-contaminated water. These two studies would suggest that if the corn and bean plants were collected were grown in PFAS-contaminated soil and water, little to no PFAS, other than small amounts of PFBA, would be found in their seeds. The PFAS concentrations found in the tested corn grain and bean pod samples (Table 3) would suggest that the source of these PFAS was not the soil or water they were grown in. Targeted analysis of the tested insecticide samples (Table 1) could account for the PFOS concentrations observed in the corn and bean samples, but not for the other 5 PFAS we observed (PFBA, PFHxA, PFHxS, PFHpA, and PFOA).

The tested formulations in Table 1 are only those found in the complete record of the pesticides applied to the fields in 2017. The tested insecticides likely do not encompass all the potential PFAS sources that could be applied to the site historically. The site is located near third party fields that could contribute pesticide and other product drift. Additionally, the site is located in a city that experiences dust storms several times a year. PFAS have been observed in a variety of dusts (Murakami and Takada, 2008; Wang et al., 2010; Fromme et al., 2009), and dust storms could result in environmental transport of top soil PFAS in dry environments.

The soil samples collected were surface samples. Surface level PFAS distribution often does not match distribution at lower levels (Sepulvado et al., 2011). The roots of the three plants species likely have access to soils whose PFAS concentrations and distribution may not match that of the surface samples collected for this study. This could explain why the peanut samples had concentrations of PFBA, PFHxS, and PFHpA, while none of the sampled surface soil had significant concentrations of those analytes.

4.3. Significance of PFAS in pesticides

Major PFAS contamination has mostly been associated with industrial production and use of PFAS, sites with the use of aqueous firefighting foams, and municipal and industrial waste. While the insecticides tested are commonly used on cotton, a non-consumptive agricultural product, PFAS are generally believed to not significantly degrade environmentally. Years of continuous use of PFAS and PFAS precursor-containing pesticides could lead to significant concentration of PFAS in the soil. Future use of soils treated with PFAS contaminated pesticides for other crops or pesticide drift could lead to PFAS concentrations being found in crops used for human or animal consumption. This potential was observed in three samples of foodstuff crops (corn, beans, and peanuts) that were grown on site, although the source of the PFAS in these crop samples does not appear to be the soil.

One PFAS, N-ethyl perfluorooctane sulfonamide or Sulfluramid (EtFOSA; $C_8F_{17}SO_2NHC_2H_5$), has been used in ant and roach insecticides. EtFOSA is known to degrade into PFOS and FOSA and contribute to environmental concentrations of these chemicals (Nascimento et al., 2018). EtFOSA was not detected in targeted analysis or suspect screening of this study's 10 test insecticides. Applied EtFOSA containing insecticides are currently known to be used in South America to deal with leaf cutter ant, an issue unlikely to occur at the test site.

Insecticide 6′s active ingredient is malathion. Malathion was, at one point, the most commonly used organophosphate insecticide in North America (Bonner et al., 2007). Only one specific formulation was tested. If many malathion formulations, for all of their many uses, contained PFOS concentrations similar to those found in insecticide formulation 6, many people around the world could be expose to PFOS through malathion use.

Suspect screening of all 10 insecticides and TOP assay on insecticides

6 and 10 showed potential for PFAS concentrations outside of the 24 targeted PFAS. Insecticide 10 showed no PFAS concentrations when run for target PFAS analysis, but both suspect screening and the TOP assay showed potential PFAS in the insecticide.

5. Conclusions

In the present work we have discovered PFOS in 6 out of 10 tested insecticides commonly used to treat cotton. In doing so, we identified a source of PFAS environmental contamination for rural and agricultural areas that potentially has been, and could continue to, impact PFAS concentrations in human and animal foodstuff crops grown in these areas. Suspect screening and PFAA-precursor oxidation tests showed evidence PFAS outside of the 24 PFAS included in the targeted analysis in 7 of 10 of the insecticides we tested. Our research also detected multiple PFAS species in soil and plant grab samples beyond what was observed in the insecticides we tested (PFOS). Results from our suspect screening and PFAA-precursor oxidation tests could offer a possible explanation for these concentrations. In this study, we only characterized PFAS concentrations in 10 different insecticides. Further investigation of a wider variety of pesticides as potential PFAS contamination sources should be done to better understand the PFAS exposure risk pesticides could present.

Environmental Implications

(a) The studied material concerns the chemical group per- and polyfluorinated substances (PFAS) which are of utmost regulatory concern around the world.
(b) The work describes a previously unknown source, pesticides, for environmental PFAS contamination.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The corresponding author (Steven Lasee) currently is a research fellow for the Oak Ridge Institute for Science and Education that works with the United States Environmental Protection Agency. The Author also runs the consulting firm “Lasee Research and Consulting”.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.hazl.2022.100067.

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Bioaccumulation and metabolic impact of environmental PFAS residue on wild-caught urban wetland tiger snakes (*Notechis scutatus*)

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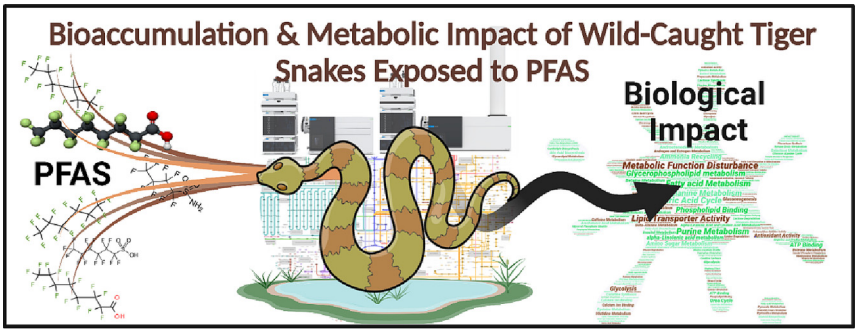
HIGHLIGHTS

- Liver PFAS concentrations were associated with lower snake body condition.
- Highest reported liver PFAS concentration in reptile and Australian vertebrate.
- Energy production pathways impacted in muscle tissues of PFAS-exposed snakes.
- PFAS liver concentrations were higher in males when compared to females snakes.

GRAPHICAL ABSTRACT



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ABSTRACT

PFAS contamination of urban waters is widespread but understanding the biological impact of its accumulation is limited to humans and common ecotoxicological model organisms. Here, we combine PFAS exposure and bioaccumulation patterns with whole organism responses and omics-based ecosurveillance methods to investigate the potential impacts of PFAS on a top predator of wetlands, the tiger snake (*Notechis scutatus*). Tiger snakes (18 male and 17 female) were collected from four wetlands with varying PFAS chemical profiles and concentrations in Perth, Western Australia. Tiger snake livers were tested for 28 known PFAS compounds, and Σ28PFAS in liver tissues ranged between 322 ± 193 μg/kg at the most contaminated site to 1.31 ± 0.86 μg/kg at the least contaminated site. The dominant PFAS compound detected in liver tissues was PFOS. Lower body condition was associated with higher liver PFAS, and male snakes showed signs of high bioaccumulation whereas females showed signs of maternal offloading. Biochemical profiles of snake muscle, fat (adipose tissue), and gonads were analysed using a combination of liquid chromatography triple quadrupole (QqQ) and quadrupole time-of-flight (QToF) mass spectrometry methodologies. Elevated PFAS was associated with enriched energy production and maintenance pathways in the muscle, and had weak associations with energy-related lipids in the fat tissue, and lipids associated with cellular genesis and spermatogenesis in the gonads. These findings demonstrate the bioavailability of urban wetland PFAS in higher-order reptilian predators and suggest a negative impact on snake health and metabolic processes. This research expands on omics-based ecosurveillance tools for informing mechanistic toxicology and contributes to our understanding of the impact of PFAS residue on wildlife health to improve risk management and regulation.

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1. Introduction

Poly- and perfluoroalkyl substances (PFAS) are a known contaminant class of concern that are pervasive in most aquatic environments (Podder et al., 2021). Compared to aquatic invertebrates and small fish, relatively high concentrations of PFAS (i.e., the common and well-studied perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) constituents) are required to induce acute toxicity (i.e., mortality) in larger vertebrates such as reptiles, mammals, birds, etc. Instead, environmentally relevant concentrations of PFAS tend to pose sublethal health effects and result in metabolic perturbations (Ankley et al., 2021; Sinclair et al., 2020). For example, chronic PFAS exposure has been shown to interfere with the growth, development, or body weight of amphibians (Flynn et al., 2021; Hoover et al., 2017), birds (Newsted et al., 2008), fish (Suski et al., 2021), mammals (Martin et al., 2007) and reptiles (Beale et al., 2022a; Furst et al., 2019; Zhang et al., 2020). While the mechanisms and pathways of PFAS toxicity are still poorly understood, PFOS and PFOA have been repeatedly shown to influence nuclear receptors involved in lipid metabolism in fish (Cheng et al., 2016; Yang et al., 2014) and in mammals (Seacat et al., 2002; Tan et al., 2012), and the genes involved in lipid metabolism in birds (O'Brien et al., 2011) suggests PFAS may exhibit a common trend of disrupting lipid modulation in these organisms. The uptake, internal distribution, and toxicokinetics of PFAS, however, are dependent on the organism and chemical-specific attributes (Abercrombie et al., 2021; Ankley et al., 2021). As such, the toxicological effects and ecological risk from PFAS for most species is still unknown (or any other chemical contaminant or contaminant mixture).

Like most top predators, higher trophic-tier wetland snakes are susceptible to accumulating anthropogenic contaminants (Gerke et al., 2020; Heinz et al., 1980; Lettoof et al., 2020a). Snakes have relatively long lifespans, small home ranges, a multi-trophic tier life history and low metabolic rates; consequently, the use of snakes as suitable bioindicators of environmental pollution is gaining momentum (Haskins et al., 2021; Lettoof et al., 2020b; Lettoof et al., 2021b; Wu et al., 2020). Investigations of PFAS exposure, bioaccumulation and toxicity in reptiles is limited (Ankley et al., 2021; Bangma et al., 2019; Beale et al., 2022a; DeWitt et al., 2012; Wang et al., 2013); and published PFAS contamination in snakes has only been investigated in the context of an indigenous food source (Food Standards Australia New Zealand, 2018) as opposed to understanding biological impact. Although reptiles are beginning to receive more consideration for ecotoxicological assessments (Chen et al., 2019; dos Santos et al., 2021; Hopkins, 2000), their low metabolism and the relatively limited understanding of their dynamic physiology can result in studies failing to detect toxicological susceptibility (Cunningham et al., 2021; Finger et al., 2016; Pauli et al., 2010; Weir et al., 2010) and thus reptiles as a taxon present a challenge when trying to assess sublethal impacts from chronic contamination.

Metabolomics—the abundance measurement of hundreds of metabolites in a tissue—has shown to be a sensitive tool in ecotoxicological or environmental impact assessments (Beale et al., 2022b; Hines et al., 2010; Malinowska and Viant, 2019; Sinclair et al., 2019). By screening a suite of biomolecules, metabolomics can identify the molecular responses to chemicals and help determine the mechanistic pathways of toxicity which may not be detected when assessing traditional physical or physiological parameters. Further, by combining targeted and untargeted metabolomic (i.e., polar metabolites) and lipidomic (i.e., non-polar metabolites/lipids) datasets with quantitative bioaccumulated PFAS measurements, we can begin to understand system-wide performance or disruption—contributing towards the transition from static environmental monitoring metrics towards holistic omics-based ecosurveillance approaches (Beale et al., 2022b). In this study, we quantified the concentrations of 28 PFAS in surface waters of four wetlands in Perth, Western Australia, and in the livers of resident western tiger snakes (*Notechis scutatus occidentalis*). In order to test the hypothesis that PFAS exposure impacts snake health and metabolic processes, we assessed the patterns of total PFAS exposure in snakes and its impact on body condition coupled to the identified biochemical response of total PFAS exposure in these wild snakes using metabolomics and lipidomics.

2. Materials and methods

2.1. Ethics

Tiger snakes were humanely euthanised as per Curtin University Animal Ethics Committee approval ARE2020–15. Snakes were collected under the Department of Biodiversity, Conservation and Attractions permit no. 08–002624-02. Samples herein were obtained from the preserved cadavers stored at -20 °C for up to two months.

2.2. Study sites and species

Western tiger snakes are ~1 m terrestrial, viviparous elapid, typically associated with wetlands and wet forests on the Australian mainland and show a dietary preference for frogs (Lettoof et al., 2020c). Once attaining adult size there is no evidence of tiger snake predation in Perth, Western Australia, so we consider this species a top predator. No longevity data exists for this species in this study area.

Tiger snakes are only known to persist in a few wetlands in the Perth region, and we collected snakes from four wetland sites with recent contaminant data (Fig. 1). Herdsman Lake (31 55° 12′ S, 115 48° 19′ E), which is located close to Perths' Central Business District and is heavily modified, and has elevated concentrations of As, Cu, Pb, Zn, Th, Sr and Sb; Bibra Lake (32 5° 32′ S, 115 49° 27′ E) which is mostly surrounded by urbanisation and has elevated Se and V; Lake Joondalup (31 45° 34′ S, 115 47° 33′ E) which is located on the current edge of urbanised Perth and has elevated Hg; and Loch McNess (31 32° 44′ S, 115 40° 50′ E) which is located within Yanchep National Park—outside of urbanisation—and has elevated concentrations of As, Cs, Tl, Se and Hg (Lettoof et al., 2020a; Lettoof et al., 2021b).

2.3. Snake sampling

Adult western tiger snakes (> 0.55 m snout-vent length [SVL]) were hand caught from each site in September 2020. Five males and five females were randomly collected across each site, except for Yanchep National Park where only three males and two females were collected. Snakes were humanely euthanised via blunt-force trauma and carcasses were immediately frozen in a -20 °C freezer. In the laboratory, SVL and body mass (with prey items removed) were measured, and whole liver tissue removed for PFAS quantification. Muscle tissue, the posterior 'fat pad' (white adipose tissue) and gonads (testes and ovaries) were subsampled for omics analyses. Using the SVL and body mass data, a scaled mass index (SMI) was quantified for each snake (Peig and Green, 2010). The SMI is calculated by $W_i(L_0/L_i)^{bSMA}$ where W_i and L_i are the weight and SVL of individuals, L_0 is the arithmetic mean length of all sampled individuals, and b_{SMA} is the scaling exponent estimated by the standardised major axis regression of mass on length of all sampled individuals.

2.4. PFAS quantification in water and snake livers

The water samples were collected on two opposite sides of each lake except for Bibra Lake, which only had one accessible side by foot. Samples were collected one meter from the water's edge by submerging and opening a capped sample container (volume 500 ml) 10 cm beneath the water surface – to avoid the collection of surface films – and 10 cm above the sediment bed. Field blank samples were collected at three of the six sampling locations to verify that cross-contamination was avoided, and field replicates were collected at three sampling locations to verify the reproducibility of analytical results. Water samples were analysed for a suite of 30 PFAS by Eurofins Australia as per USEPA Method 537. The concentration of each analyte was determined using the isotope dilution technique. Quantification of linear and branched isomers was conducted as a single total response

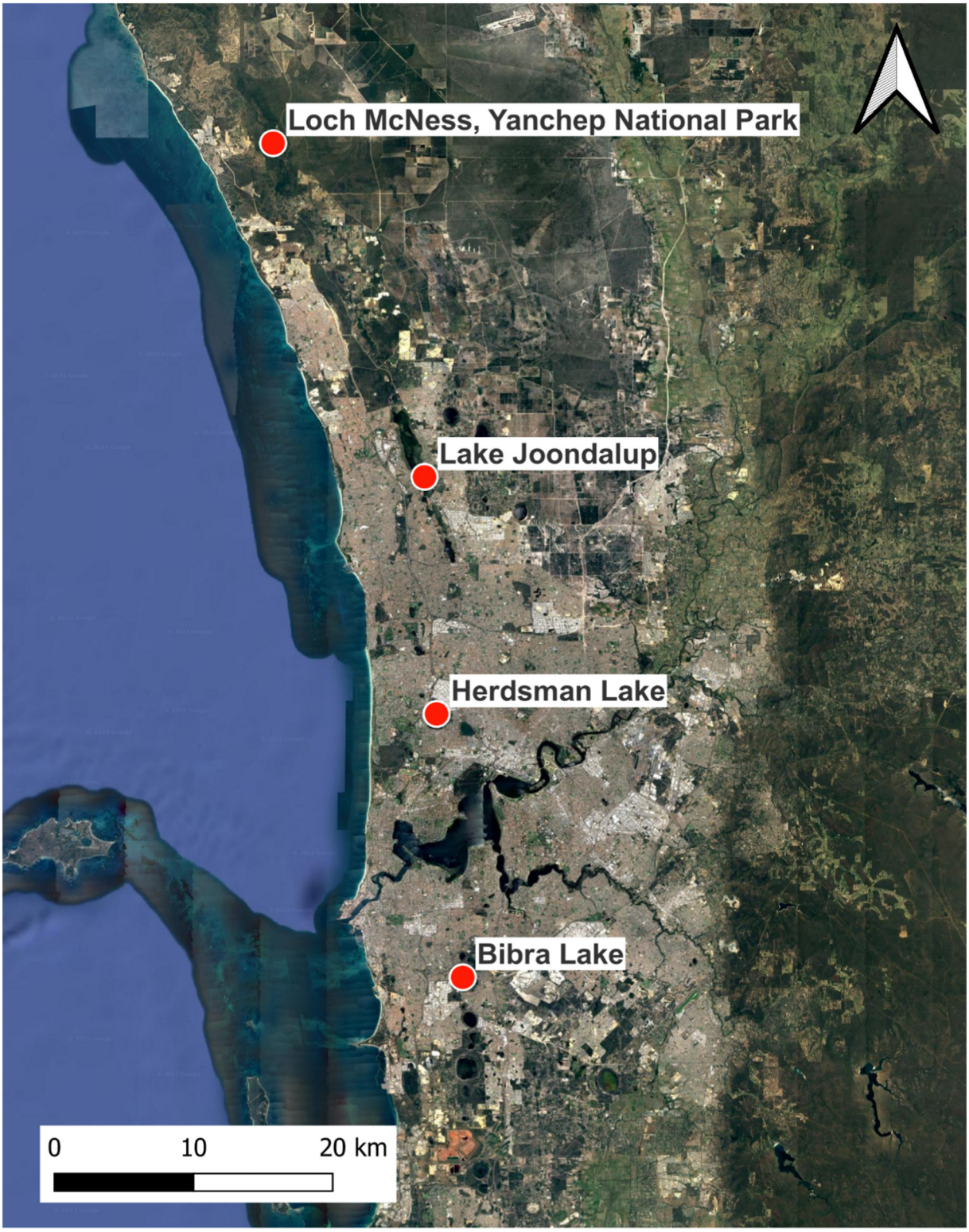

Fig. 1. Map of Perth, Western Australia, and the four study wetlands where tiger snakes were collected for liver PFAS analysis. Satellite images were obtained by Google Earth Pro in 2023.

using the relative response factor for the corresponding linear standard. A branched PFOS standard and branched PFHxS standards were used for the quantification of PFOS and PFHxS, respectively. Full analytical results for the water samples and the results of field QA/QC are provided in Table S1.

Dissected liver tissue was stored at −20 °C for a maximum of six weeks before analysis. PFAS analysis of the liver tissues was carried out by a commercial laboratory (Symbio Laboratories, Brisbane) for a suite of 28 PFAS using a in-house UHPLC/HR-MS method (Method CR148, commercial in confidence). Noting, PFNS and PFPrS were not included in the suite of

analytes offered by the laboratory analysing the liver tissue. In addition, analysis of the liver total fat content was also carried out by Symbio Laboratories using an in-house acid hydrolysis method followed by solvent extraction and gravimetric analysis (Method CF008.2, commercial in confidence).

2.5. Metabolomics and lipidomics

2.5.1. Metabolite and lipid extraction

Snake tissues were freeze-dried for 48 h at 105 °C and 0.01 mBar (FreeZone 4.5 L, Labconco Corp., Kansas City, MO, USA). The freeze-dried tissues (20 mg) were extracted with 100 μl water and 450 μl methanol-ethanol solution (1/1, *v*/v) containing 0.5 ppm *L*-phenylalanine (^{13}C) as the first internal standard. The samples were homogenized with beads (Bead Lysis Kit, CAP7100: Next Advance, Australia) using a Precellys® Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 5800 rpm (cycle: 2 × 15 s, pause: 30s). The tubes were centrifuged at 15,000 RCF at 4 °C for 10 min (Centrifuge 5430 R: Eppendorf, Hamburg, Germany). The supernatant (400 μl) of each sample was collected into a new vial and the pellet was resuspended in 50 μl water and 200 μl above methanol-ethanol solution. The mixtures were homogenized and then centrifuged as specified above. The volume of 200 μl supernatant was collected and mixed with the previous extraction supernatant. The remaining supernatant from all samples was collected into a 5 ml tube to make pooled quality control (QC) samples which contain 600 μl mixture per QC.

The extracted samples and pooled QC samples were transferred into 1 ml Captiva EMR-lipid cartridges (Agilent Technologies, USA) and filtered into 1.5 ml high recovery vials (Part Number: 5183–2030: Agilent Technologies, USA) using a positive pressure manifold 48 processor (PPM-48: Agilent Technologies, USA) at low pressure for 10 min. Subsequently, 100 μl of water-methanol-ethanol solution (2/1/1, v/*v*/v) was added into lipid cartridges to wash samples at low- and high-pressure vacuum, respectively. The vials containing filtered metabolites were dried in a Speedvac for 4 h. To collect the captured lipids, 500 μl DCM-methanol solution (1/2, v/v) was added into each lipid cartridge placed on a new high recovery vial and filtered at low pressure for 10 min. Another 200 μl DCM-methanol solution (1/2, v/v) was added into the lipid cartridge and filtered at low and high pressure, respectively. The filtered lipids were dried in a dry block heater (DBH4000D: Ratex, Australia) under a stream of nitrogen at 30 °C for 30 min.

The dried metabolites in each sample were recovered by resuspending in 100 μl water-methanol solution (80/20, v/v) containing 0.5 ppm labelled L-succinic acid ($^{13}C_2$) as the second internal standard. The blank samples were prepared by adding the 100 μl above water-methanol solution into empty vials. In addition, 100 μl of 1 ppm amino acid and organic acid standard mixture (Sigma Aldrich, Mulgrave, Australia) was added into empty vials for QC purposes. All samples were incubated in a ThermoMixer® C (Eppendorf, Hamburg, Germany) at 40 °C and a speed of 700 rpm for 30 min.

2.5.2. Metabolite analysis

Central carbon metabolism metabolites were analysed on an Agilent 6470 LC-QqQ-MS coupled with an Agilent Infinity II Flex UHPLC system using the Agilent Metabolomics dMRM Database and Method following Sartain (2016) and Gyawali et al. (2021). This is an ion-pair reversed-phase (IP-RP) chromatographic method, which uses an Agilent ZORBAX Extend C18 column with the ion-pairing agent tributylamine (TBA). A standard method gradient was applied comprising solvent A (97:3 water/methanol with 10 mM tributylamine +15 mM acetic acid) and solvent B (methanol with 10 mM tributylamine +15 mM acetic acid).

Untargeted polar metabolites were analysed using an Agilent 6546 Liquid Chromatography Time-of-Flight Mass Spectrometer (LC-QToF) with an Agilent Jet Stream source coupled to an Agilent Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA) following Shah et al. (2021) and Beale et al. (2021). Chromatographic separation was achieved by injection (2 μl) of sample onto an Agilent Zorbax SB-Aq column (2.1 × 50 mm, 1.8 μm) fitted with a Zorbax-C8 guard column (2.1 × 30 mm, 3.5 μm). Each sample was analysed in positive and negative ionization modes. The mobile phase was (A) 0.2 % acetic acid in water and (B) 0.2 % acetic acid in methanol 19 min with a nonlinear gradient starting at 2 % B. The column temperature was set at 60 °C. The detector gas temperature was 325 °C with a drying gas rate of 9 L min^{-1}. The sheath gas temperature and flow were 225 °C and 10 L min^{-1}; the nebulizer pressure was also 45 psi. The acquisition range was 100 to 1700 *m/z*, at 3.5 spectra per second. Reference mass ions were 121.050873 and 922.009798 for the positive mode and 119.036320 and 966.000725 for the negative mode. Auto MSMS data on pooled PBQC samples were obtained at collisions of 10 eV, 20 eV and 40 eV. The PBQC AutoMSMS data was used to generate a curated PCDL for further interrogation of acquired samples using accurate mass, MS2 spectra and retention time. Collected data were processed using MassHunter Profinder software (Version 10.0, Agilent Technologies, Santa Clara, CA, USA), normalized to IS, and putatively identified against the Agilent METLIN (AMRT MS/MS) Metabolite PCDL (G6825–90008, Agilent Technologies, Santa Clara, CA, USA) and a curated in-house PCDL based on MSMS spectra and library threshold score of 0.8.

2.5.3. Lipid analysis

For lipids, dried samples were recovered by resuspending in 100 μl methanol-butanol solution (50:50, *v*/v) containing 0.1 ppm d5-TG ISTD Mix (d5-Triacylglyceride internal standard mixture) (LM6000-1EA: Sigma-Aldrich, St. Louis, MO, USA). The blank samples were prepared by adding 100 μl of the above methanol-butanol solution into empty vials. All samples were incubated in a ThermoMixer® C (Eppendorf, Hamburg, Germany) at 20 °C and a speed of 700 rpm for 30 min. Untargeted lipids were analysed using an Agilent 6546 Liquid Chromatography Time-of-Flight Mass Spectrometer (LC-QToF) with an Agilent Jet Stream source coupled to an Agilent Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA) following Beale et al. (2021). Chromatographic separation was achieved by injection (1 μl) of the sample onto an Agilent InfinityLab Poroshell HPH-C18 column (2.0 × 150 mm, 2.7 μm). Each sample was analysed in positive and negative ionization modes. The mobile phase was (A) 10 mM ammonium acetate and 10 μM medronic acid in water/methanol (90:10, v/v) and (B) 10 mM ammonium acetate in acetonitrile/methanol/isopropanol (20:20:60, v/v/v) operated for 30 min with a nonlinear gradient starting at 55 % B. The column temperature was set at 60 °C. The detector gas temperature was 250 °C with a drying gas rate of 11 L min^{-1}. The sheath gas temperature and flow were 300 °C and 12 L min^{-1}; the nebulizer pressure was also 35 psi. The acquisition range was 50 to 1600 *m/z*, at 3 spectra per second. Capillary voltages for the positive and negative ionization modes were 3500 V and 3000 V, respectively. Reference mass ions were 121.060873 m/z and 922.009198 m/z (positive mode), and 119.036320 and 980.016375 m/z (negative mode). AutoMSMS data on pooled PBQC samples were obtained at collisions of 20 eV and 35 eV. Collected data were processed using MassHunter Profinder software (Version 10.0, Agilent Technologies, USA), normalized to IS, and putatively identified against the Agilent METLIN Lipids PCDL (G6825–90008, Agilent Technologies, Santa Clara, CA, USA) and a curated in-house PCDL based on MSMS spectra and library threshold score of 0.8.

2.6. Data analysis

PFAS which were not measured above the limit of reporting (LOR) in one or more samples (n-1) were assigned half the LOR concentration threshold to facilitate downstream statistical analysis (Zeghnoun et al., 2007). If a PFAS was not measured in all the samples (n) per site/group, then it was excluded. There was no correlation between liver lipid concentration and total PFAS ($r^2 = 0.06$, $p = 0.75$), so we did not normalise our PFAS to liver lipid contents (Hebert and Keenleyside, 1995). Further, as the majority of measured PFAS in the liver tissues was PFOS, with total PFAS highly correlated with PFOS ($r^2 = 0.99$), all statistical tests used total PFAS data in order to account for any minor PFAS constituent additive

effects. We used univariate generalised linear mixed models (GLMMs; *lme4* package (Bates et al., 2014)) to assess the relationships between total liver PFAS concentration and snake parameters of health. First, we fitted a GLMM (Gaussian error structure) with total PFAS as the response variable, an interaction between SVL and sex as the predictor variables, and site as the random effect. We then fitted univariate GLMMs to assess total PFAS influences on body condition. Models were fitted with total PFAS as the predictor variable, and site and sex as the random effects. Gaussian error structures were used for body condition. Model residuals were used to assess best fit, and variables were scaled to improve model fit if needed.

The omics datasets (metabolites and lipids) were log-transformed and multivariate data analysis was conducted using SIMCA (v17.0.2, Sartorius Stedim Biotech, Umeå, Sweden) and MetaboAnalyst 5.0 (Pang et al., 2022; Pang et al., 2021). All data were normalized via a combination of normalising by median, log transforming and auto-scaling, until data visually resembled a normal distribution. Using regression against liver PFAS, functional omics outputs were enriched, and pathway impact assessments were undertaken using MetaboAnalyst 5.0. Of the available metabolic pathways, we used the *Gallus gallus* (chicken) pathway library as birds and reptiles share similar physiology. A false discovery rate of ≤ 0.05 of the enriched outputs was set as the minimum cut-off threshold for discussion.

3. Results and discussion

3.1. PFAS in wetland waters

Of the 30 PFAS tested in wetland surface water, 12 were detected above LOR; the PFAS concentrations are presented in Table S2. No PFAS were detected above LOR in the water samples from Yanchep National Park. Herdsman Lake waters had the highest concentrations of total PFAS. Concentrations were similar at the east and west sites (0.122 and 0.101 μg/l respectively). Further, it was the only wetland analysed where PFNA was detected (0.002 μg/l). Lake Joondalup waters had similar concentrations of total PFAS (0.104–0.107 μg/l) to Herdsman Lake, and between sampling locations. The total PFAS detected in Bibra Lake waters was roughly half the concentration of the other lakes, and no PFHxS nor PFHpS were detected in Bibra Lake water samples. Proportional PFAS mixtures are visualised in Fig. 2.

Total PFAS concentrations were in the same general range as previously reported PFAS data for Herdsman Lake (0.129 and 0.102 μg/l), Lake Joondalup (0.132 and 0.076 μg/l) and Bibra Lake (0.089 and 0.034 μg/l) in Autumn and Spring 2019, respectively (Richmond, 2022). This study found that PFAS concentrations in surface water reflect the age and the intensity of urban development in Perth. Lakes located close to the city centre tend to contain a wider range of detectable PFAS compounds and higher concentrations of PFAS compared to lakes on the urban fringe. The high PFAS concentrations in Herdsman Lake, relative to other sites included in this study, are not surprising; this wetland is located within an older part of the metropolitan area, receives stormwater drainage from surrounding industrial and residential land, and is the recipient of leachate seepage from landfill (Foulsham, 2009). The higher concentration of PFOA and longer chain PFAS such as PFOS, PFHpA and PFNA reflects a broad range of compounds that have been used in various products over the last 50 years (Buck et al., 2011; Sznajder-Katarzyńska et al., 2019). The PFAS concentration in Lake Joondalup, however, is comprised of more short-chain PFAS compounds—likely reflecting the PFAS usage over the last 20 years (Ahrens and Bundschuh, 2014). Despite being located on the edge of the urban-matrix, the wetland features stormwater drains from surrounding residential land and two fire stations within its catchment. Post-2000 firefighting foams contain fluorotelomers that decompose into predominately PFHxA and PFPeA (Ahrens and Bundschuh, 2014), and are likely the primary cause of contamination – as these compounds are dominant in Lake Joondalup waters.

Bibra Lake waters had relatively low PFAS concentrations, despite being in a historically urbanised area. The wetland is mostly surrounded by remnant vegetation which likely buffers the lake from contaminant impacts through surface runoff, and it only receives urban stormwater from a single drain (City of Cockburn, 2015). The PFAS profile is mostly short-chained compounds, reflecting more recent impacts from minor incidental firefighting foam discharges transported via stormwater. Moreover, it is important to note that all these lakes are surface expressions of groundwater, so groundwater transport is another potential pathway for PFAS inputs into

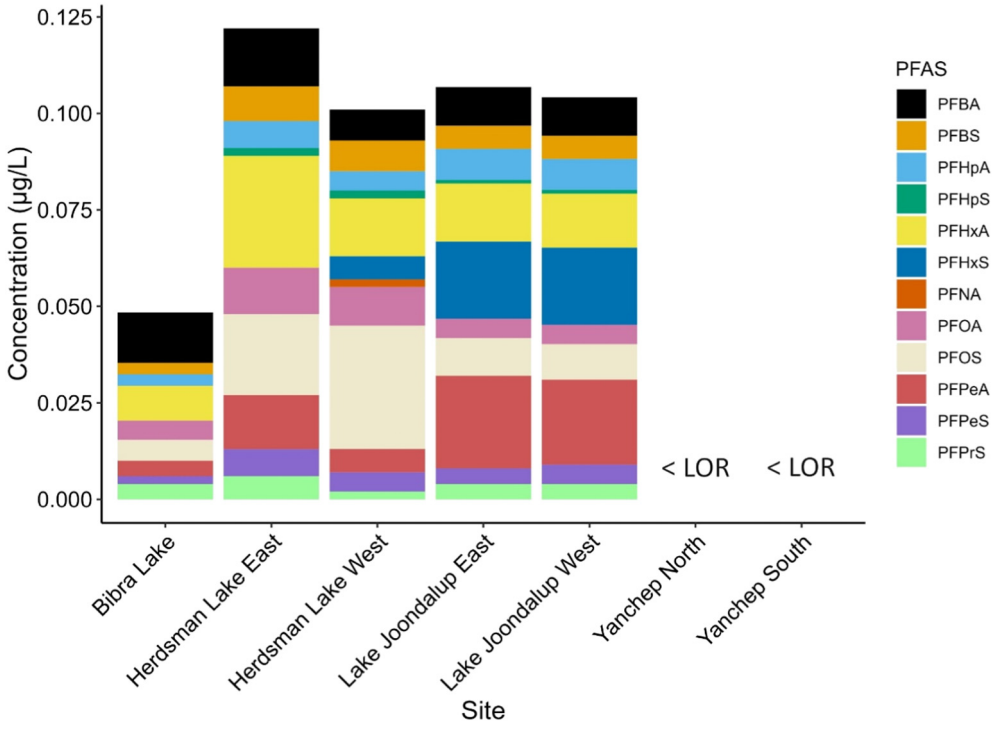

Fig. 2. Detected PFAS mixtures in the surface waters of the four studied wetlands in Perth, Western Australia. <LOR = below reporting limit of 0.001 μg/l.

these sites. Since Loch McNess in Yanchep National Park has no upstream urban development, it is not surprising that we did not detect any PFAS in its surface waters.

3.2. PFAS in snake livers

The prevalence of exposure and concentrations of 12 PFAS in tiger snake livers are presented in Table 1. PFOS was the dominant PFAS in snakes from all sites and was detected in all snakes except a single female from Yanchep National Park. Herdsman Lake snakes had the highest prevalence of contamination and mean concentration of 11 PFAS, including the only detection of 8:2 FTSA and 10:2 FTSA in a single snake. Lake Joondalup snakes were contaminated with 10 PFAS, including the only detection of PFTeDA in two snakes. Bibra Lake snakes had a low (0.6–31 μg/kg) liver concentrations for three PFAS and Yanchep snakes only had trace amounts of PFOS (≤ 2.5 μg/kg). Total PFAS concentrations were highest in Herdsman Lake snakes (322 ± 193 μg/kg) followed by Lake Joondalup snakes (93.6 ± 53.4 μg/kg), and were low in Bibra Lake (13.98 ± 7.85 μg/kg) and Yanchep snakes (1.31 ± 0.86 μg/kg). The other PFAS compounds were not detected above LOR in any snake livers.

PEPrS, PFBS, PFBA, PFPeS, PFPeA, PFHxA and PFHpA were detected in water samples but not snake livers, suggesting these PFAS do not bioaccumulate or are not in high enough concentrations to bioaccumulate in higher order vertebrates at these sites. PFDS, PFNA, PFDA PFUnDA, PFDoDA, PFTrDA, PFTeDA, 8:2 FTSA and 10:2 FTSA were detected in snake livers but not water samples. As tiger snakes at these sites feed predominantly on frogs (Lettoof et al., 2022) and show little emigration (isolated from urbanisation (Lettoof et al., 2021c)), detection of these PFAS suggest local exposure and that they have the potential to bioaccumulate in higher order vertebrates. Despite Yanchep National Park being far from urbanisation and the lack of PFAS detection in the waters, snakes were impacted with PFOS residue. As our water sampling was only sufficient in offering a snapshot of PFAS concentrations, the frequency of contamination and higher concentration in snake livers supports the use of wetland snakes as bioindicators of wetland PFAS contamination. PFOS occurring as the dominant PFAS in snake livers is consistent with previous studies reporting PFAS accumulation in tissues of other vertebrate taxa (Beale et al., 2022a; Food Standards Australia New Zealand, 2018; Stahl et al., 2012; Wang et al., 2013).

The concentrations of PFOS in snake livers from the most contaminated site, Herdsman Lake (mean: 279.9, max: 700 μg/kg), is lower than the single other published detections of PFOS in an anurophagous wetland snake—the Australian keelback (*Tropidonophis mairii*; mean: 2650 μg/kg, max: 2800 μg/kg, $n = 2$); however, the Australian keelback tissue that was analysed and its contamination/exposure history is not specified (Food Standards Australia New Zealand, 2018). To the best of our knowledge, no peer-reviewed published studies have previously reported PFAS concentrations in reptile livers and further research is needed to identify the tissue-specific partitioning of PFAS in wild reptiles; however, the PFAS concentrations detected in Perth's urban tiger snake livers are substantially higher than those reported in duck livers (range: BDL – 9.5 μg/kg, max: BDL – 340 μg/kg) from South-Eastern Australia (Sharp et al., 2021) and fish liver tissue (range: BDL – 70, max: BDL – 107 μg/kg) from the harbour of Australia's largest city—Sydney, New South Wales (Thompson et al., 2011). Currently, it appears the concentrations of PFAS detected in tiger snake livers from both Herdsman Lake and Lake Joondalup are the highest reported in Australian freshwater vertebrates.

3.3. The relationships among PFAS and snake physical condition

A summary of the snake's physical measurements at the time of sampling and their total (liver) lipid concentrations are presented in Table 2. The collected snakes were all adults and considered 'healthy' on visual inspection (i.e., no defects or abnormalities). We found no significant relationship between SVL and total liver PFAS ($r^2 = 0.71$, $X^2 = 0.07$, $p = 0.79$); however, we found males had higher but not statistically significant total PFAS liver concentrations relative to female snakes ($r^2 = 0.71$, $X^2 = 2.72$, $p = 0.10$), and a strong but not statistically significant interaction effect between SVL and sex, and total PFAS ($r^2 = 0.71$, $X^2 = 2.89$, $p = 0.10$). The relationship between male SVL and total PFAS was positive, and negative between female SVL and total liver PFAS (Fig. 3). We suspect the non-

Table 1
Exposure and concentration of PFAS (μg/kg) in Western tiger snake (*Notechis scutatus occidentalis*) livers from four wetlands around Perth, Western Australia. Prev. = prevalence of snakes with PFAS above detection limit; <LOR = below reporting limit of 0.5 μg/kg.

PFAS	Herdsman Lake		Bibra Lake		Lake Joondalup		Yanchep NP	
	Prev.	Mean ± SD (Range)	Prev.	Mean ± SD (Range)	Prev.	Mean ± SD (Range)	Prev.	Mean ± SD (Range)
PFHxS	9/10	7.04 ± 16.22 (<LOR – 53)	0/10	<LOR	2/10	0.34 ± 0.20 (<LOR – 0.8)	0/5	<LOR
PFHpS	5/10	1.66 ± 2.42 (<LOR – 7.7)	0/10	<LOR	0/10	<LOR	0/5	<LOR
PFOS	10/10	279.9 ± 174.2 (99–700)	10/10	13.45 ± 7.52 (5.2–31)	10/10	86.5 ± 52.8 (30–170)	4/5	1.31 ± 0.86 (<LOR – 2.5)
PFDS	10/10	3.65 ± 2.27 (0.7–7.5)	0/10	<LOR	2/10	0.34 ± 0.21 (<LOR – 0.9)	0/5	<LOR
PFNA	8/10	1.93 ± 1.94 (<LOR – 6.1)	0/10	<LOR	3/10	0.50 ± 0.47 (<LOR – 1.6)	0/5	<LOR
PFDA	10/10	7.08 ± 4.53 (2.3–15)	1/10	0.29 ± 0.11 (<LOR – 0.6)	10/10	2.04 ± 1.12 (0.6–3.9)	0/5	<LOR
PFUnDA	9/10	2.80 ± 1.79 (<LOR – 6.1)	0/10	<LOR	2/10	0.43 ± 0.40 (<LOR – 1.4)	0/5	<LOR
PFDoDA	10/10	9.6 ± 6.77 (0.9–26)	5/10	0.53 ± 0.30 (<LOR – 0.9)	9/10	2.46 ± 1.91 (<LOR – 5.2)	0/5	<LOR
PFTrDA	9/10	6.53 ± 10.68 (<LOR – 34)	0/10	<LOR	6/10	1.4 ± 1.93 (<LOR – 6.3)	0/5	<LOR
PFTeDA	0/10	<LOR	0/10	<LOR	2/10	0.78 ± 1.27 (<LOR – 4.2)	0/5	<LOR
8:2 FTSA	1/10	0.38 ± 0.40 (<LOR – 1.5)	0/10	<LOR	0/10	<LOR	0/5	<LOR
10:2 FTSA	1/10	0.35 ± 0.30 (<LOR – 1.2)	0/10	<LOR	0/10	<LOR	0/5	<LOR
Total	10/10	322 ± 193 (100–750)	10/10	13.98 ± 7.85 (5.2–32)	10/10	93.6 ± 53.4 (38–180)	4/5	1.31 ± 0.86 (<LOR – 2.5)

Table 2
Physical measurements (mean ± SD) of the sampled tiger snakes.

Site	Sex (*n*)	Size (SVL; cm)	Body mass (g)	Liver lipids (g/100 g)
Herdsman Lake	Female (5)	75.9 ± 2.4	170.3 ± 34.6	3.6 ± 1.7
	Male (5)	83.3 ± 6.6	226.6 ± 70.5	3.1 ± 0.7
Bibra Lake	Female (5)	69.4 ± 10.3	197.4 ± 84.7	3.3 ± 1.2
	Male (5)	80.4 ± 3.0	234.4 ± 71.6	2.1 ± 0.3
Lake Joondalup	Female (5)	68.0 ± 2.8	158.5 ± 45.8	2.9 ± 0.9
	Male (5)	78.8 ± 5.3	251.4 ± 59.5	2.6 ± 0.6
Yanchep	Female (2)	68.0 ± 4.2	160 ± 0	2.2 ± 0.2
	Male (3)	74.4 ± 9.5	184.2 ± 38.3	3.0 ± 1.1

significant results are an artifact of the small sample size of contaminated snakes, as these observations are supported by previous findings. The inverse relationship between PFAS concentrations and size (a proxy for age (Waye, 1999)) in male and female tiger snakes could be easily attributed to females eliminating PFAS via maternal transfer—a phenomenon that has been shown in snakes such as *Enhydris chinensis* with organophosphorus flame retardants and plasticizers (Liu et al., 2019) and metals in tiger snakes (Lettoof et al., 2021d), *Nerodia sipedon* (Chin et al., 2013) and *Lamprophis fuliginosus* (Hopkins et al., 2004). The positive relationship between total liver PFAS and the size of male snakes suggests PFAS is bioaccumulating in male tiger snakes from contaminated sites. Of the only other comparable reptile studies, males have also been reported with higher concentrations of PFAS in snapping turtles, *Chelydra serpentina* (Kannan et al., 2005), and Chinese alligators, *Alligator sinensis* (Wang et al., 2013). Additionally, Zhang et al. (2020) found female *Eremias argus* lizards exposed to PFOA had associations with higher investment of physiological resources into self-maintenance than males; if female tiger snakes respond similarly—in conjunction with the capacity to maternally transfer contaminants—the long-term exposure and accumulation of PFAS may impact the fitness and survival of male snakes more than females. Testing of PFAS burdens on new-born offspring compared to mothers of different sizes is needed to clarify maternal transfer of PFAS in snakes and warrants further investigation into the potential impacts on juvenile development and survival.

We found a weak yet significant negative relationship between liver total PFAS and snake body condition ($r^2 = 0.12$, $X^2 = 4.84$, $p = 0.03$; Fig. 4), predicting liver total PFAS of 750 μg/kg is associated with approximately 30 % loss in mean body condition. Roughly 50 % of the variation in snake body condition estimates is caused by stored fat bodies (Weatherhead and Brown, 1996), while the remaining unexplained

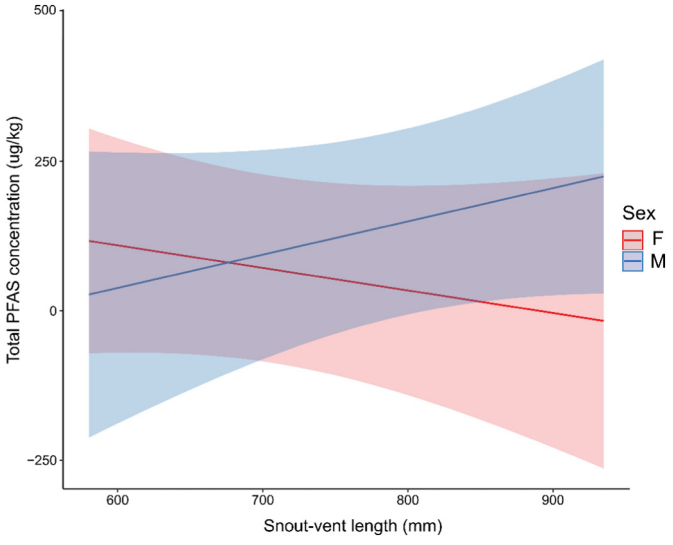

Fig. 3. The relationship between total liver PFAS concentration and snout-vent length in male and female tiger snakes collected from four wetlands around Perth, Western Australia. Predicted values were extracted from a GLMM which included site as a random effect. Shaded area represents 95 % confidence intervals.

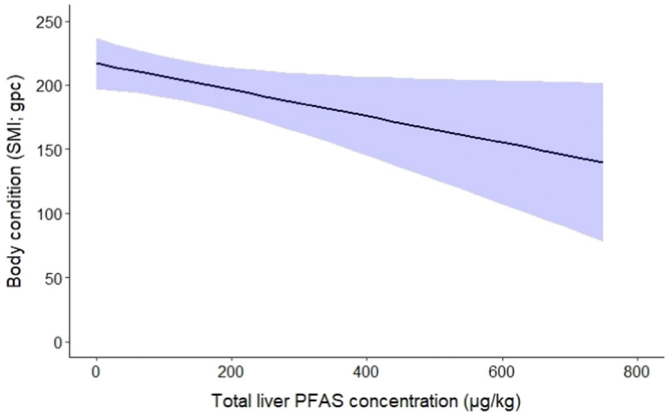

Fig. 4. The relationship between total liver PFAS concentration and body condition (reported as scaled mass index SMI, grams per cm) in western tiger snakes collected from four wetlands around Perth, Western Australia. Predicted values were extracted from a GLMM which included site and sex as random effects. Shaded area represents 95 % confidence intervals.

variance can likely be attributed to larger organs such as muscle and liver tissue (Madsen and Shine, 2002). We acknowledge that the PFAS concentrations in these snakes only explain 12 % of the variation in poor body condition and the model was built from a small sample size, but the results are unsurprising given the ability for PFAS compounds to bioaccumulate in predators, and interfere with lipids and metabolic energy pathways. We suspect body condition is also being impacted from a cocktail of other urban contaminants—as 20 % of the variation in snake body condition at these sites is explained by metal(loid) contamination (Lettoof et al., 2022). For example, exposure to PFAS has been shown to magnify the impact of cadmium and lead on kidney function (Jain, 2019), but otherwise, the relationship between PFAS and metal toxicity is largely unknown and warrants further investigation. Importantly, these potential co-contributing impacts to snake body condition makes them particularly vulnerable to predation (Mattisson et al., 2016) and mortality (Shine et al., 2001), and a reduction in reproductive frequency and outputs (Madsen and Shine, 1996; Milenkaya et al., 2015) which may lead to population declines.

3.4. Impact of PFAS on snake muscle biochemical profiles

Muscle biochemical profiling showed clear significant site-specific groupings ($R^2x = 0.22$, $R^2y = 0.48$, $Q^2 = 0.3$, $p < 0.01$), but high variation among individuals (Fig. 5). The axis orientation does not follow a linear relationship with PFAS concentrations; snake profiles are likely impacted by the unique metal mixtures accumulated in each population (Lettoof et al., 2020a; Lettoof et al., 2021b) and potentially the differences in parasite infection in these populations (Lettoof et al., 2022; Wang et al., 2004). The high variation of individuals within a site is likely attributed to the high variation of metabolic and physiological states of reptile individuals within a population (Coz-Rakovac et al., 2011; Lettoof et al., 2021a; Moon et al., 1999), and suggests in situ metabolomics studies on reptiles needs to sample more individuals to capture the high variability within sites. Categorising snakes by sex showed no obvious groupings so sexes were pooled for further analysis.

After controlling for the influence of site, linear mixed models identified 52 muscle metabolites and lipids showing significant ($p = 0.001$–0.049) positive relationships with liver PFAS and eight showing significant ($p = 0.027$–0.042) negative relationships with liver PFAS; however, after a false discovery rate adjustment, none of these were significantly perturbed (FDR = 0.068–0.383; Table S3). This indicates many snake muscle metabolites and lipids have relationships with PFAS, but the effect of site and variation of individual physiological states in wild snakes creates substantial 'noise' in the data and a larger sample size could be required to confirm if

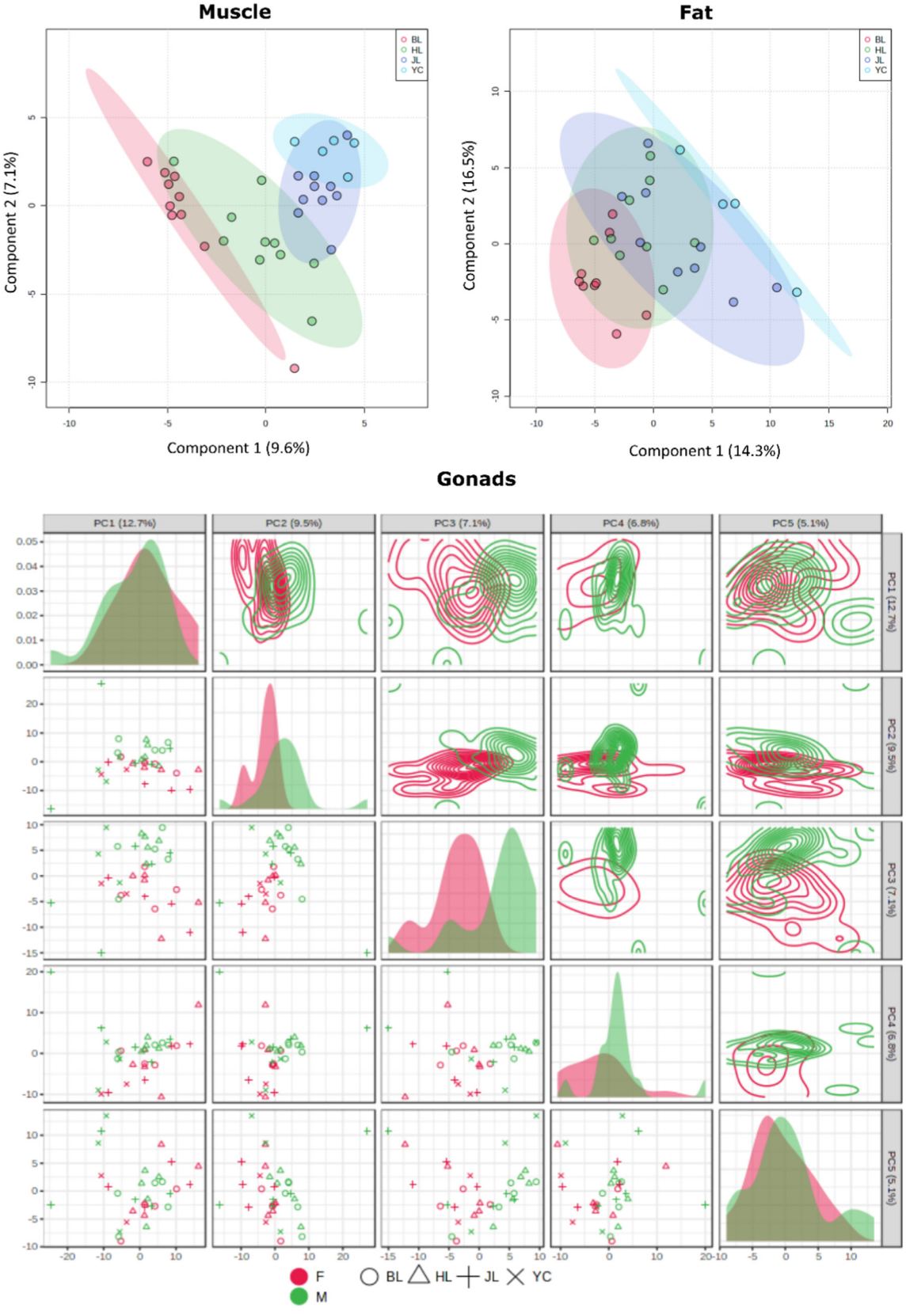

Fig. 5. PLS-DA plots for tiger snake muscle and fat, and an iPCA plot series for tiger snake gonad (testes and ovaries, pooled) metabolome and lipidome. Grouped by site; BL = Bibra Lake; HL = Herdsman Lake; JL = Lake Joondalup; YC = Yanchep National Park; F = female; M = male.

these metabolites/lipids are useful biomarkers of PFAS accumulation in snakes. When assessing the chemical groups, PFAS accumulation did not significantly (FDR = 0.56–0.76) enrich any muscle lipidome groups (Table S4) that other studies reported (Beale et al., 2022c) but did significantly (FDR = 0.01–0.05) enrich 11 metabolome groups (Table S5): pyrimidines, indoles, fatty acids and conjugates, amino acids and peptides,

phenylacetic acids, TCA acids, pyridines, purines, benzoic acids, benzenes and fatty amines.

Enrichment of these chemical groups suggests perturbation of their related pathways, all of which have been repeatably associated with PFAS exposure in humans (Guo et al., 2022) and other biota (Beale et al., 2022c), suggesting a common effect of these contaminants on organisms. Pyrimidines, pyridines and purines are involved in nucleotide metabolism (Hubert and Sutton, 2017; Nyhan, 2005), and TCA acids (i.e. tricarboxylic acid cycle), fatty acids and conjugates, amino acids and peptides are involved in carbohydrate metabolism which are critical for cellular homeostasis and energy generation related mechanisms (Martínez-Reyes and Chandel, 2020; Nyhan, 2005). In uricotelic animals specifically (e.g. birds and land reptiles), these chemical groups are involved in the uric acid cycle which disposes of nitrogenous waste from protein metabolism, including forming chemicals involved in the 'salvage pathway' for recycling purines and pyrimidines (Balinsky, 1972; Salway, 2018). Although the understanding of these mechanisms is relatively sparse in reptiles, impacts on the uric acid cycle can cause renal disease and gout which can be fatal for reptiles (Campbell, 2006). In addition, the enrichment of benzoic acids, phenylacetic acids, benzenes and fatty amines—common metabolites of xenobiotics (Williams, 1974)—suggests a relationship between PFAS and detoxification processes in the muscle and warrants further research.

The significantly impacted and enriched muscle metabolite and lipid pathways were synthesis and degradation of ketone bodies (FDR = 0.02), valine, leucine and isoleucine degradation (FDR = 0.03), biosynthesis of unsaturated fatty acids (FDR = 0.03), and selenocompound metabolism (FDR = 0.03; Table S6; Fig. 6). Selenium is an essential trace element for organisms, but is toxic in excessive concentrations (Mézes and Balogh, 2009). Perturbation of selenocompound metabolism pathways could increase selenium toxicity in tiger snakes and could be evidence of a synergistic impact from PFAS and legacy metalloid contamination, which tiger snakes from these sites are exposed to.

Ketone bodies, valine, isoleucine, and leucine (branched-chain amino acids; BCAAs) and unsaturated fatty acids are all crucial for energy production. Specifically, BCAAs catabolism in the muscle yields compounds that can be used for ATP generation, protein synthesis and regulators of glucose transport proteins, and synthesis of ketones (Holecek, 2021; Zhang et al., 2017), while fatty acids are the precursors of ketones and aerobic production of ATP by fatty acid metabolism fuels gluconeogenesis (Fukao et al., 2004; Mayes et al., 2003). Compared to birds and mammals, squamate reptiles (lizards and snakes) have limited energy stores which are slowly replenished from a low metabolism. Bursts of vigorous activity, such as hunting and subduing prey or escaping a predator, is primarily fuelled by muscle glycogen stores and gluconeogenesis is performed in the muscle to quickly refuel these stores (Gleeson, 1991; Hancock et al., 2001; Hitchcox, 2009). PFAS-induced perturbation of these energy production pathways could result in muscle wasting (Holecek and Vodenicarovova, 2018) and is a likely explanation for the low snake body condition we detected (Fig. 4), which can translate into a reduced ability to hunt or escape predators.

Other important pathways that were impacted and enriched ($-LOG_{10}$ (p) = 1.33–2.06) but not identified as significantly perturbed (FDR = 0.11–0.17) include butanoate metabolism, glutathione metabolism, steroid hormone biosynthesis, thiamine metabolism, tyrosine metabolism, retinol

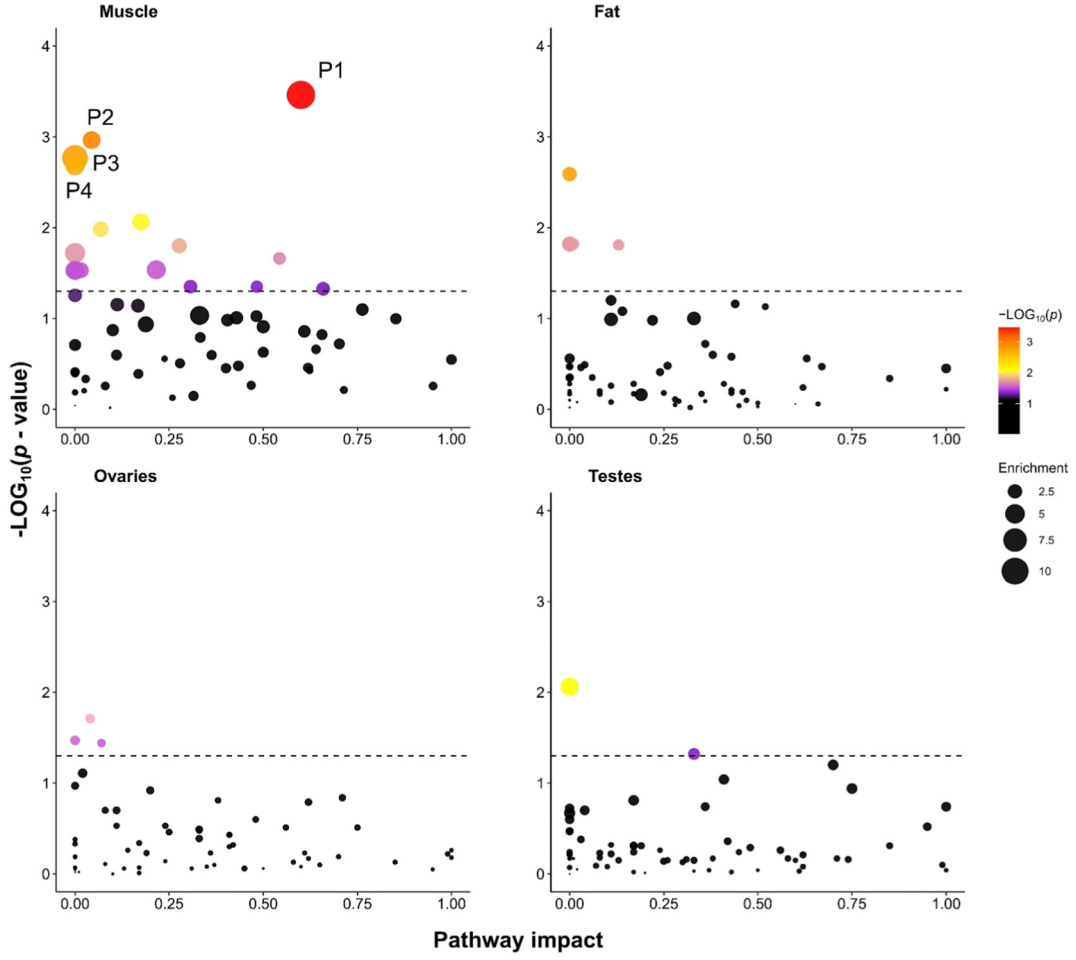

Fig. 6. Significant metabolic pathways identified in each tissue via enrichment and impact analysis using MetaboAnalyst 5.0 (Enrichment Analysis and Pathway Impact Toolbox). Labelled bubbles are significant pathways after a false discovery rate adjustment; P1 = synthesis and degradation of ketone bodies; P2 = valine, leucine and isoleucine degradation; P3 = selenocompound metabolism; P4 = biosynthesis of unsaturated fatty acids.

metabolism, fatty acid biosynthesis, fatty acid elongation, fatty acid degradation, propanoate metabolism, terpenoid backbone biosynthesis, citrate cycle (TCA cycle), sphingolipid metabolism, and nicotinate and nicotinamide metabolism (Table S6; Fig. 6).

3.5. Impact of PFAS on snake fat

Unlike the muscle, the fat biochemical profiling showed non-significant ($R^2x = 0.34, R^2y = 0.32, Q^2 = -0.02, p = 1$) site-specific groupings, with substantial overlap and high variation among individuals—especially the snakes from Yanchep National Park where one was identified as an outlier (Fig. 5). The axis-gradient site groups also do not follow a linear relationship with population PFAS accumulation, as the biochemical profiles are likely influenced by the same environmental stressors as suggested in the muscle data. Categorising snakes by sex showed no obvious groupings so sexes were pooled for further analysis.

After controlling for the influence of site, linear mixed models identified 25 fat metabolites and lipids showing significant (p = 0.001–0.049) positive relationships with liver PFAS and two showing significant (p = 0.012–0.038) negative relationships with liver PFAS; however, after a false discovery rate adjustment, none of these were significantly (FDR = 0.09–0.86) perturbed (Table S3). This suggests many snake metabolites and lipids stored in adipose tissue may have relationships with PFAS, but the effect of site and variation of an individual creates 'noise', and a larger sample size is required. When assessing the chemical groups, PFAS accumulation did not significantly enrich adipose lipidome groups (FDR = 0.5–0.95; Table S4), but did significantly enrich monoradylglycerols, fatty aldehydes and bile acids of the metabolome groups (FDR = 0.02–0.05; Table S5). The enrichment of monoradylglycerols and fatty aldehydes is unsurprising, as these are the primary constituents of reptile adipose tissue (Azeez et al., 2014; Price, 2017) and PFAS impacting lipid accumulation is a common response in vertebrates (Beale et al., 2022c). Similarly, PFAS interfering with bile acids—lipids with regulatory roles in metabolic and cellular homeostasis (Chiang and Ferrell, 2019)—and their syntheses has been frequently reported in humans and rats (Zhao et al., 2015) and an increase in these lipids is often a biomarker of fatty liver disease (Puri et al., 2018; Sen et al., 2022).

There were no significantly impacted and enriched fat metabolite and lipid pathways; however, important pathways that were impacted and enriched ($-\text{LOG}_{10}(p)$ = 1.81–2.59) but not identified as significantly perturbed (FDR = 0.16–0.2) include biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, fatty acid elongation, fatty acid degradation and primary bile acid biosynthesis (Table S6; Fig. 6). Besides potentially impacted fatty acid pathways, it is not surprising that the adipose tissue had no significantly perturbed pathways as its function in reptiles is purely storage of triglycerides/lipids/energy until mobilisation for reproduction or winter maintenance (Price, 2017).

3.6. Impact of PFAS on snake gonads

Due to the limited sampling of female snakes from Yanchep National Park (n = 2), we did not have the minimum samples required for groups in a PCA analysis (n = 3), so data from both sexes tissues (ovaries and testes) had to be pooled. The iPCA showed large overlapping among sites but a clear separation between sexes (Fig. 5), indicating that these two organs have different metabolic profiles and should be further assessed separately. Besides site-specific stressors influencing variation in gonad biochemical profiles, snakes were collected in peak breeding season so testes could have been in different stages of use (e.g. recently mated, not yet mated for the season). Despite no females having fertilised or enlarged ova, they may have also been in variable states of early reproduction.

After controlling for the influence of site, linear mixed models identified 18 ovary metabolites and lipids showing significant (p = 0.001–0.047) positive relationships with liver PFAS and six showing significant (p = 0.012–0.044) negative relationships with liver PFAS (Table S3); however, after a false discovery rate adjustment, none of these were significantly (FDR = 0.37–0.94) perturbed (Table S3). The ovaries had triradylglycerols and sterols as lipidome chemical groups significantly (p = 0.01–0.02) enriched from PFAS accumulation (Table S4), and octadecanoids, benzenediols, fatty amines, purines and benzoic acids as metabolome chemical groups significantly (p = 0.001–0.04) enriched from PFAS accumulation (Table S5); however, after a false discovery rate adjustment none of these were significantly (FDR = 0.1–0.55) enriched. Although we do not have the statistical power to confidently link these chemical groups with PFAS accumulation, these chemicals are used for cellular genesis (Liu et al., 2022; Nagle et al., 1998; Quaranta et al., 2022; van Meer et al., 2008) and their potential enrichment from PFAS could impact embryo development and warrants further investigation with bigger sample sizes.

Only nine metabolites and lipids in snake testes showed a significant (p = 0.002–0.033) positive relationship with liver PFAS; however, after a false discovery rate adjustment, none of these were significantly perturbed (FDR = 0.59–0.99; Table S3). The testes had sphingomyelins, cardiolipins and glycerophosphoethanolamines lipidome chemical groups significantly (p = 0.02–0.04) enriched from PFAS accumulation (Table S4), however, after a false discovery rate adjustment, none of these were significantly (FDR = 0.12–0.17) enriched. No metabolome chemical groups were significantly enriched (FDR = 0.99; Table S5). As previously stated, a larger sample size would help identify if these lipid groups are impacted by PFAS and warrants investigation as these they represent large components of spermatozoa and spermatogenesis (Alvarez et al., 1987; Ren et al., 2019), and enrichment could result in impacts on sperm motility and overall quality (Li et al., 2022; Otala et al., 2005) or represent a perturbation of spermatogenesis (Furland et al., 2011).

The ovaries and testes had no significantly impacted and enriched fat metabolite and lipid pathways; however, important pathways that were impacted and enriched ($-\text{LOG}_{10}(p)$ = 1.44–2.06) but not identified as significantly perturbed (FDR = 0.58–0.98) in the ovaries included propanoate metabolism, drug metabolism - other enzymes, and valine, leucine and isoleucine degradation, and in the testes included selenocompound metabolism, and ubiquinone and other terpenoid-quinone biosynthesis (Table S6; Fig. 6). The potential relationship between these pathways and PFAS could lead to reproduction perturbation and warrants further investigation.

4. Concluding remarks and future research needs

This research provides evidence for PFAS accumulation in top predator snakes, and the observed associations between PFAS and the whole organism and its metabolic health. Notably, some long-chain PFAS were detected in snake livers that were not detected in the water samples from the same site. This finding is particularly important for environmental management and regulation decisions as it demonstrates that reliance on PFAS measurement of aqueous media alone is inadequate for predicting accumulation and ecological impacts to higher-order species.

Consistent with other PFAS studies, we found higher PFAS concentrations were loosely associated with perturbation of the lipidome (particularly lipids involved in cellular genesis in the ovaries and spermatogenesis in the testes) and strongly associated with energy production and maintenance pathways in the muscles metabolome, specifically. These mechanistic disruptions likely contribute to the lower body condition found in snakes with high PFAS. As the PFAS-impacted sites were urban wetlands likely exposed to PFAS residue from stormwater, a greater impact to snakes will possibly be observed in wetlands containing higher concentrations of PFAS (e.g. those adjacent to airports or military bases where a known PFAS contaminant plume exists). This study further supports the use of tiger snakes or other top predator wetland snakes as bioindicators of wetland health and function.

Metabolomics and lipidomics give detailed insight into how pollutants change the biochemical pathways and mechanisms of an organism—a tool of great value for studying reptile ecotoxicology; however, given the fluctuation in metabolism and physiological states in wild populations, and the likely interference from other legacy contaminants in our study

wetlands (expected in most urban wetlands), we did not find many statistically robust relationships with PFAS accumulation in tiger snakes. To improve these studies, we recommend testing biota for common legacy metals to tease out potential antagonistic effects and sampling more individuals to capture the natural variation. Moreover, more reptiles need to have their metabolic pathways mapped and made available for metabolomics analysis software to create more accurate inferences. Nonetheless, following a functional omics-based approach we identified the biological response (phenotype) of four populations of tiger snakes exposed to a range of PFAS concentrations and strong associations between PFAS concentrations and biochemical pathways. With more research, utilising a larger sample size, these data have the potential to be incorporated into omics-based ecosurveillance techniques to monitor these snake populations and contribute a biological line of evidence to the environmental risk assessment and management of PFAS into the future.

CRediT authorship contribution statement

D.C. Lettoof: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **T.V. Nguyen:** Methodology, Data curation, Writing – original draft. **W.R. Richmond:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition, Resources. **H.E. Nice:** Investigation, Writing – review & editing. **M.M. Gagnon:** Writing – review & editing. **D.J. Beale:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Resources.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165260.

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Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds

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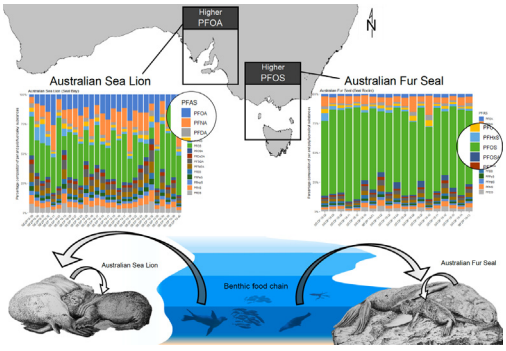
HIGHLIGHTS

- Identification of PFAS concentrations in Australian pinnipeds from multiple colonies
- Elevated concentrations of PFAS in *A. p. doriferus* compared to *N. cinerea* pups
- Geographical foraging range is the suspected factor for interspecies differences
- Maternal transfer of PFAS in Australian pinnipeds demonstrated
- High PFAS concentrations in neonatal pups warrant health investigation

GRAPHICAL ABSTRACT



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ABSTRACT

Per and polyfluorinated substances (PFAS) exposure was investigated in Australian pinnipeds. Concentrations of 16 PFAS were measured in the livers of Australian sea lion (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*) and a long-nosed Fur Seal (*Arctocephalus forsteri*) pup sampled between 2017 and 2020 from colonies in South Australia and Victoria. Findings reported in this study are the first documented PFAS concentrations in Australian pinnipeds.

Median and observed range of values in ng/g wet weight were highest for perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) in the liver of *N. cinerea* (PFOS = 7.14, 1.00–16.9; PFOA = 2.73, 0.32–11.2; PFNA = 2.96, 0.61–8.22; n = 28), *A. forsteri* (PFOS = 15.98, PFOA = 2.02, PFNA = 7.86; n = 1) and *A. p. doriferus* (PFOS = 27.4, 10.5–2119; PFOA = 0.98, 0.32–52.2; PFNA = 2.50, 0.91–44.2; n = 20). PFAS concentrations in *A. p. doriferus* pups were significantly greater ($p < 0.05$) than in *N. cinerea* pups for all PFAS except PFOA and were of similar magnitude to those reported in northern hemisphere marine animals. These results demonstrate exposure differences in both magnitude and PFAS profiles for *N. cinerea* in South Australia and *A. p. doriferus* in Victoria.

This study reports detectable PFAS concentrations in Australian pinniped pups indicating the importance of maternal transfer of these toxicants. As *N. cinerea* are endangered and recent declines in pup production has been reported for *A. p. doriferus* at the colony sampled, investigation of potential health impacts of these toxicants on Australian pinnipeds is recommended.

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1. Introduction

Per and polyfluoroalkyl substances (PFAS) are a group of over 4000 anthropogenic compounds produced for a multitude of industrial and consumer applications. Several PFAS including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), and substances that degrade to PFOA or PFOS, are considered persistent organic pollutants (POPs) with long-term environmental persistence, as well as bioaccumulation potential (UNEP, 2009). Per and polyfluoroalkyl substances are used or produced in many industries including electronics manufacture, metal plating paints, textiles, surface treatments, fluorochemical manufacture and in aqueous film forming foam (AFFF) used for fighting liquid fuel fires (Buck et al., 2011; Lindstrom et al., 2011; Prevedouros et al., 2006). Environmental contamination by PFAS occurs via industrial emissions, degradation of PFAS-containing products in landfill or from direct run-off of PFAS into water bodies (Buck et al., 2011; Houde et al., 2006b). Per and polyfluoroalkyl substances are ubiquitous in the ocean, assisted by widespread ocean current distribution and atmospheric transport (Butt et al., 2010; Giesy and Kannan, 2001). Abiotic and biotic breakdown of longer chain derivatives from PFAS sources allows the formation of the more commonly known compounds, including the aforementioned PFOS and PFOA (Buck et al., 2011).

The toxic effects of PFAS have been reported in both human and animal studies (Dong et al., 2009; Kennedy et al., 2004; Lau et al., 2004; Pachkowski et al., 2019; Yang et al., 2002). Awareness of their environmental and health effects has resulted in a gradual phase out of selected compounds (Johnson et al., 2020; Kennedy et al., 2004; Pachkowski et al., 2019; USEPA, 2016). In Australia, these compounds are still employed in specialised fields and industries including in AFFFs, carpet manufacture and can be found in imported products (AHPPC, 2016; Enrisks, 2018; HEPA, 2020).

In both human and laboratory animals, PFOS and PFOA have been reported to cause effects ranging from immunosuppression and immunotoxicity including decreased immune response to vaccines, reduction of serum thyroid hormones, neurotoxicity, decreased body weight, developmental defects, and liver pathology (Dong et al., 2009; Kennedy et al., 2004; Lau et al., 2004; Pachkowski et al., 2019; Yang et al., 2002). In laboratory mice, immunotoxic effects of PFOS and PFOA include splenic and thymic atrophy and suppression of the adaptive immune response (Dong et al., 2009; Yang et al., 2002). Developmental effects in laboratory animals have also been identified, with pregnant mice and rats exposed to PFOS having litters with reduced survival and stunted growth (Lau et al., 2004). Delayed growth and development in mice pups have also been associated with low concentrations of PFOA (3000 ng/g body weight), and high mortality has been associated with high dosage (10,000–20,000 ng/g body weight) (Lau et al., 2006).

Per and polyfluoroalkyl substances are commonly detected in marine environments, even in remote regions such as the Arctic and Antarctic (Bengtson-Nash et al., 2010; Butt et al., 2010; Dreyer et al., 2009; Giesy and Kannan, 2001; Muir et al., 2019). Upper trophic marine predators are particularly vulnerable to bioaccumulation of these substances due to the high affinity of PFAS for protein rich tissue and their slow elimination by the body (Kelly et al., 2009), although little is known of the effects of these substances in marine organisms. Investigations of PFAS in marine wildlife are predominantly limited to studies in the northern hemisphere and in Antarctica (Brown et al., 2018; Gebbink et al., 2016; Giesy and Kannan, 2001; Gui et al., 2019; López-Berenguer et al., 2020; Muir et al., 2019; Routti et al., 2015) and include a wide range of marine and freshwater biota such as pelicans (*Pelecanus occidentalis*), sea otters (*Enhydra lutris*), and several pinniped and cetacean species (Fair and Houde, 2018; Ishibashi et al., 2008; Kannan et al., 2002a, 2002b; Muir et al., 2019; Olivero-Verbel et al., 2006; Spaan et al., 2020; Van de Vijver et al., 2005). Studies also report associations of elevated PFAS concentrations with disease (Fair and Houde, 2018). For example, significantly higher concentrations of PFOS and PFOA were reported in sea otters determined to have died from infectious aetiology, compared to sea otters that died from trauma (Kannan et al., 2006). Other effects reported include increased Peroxisome Proliferator-Activated Receptor α (PPAR α) activation, a nuclear hormone receptor important for metabolic processes, and immunomodulation in Baikal seals (*Phoca sibrica*) (Ishibashi et al., 2008) and bottlenose dolphins (*Tursiops truncatus*) (Fair et al., 2013), and thyroid hormone disruption in polar bears (*Ursus maritimus*) (Bourgeon et al., 2017). The most likely source of these toxicants for pinnipeds is dietary (De Silva et al., 2021) with neonatal animals acquiring PFAS through placental and lactational transfer (Grønnestad et al., 2017). Greater PFOS concentrations have been reported in melon headed whale (*Peponocephala electra*), bottlenose dolphin and harbour seal (*Phoca vitulina*) offspring compared to their mothers (Hart et al., 2008; Houde et al., 2006a; Shaw et al., 2009).

In Australia, there is increasing concern about potential human health impacts from environmental PFAS contamination, which have been identified across the country from fire training grounds, oil and gas industries, defence bases, airports and chemical manufacturers (Enrisks, 2018; HEPA, 2020). Perfluorooctane sulfonate concentrations have been detected in freshwater fish and prawns (2.50–21.0 ng/g) sampled in waterways and in surface and ground water in proximity to a defence base (Enrisks, 2018; NSW Government, 2017; Teo and Goldsworthy, 2019). Per and polyfluoroalkyl contamination has also been identified in soil (PFOS = 2 kg in 9,800,000 m^3, PFOA = 0.1 kg in 2,700,000 m^3), surface and ground water (PFOS = 60 kg in 9,800,000 m^3, PFOA = 0.06 kg in 2,700,000 m^3) at several defence bases and river systems (PFOS = 0.045 μg/L, PFOA = 0.014 μg/L) in Victoria and South Australia (Allinson et al., 2019; Aurecon, 2018; Cowin, 2019; Senversa, 2017). Reporting of PFAS contamination in marine species in Australia is limited to fish, crustaceans and bottlenose dolphins (Gaylard, 2016; Taylor, 2018) while concentrations have been reported in the blood, skeletal muscle and liver of Antarctic pinnipeds (Bengtson-Nash et al., 2010; Kannan et al., 2001; Routti et al., 2015; Schiavone et al., 2009; Tao et al., 2006). Elevated concentrations of POPs including dioxin-like PCBs (dl-PCBs) have been reported in Australian fur seals, (*Arctocephalus pusillus doriferus*) (Taylor et al., 2018) associated with an alopecia syndrome (Lynch et al., 2011); however, PFAS concentrations have not been reported in any Australian pinniped.

The Australian sea lion (*Neophoca cinerea*) has a limited geographical range in South Australia and Western Australia with a declining population trajectory and low population size estimated at 12,690 individuals (Goldsworthy, 2015, 2020). Australian fur seals are distributed along the southeast coast of Australia with a population estimate of 120,000 individuals; the majority of pup production (78%) is in Victoria (Kirkwood et al., 2010). The long-nosed fur seal (*Arctocephalus forsteri*) is distributed along the east, south and west Australian coasts, with the majority of the population found in South Australia with an estimated population of 100,000 individuals in South Australia (Shaughnessy et al., 2015).

All pinniped populations in Australia were impacted by historical commercial harvesting in the eighteenth and nineteenth centuries (Ling, 1999). Both fur seal populations have somewhat recovered from commercial harvesting, and both species are listed as 'Of Least Concern' by the International Union for Conservation of Nature (Chilvers and Goldsworthy, 2015; Hofmeyr, 2015). However, while the *A. forsteri* population continues to increase, recent declines in pup numbers have been identified at key breeding sites of *A. p. doriferus*, including at Seal Rocks near Phillip Island, Victoria where a 4.2% per annum decline in pup production has been observed since 2007 (McIntosh et al., 2018). The cause of this decline is considered to be a combination of factors including: changes in the food web, disease and climate change impacts including the inundation of low-lying breeding areas (McIntosh et al., 2018; McLean et al., 2018).

Neophoca cinerea is classified as endangered on the IUCN Red List (Goldsworthy, 2015) and in late 2020 was listed as endangered under Australian legislation, the Environmental Protection and Biodiversity Conservation Act of 1999 (EPBC Act). High pup mortality is reported

in this species (DSEWPC, 2013; Goldsworthy, 2015, 2020). At Seal Bay on Kangaroo Island, South Australia, total pup production is declining by 2.3% each 18-month breeding season (Goldsworthy et al., 2019). Identifying the threats impacting the recovery of *N. cinerea* including disease and pollutants is a key objective of the species' Recovery Plan (DSEWPC, 2013). Endemic hookworm (*Uncinaria sanguinis*) infection occurs at 100% prevalence in *N. cinerea* pups and causes haemorrhagic enteritis (Marcus et al., 2014) which contributes considerably to pup morbidity and mortality. Given the significance of this pathogen on pup survival, investigating pup exposure to the immunomodulatory impacts of PFAS is a critical component of disease investigation in this species.

This study will determine the concentrations of 25 PFAS in *N. cinerea*, *A. p. doriferus* and a single *A. forsteri* pup and in a small number of older aged individuals. The potential impact of PFAS in these species will be discussed in the context of effects on the immune system and on neonatal development, as well as the importance of maternal transfer. As upper trophic species, pinnipeds are key sentinels of the health of the marine ecosystem. Knowledge of toxicant concentrations and the potential impact of exposure can enhance our understanding of the resilience of pinnipeds to other key threatening processes, such as the effects of climate change, the alteration of food webs and the ability of individuals and populations to combat disease.

2. Materials and methods

2.1. Sample collection

All sample collection for this study took place between July 2017 and January 2020. Samples were collected from *N. cinerea* pups (n = 28), juveniles (n = 3) and an adult male (n = 1) at Seal Bay, Kangaroo Island (35.96°S, 137.32°E) between February–March 2018 and July 2019–January 2020, from pups at Dangerous Reef (34.82°S, 136.23°E; n = 6, July–August 2017) and Olive Island (32.72°S, 133.97°E; n = 2, September 2017). Samples were collected from *A. p. doriferus* pups (n = 20) and juveniles (n = 2) sampled at Seal Rocks, Western Port, Victoria (38.52°S, 145.112°E, between December 2018–January 2019 and December 2019–January 2020) and from an *A. forsteri* pup sampled at Cape Gantheaume, Kangaroo Island (36.01°S, 137.47°E; n = 1, in January 2018). Neonatal pups were aged between 2 and 12 weeks at the time of sampling based on standard length for *N. cinerea* (Stokes et al., 2020) and time of sampling (December and January respectively) for *A. p. doriferus* and *A. forsteri* pups (Kirkwood et al., 2010; McKenzie et al., 2005). Liver samples were collected from fresh carcasses (no or mild autolysis as determined by a veterinary pathologist) as part of full necropsy examination on animals found dead at each site. Sampling sites are shown in Fig. S1.

Approximately 50 g of liver was collected from each individual using a sterile scalpel blade. Samples were stored in individual PFAS free commercial zip-lock bags and frozen at −20 °C when freezer storage was available. At more remote sites, approximately 7 g of liver was collected into multiple 1.5 mL PFAS free cryogenic vials and frozen in liquid nitrogen at −196 °C. Longer term storage of all samples prior to analysis was at −20 °C. Morphometric details including standard length and sex were determined during necropsy.

Sample collection from *N. cinerea* and *A. forsteri* was approved by the University of Sydney Animal Ethics Committee (Approval 2017/1260). Sample collection for *A. p. doriferus* was approved by Phillip Island Nature Parks Animal Ethics Committee (Approval No. 2.2016).

2.2. Sample preparation and analysis

All sample preparation and analysis were undertaken at the National Measurement Institute (NMI), North Ryde, NSW, Australia utilising an optimised protocol based on reference United States Environmental Protection Agency (USEPA) methods, USEPA 537.1 (drinking water method) (Shoemaker and Tettenhorst, 2018) and USEPA-821-R-11-007 (draft biosolid method) (USEPA, 2011). There is no USEPA reference method available for biota.

Liver samples were homogenised using a handheld blender (Stuart SHM-1, Cole-Parmer Ltd., Stone, United Kingdom). The blender head was cleaned between each sample using Extran® MA05 detergent and subsequently rinsed with deionised water.

Approximately 0.7 g of homogenised liver was weighed into 50 mL Falcon® polypropylene (PP) tubes to which 20 μL of ^{13}C isotopically labelled perfluorinated analogues of the target compounds (Wellington Laboratories, Canada, Table S1) was added to act as internal standards. Ten millilitres of a 0.01 N methanolic potassium hydroxide (KOH) solution was added to each sample followed by saponification via tumbling for 12 h (REAX-2 Overhead Shaker, Heidolph Instruments, Schwabach Germany).

Following saponification, samples were centrifuged at 3500 RPM for 10 min. The supernatant was removed and purified using solid phase extraction (ENVI-Carb 500 mg, 120–400 Mesh, Supelco, Bellefonte USA), eluted with 100% LCMS grade methanol (Merck, Darmstadt, Germany), then concentrated under nitrogen (Thermo/Pierce, ReactiTherm III Evaporation Block, Waltham, USA), followed by centrifugal filtration. The final extracts were pipetted into 700 μL LCMS vials and ^{13}C isotopically labelled injection standard was added (Wellington Standards, Canada, Table S1). Prior to analysis, the samples were vortexed using an MX-S mixer (DLAB Scientific, Beijing, China).

Samples were analysed on a high-performance liquid chromatograph/triple quadrupole mass spectrometer (ABSciex Exion AD UPLC, ABSciex QTRAP® LC-MS/MS (6500+), Framingham, USA). Extract (2 μL) was injected and eluted from a C18 column (Acquity BEH XBridge, 2.1 × 100 mm × 1.7 μm (130 Å), Waters, USA) using a solvent gradient transitioning from water to methanol, with a modifier of 20 mM ammonium acetate. The mass spectrometer was operated in negative mode with electrospray ionisation, with multiple reaction monitoring (MRM) of the two characteristic transitions with quantification based on ^{13}C isotopically labelled surrogates and certified calibration standards (Wellington Standards, Canada, Table S1). The concentrations of 25 PFAS were determined and are adjusted for the recovery of the labelled internal standards. Compounds included, perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonamide (PFOSA), perfluorodecane sulfonate (PFDS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoA), Perflurotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorobutanesulfonic acid (PFBS), perfluoropentane sulfonate (PFPeS), perfluoroheptane sulfonate (PFHpS), perfluorononane sulfonate (PFNS), perfluorohexanoic acid (PFHxA), pentafluorobenzoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluoroheptanoic (PFHpA), fluorotelomer sulfonic acid (4:2 FTS; 6:2 FTS; 8:2 FTS; 10:2 FTS), *N*-methylperfluorooctane sulfonamidoacetic acid (N-MeFOSAA (2355-31-9)) and *N*-ethyl perfluorooctane sulfonamido acetic acid (N-EtFOSAA (2991-50-6)).

2.3. Quality assurance

Several sample batches were analysed over a 3-month period. Each batch of samples included four controls - a blank (10 mL of a 0.01 N methanol and potassium hydroxide solution), a blank spike (10 mL of a 0.01 N methanol and potassium hydroxide solution containing 20 μL of native PFAS, Table S1), duplicate samples, and PFAS spiked samples. Method background contamination was monitored with batch blanks, which were all below limits of reporting. Method verification for each batch is by recovery of PFAS from a blank spike, ranging from 84 to 112%. Method precision was verified through duplicate sample analysis to ensure relative percentage difference of results was within ±20%. Recovery of PFAS analytes was monitored with PFAS spiked samples, with recoveries in the range 84–160% for all PFAS reported. Method accuracy

has been determined previously within the laboratory by analysis of certified reference materials within 10% of PFOS reference value (Trout, SRM1946, NIST, USA), and ongoing participation in international proficiency studies (Interlaboratory Comparison of POPs in Food, Norwegian Institute of Public Health, and PFAS in Biota, National Measurement Institute, Australia). All quality assurance samples were analysed using the methodology detailed in Section 2.2.

Limits of Reporting (LORs) have been established for each compound based on the instrument and method performance at NMI Australia. For the majority of the compounds, the LOR was 0.5 ng/g except for PFTrDA, PFTeDA, PFNS and PFDS for which the LOR was 1.0 ng/g. Perfluorohexane sulfonate and PFOS were quantified using combined branched and linear standards. All PFAS linear and branched isomers in samples were totalled for reporting. The calibration standards used were certified reference materials including PFAS combined surrogates, a native standard and a PFAS recovery standard (Wellington Standards, Canada; Table S1).

2.4. Statistical analysis

Sample size limitations restricted statistical modelling to *N. cinerea* (n = 28) and *A. p. doriferus* (n = 20) pups sampled at Seal Bay and Seal Rocks, respectively. Statistical analysis of all remaining results is limited to descriptive statistics.

Data for both breeding seasons were combined for each species to ensure a suitable sample size for interspecies comparisons of PFAS concentrations in pups. For the majority of PFAS, the data were highly skewed, necessitating natural log transformation of the data. Post-transformation, data was still not normally distributed based on the Shapiro-Wilks test. For this reason, all statistical analyses were performed using non-parametric methods on natural log transformed data. An outlier was detected in the *A. p. doriferus* pup dataset based on Mahalanobis distance and was excluded from all interspecies statistical analyses but included in the descriptive results. As a result, the sample size for *A. p. doriferus* for multivariate analysis is n = 19. All statistical analyses were performed using R 3.2.2 for Windows (R Core Team, 2015). Statistical significance for all analysis was established at $p < 0.05$. Multivariate statistical analyses were carried out using the vegan-package (Oksanen et al., 2018). Reference intervals were produced using the 'boot' R package (Canty and Ripley, 2019). All PFAS concentrations are reported in ng/g wet weight.

2.4.1. Development of reference intervals and interspecies comparison of PFAS concentrations in N. cinerea, A. p. doriferus and A. forsteri

Robust 95% reference intervals were developed for PFOS, PFOA and PFNA in *N. cinerea* pups sampled at Seal Bay. The remaining PFAS had insufficient numbers of samples with PFAS levels above the LOR to form reference intervals and for this reason, median and observed range of values are reported. Based on the American Society of Veterinary Clinical Pathology (ASVCP) recommendations (Friedrichs et al., 2012) for sample sizes ≥20 and <40, robust 95% reference intervals were developed for PFOS, PFOA and PFNA, with 90% confidence intervals around each limit calculated using a bootstrap approach with 10,000 replicates. Data were transformed using a Box-Cox transformation and outliers were removed prior to calculating the reference intervals. Outliers were identified using Horn's algorithm based on Tukey's interquartile fences. Two outliers were detected and removed from PFOA and PFNA data, reducing the sample size for these compounds to 26. No outliers were detected in the PFOS data (n = 28). The sample size for *A. p. doriferus* restricted reporting to medians and observed range of values (Friedrichs et al., 2012).

A Kruskal Wallis test was used to compare PFAS concentrations between *A. p. doriferus* and *N. cinerea* pups. Effect sizes (r) were calculated as the Z statistic divided by the square root of the sample size (N) and assessed as: small effect = 0.1–0.3, medium effect = 0.3–0.5, large effect ≥ 0.5. Interspecies comparison was not undertaken for PFAS when concentration data within a species was below the LOR (PFDoDA, PFTrDA, PFTeDA, PFBS, PFPeS, PFHpS, PFNS, PFDS, PFHxA, PFBA, PFPeA, PFHpA, 4:2 FTS, 6:2 FTS, 8:2 FTS, 10:2 FTS, N-MeFOSAA (2355-31-9), N-EtFOSAA(2991-50-6)). Differences in concentrations with pup sex was also compared for both *A. p. doriferus* and *N. cinerea* utilising the Kruskal Wallis test with subsequent pooling of data if no significant differences were found. If compounds were detected in some of the samples within each species, for any results below the LOR, the LOR value was substituted such that <LOR = LOR, resulting in reduced bias compared to when data below the LOR are reported as zero (Helsel and Hirsch, 2002).

To provide comparison of PFAS concentrations for each age cohort and sample site where sample size precluded statistical comparison, summed total ($\sum$PFAS) concentrations are reported. The $\sum$PFAS for each individual is calculated by the summation of the 16 PFAS that had detectable concentrations (Table 1).

2.4.2. Multivariate analysis of PFAS concentrations in N. cinerea and A. p. doriferus pups

Multivariate analyses, specifically Principal Component Analysis (PCA) and Redundancy Analysis (RDA), was used to evaluate interspecies differences (explanatory variable) in PFAS concentrations (response variable). Briefly, principal component 1 (x axis) accounts for the largest variance within the samples followed by principal component 2 (y axis) which accounts for the remaining variance. The indirect ordination method, PCA, assigns scores to each individual sample that are linear combinations of the variables. Due to sample size, this analysis was limited to PFOS, PFOA, PFNA, PFDA, PFUdA, PFHxS and PFOSA. Redundancy analysis was used to assess the influence of species in explaining the observed variance structure of PFAS identified in the PCA.

Permutation tests were used to test for significance of explanatory variables. Spearman rank correlation was employed to evaluate the relationship between individual PFAS for all pup samples included in the interspecies comparison to provide additional quantitative analysis. A Bias-corrected, accelerated (BCa) bootstrap with 1000 replicates (Efron and Tibshirani, 1993) was used to determine significance of the Spearman correlations. Significance was established at $p < 0.05$ and correlation coefficients (r_s) > 0.5. A correlation coefficient between $0.40 < r_s < 0.69$ was considered moderate, between $0.70 < r_s < 0.89$ was considered strong and between $0.90 < r_s < 1.00$ considered very strong.

3. Results

3.1. Reference intervals and interspecies comparison of PFAS concentrations in N. cinerea, A. p. doriferus and A. forsteri

All liver samples of *N. cinerea* and *A. p. doriferus* pups had detectable concentrations of PFOA, PFNA and PFOS (Table 1). Detectable concentrations of several PFAS were limited to: PFDA (*A. p. doriferus* n = 14, *N. cinerea* n = 4), PFUdA (*A. p. doriferus* n = 17, *N. cinerea* n = 9), PFHxS (*A. p. doriferus* n = 11, *N. cinerea* n = 6) and PFOSA (*A. p. doriferus* n = 15, *N. cinerea* n = 2). An additional eight PFAS (PFDoA, PFTrDA, PFTeDA, PFPeS, PFHpS, PFNS, PFDS and PFBS) were detected in liver samples of *A. p. doriferus* pups (n = 8) but were not detected in *N. cinerea* samples; PFHxA, PFBA, PFPeA, PFHpA, 4:2 FTS, 6:2 FTS, 8:2 FTS, 10:2 FTS, N-MeFOSAA (2355-31-9), N-EtFOSAA(2991-50-6) were not detected in any sample of either species. Table 1 reports reference intervals for PFOA, PFNA, and PFOS in *N. cinerea* pups sampled at Seal Bay in addition to median and observed range of values for PFDA, PFUdA, PFHxS and PFOSA for *N. cinerea*. PFAS concentrations in *A. p. doriferus* are also reported in Table 1, including the outlier (n = 20).

Significantly higher concentrations ($p < 0.05$) of PFOS, PFDA, PFUdA, PFOSA, PFDoA, PFNS and PFPeS were detected in *A. p. doriferus* pups compared to *N. cinerea* pups sampled at Seal Bay (Table 2). The concentration of PFOA was significantly higher ($p < 0.01$) in *N. cinerea* pups compared to *A. p. doriferus* pups (Table 2). The difference in PFOA,

Table 1
Concentration of PFAS in ng/g wet weight (ww) in liver samples collected in 2018–2020 from Australian sea lion (*N. cinerea*; n = 28) and Australian fur seal (*A. p. doriferus*; n = 20) pups at Seal Bay and Seal Rocks, respectively. Limits of reporting (LOR) are included for PFAS compounds. The outlier in the *A. p. doriferus* pup group is included in the median and observed range of values.

PFAS compound[b]	LOR	*A. p. doriferus*		*N. cinerea*			
		Median	Observed range of values	Median	Observed range of values	Reference intervals	
						Lower limit (90% CI)	Upper limit (90% CI)
PFOA	0.30	0.98	0.32–52.2	2.73	0.32–11.2	0.46 (0.07–0.98)	6.98 (4.73–10.3)
PFNA	0.50	2.50	0.91–44.2	2.96	0.61–8.22	0.96 (0.32–2.41)	5.77 (3.61–7.50)
PFDA	0.50	0.61	0.50–38.0	<0.50	0.50–0.90	0(0)[a]	0(0)[a]
PFUdA	0.50	0.72	0.50–11.0	<0.50	0.50–1.05	0(0)[a]	0(0)[a]
PFHxS	0.50	0.65	0.52–37.4	<0.50	0.5–3.87	0(0)[a]	0(0)[a]
PFOS	1.00	27.4	10.52–2119	7.14	1.00–16.9	0.99 (0.07–2.65)	16.7 (13.1–21.2)
PFOSA	0.50	0.79	0.50–6.43	<0.50	0.50–0.66	0(0)[a]	0(0)[a]
PFDoA	0.50	<0.50	0.50–13.2	<0.50	<0.50	Not calculated	Not calculated
PFTrDA	1.00	<1.00	1.00–3.95	<1.00	<1.00	Not calculated	Not calculated
PFTeDA	1.00	<1.00	1.00–3.28	<1.00	<1.00	Not calculated	Not calculated
PFPeS	0.50	<0.50	0.50–0.78	<0.50	<0.50	Not calculated	Not calculated
PFHpS	0.50	<0.50	0.50–70.2	<0.50	<0.50	Not calculated	Not calculated
PFNS	1.00	<1.00	1.00–8.52	<1.00	<1.00	Not calculated	Not calculated
PFDS	1.00	<1.00	1.00–23.1	<1.00	<1.00	Not calculated	Not calculated
PFBS	0.50	<0.50	0.50–1.12	<0.50	<0.50	Not calculated	Not calculated

LOR = Limit of reporting, PFOA = perfluorooctanoic acid, PFNA = perfluorononanoic acid, PFDA = perfluorodecanoic acid, PFUdA = perfluoroundecanoic acid, PFHxS = perfluorohexane sulfonate, PFOS = perfluorooctane sulfonate, PFOSA = perfluorooctane sulfonamide, PFDoA = perfluorododecanoic acid, PFTrDA = Perflurotridecanoic acid, PFTeDA = perfluorotetradecanoic acid, PFPeS = perfluoropentane sulfonate, PFHpS = perfluoroheptane sulfonate, PFNS = perfluorononane sulfonate, PFDS = perfluorodecane sulfonate and PFBS = perfluorobutanesulfonic acid.

[a] Value range contained too many LOR values and were insufficient to produce confidence intervals.

[b] Perfluorohexanoic acid (PFHxA), pentafluorobenzoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluoroheptanoic (PFHpA), fluorotelomer sulfonic acid (4:2 FTS; 6:2 FTS; 8:2 FTS; 10:2 FTS), *N*-methylperfluorooctane sulfonamidoacetic acid (N-MeFOSAA (2355-31-9)) and *N*-ethyl perfluorooctane sulfonamido acetic acid (N-EtFOSAA(2991-50-6)) were analysed in this study but were not detected in any samples.

PFDA, PFUdA, PFOS and PFOSA concentrations between the species were all categorized as a large effect (r > 0.5). Differences in PFAS were not seen with sex in both *A. p. doriferus* and *N. cinerea* pups.

Figs. 1 and 2 display percentage contribution profiles and absolute concentrations of PFAS for *A. p. doriferus* and *N. cinerea* pups, respectively. A greater contribution by PFOS was seen in *A. p. doriferus* compared to *N. cinerea* pups (65–80% and 10–57%, respectively). In *N. cinerea* pups, greater contributions were seen for PFOA (5–25%) and PFNA (7–25%) compared to *A. p. doriferus* (PFOA, 1–4%; PFNA, 2–17%).

Table 2
Interspecies comparison of PFAS concentrations in Australian sea lion (*N. cinerea*; n = 28) and Australian fur seal (*A. p. doriferus*; n = 19) pups at Seal Bay and Seal Rocks, respectively. Results in bold indicate a significant difference. Effect sizes (r–Value) were calculated where small effect = 0.1–0.3, medium effect = 0.3–0.5 and large effect ≥ 0.5.

PFAS compound	p-Value	r-Value	Effect size
PFOA	**<0.01**[a]	0.65	Large
PFNA	0.569	0.08	Small
PFDA	**<0.01**	0.59	Large
PFUdA	**<0.01**	0.56	Large
PFHxS	0.05	0.27	Small
PFOS	**<0.01**	0.98	Large
PFOSA	**<0.01**	0.72	Large
PFDoA	**<0.01**	0.35	Medium
PFTrDA	0.260	0.16	Small
PFTeDA	0.260	0.16	Small
PFPeS	**<0.01**	0.40	Medium
PFHpS	0.260	0.16	Small
PFNS	**0.015**	0.35	Medium
PFDS	0.260	0.16	Small
PFBS	0.260	0.16	Small

PFOA = perfluorooctanoic acid, PFNA = perfluorononanoic acid, PFDA = perfluorodecanoic acid, PFUdA = perfluoroundecanoic acid, PFHxS = perfluorohexane sulfonate, PFOS = perfluorooctane sulfonate, PFOSA = perfluorooctane sulfonamide, PFDoA = perfluorododecanoic acid, PFTrDA = Perflurotridecanoic acid, PFTeDA = perfluorotetradecanoic acid, PFPeS = perfluoropentane sulfonate, PFHpS = perfluoroheptane sulfonate, PFNS = perfluorononane sulfonate, PFDS = perfluorodecane sulfonate and PFBS = perfluorobutanesulfonic acid.

[a] Indicates compound which was significantly greater in *N. cinerea* compared to *A. p. dorferus*, with all others with significantly greater concentrations in *A. p. doriferus*.

Neophoca cinerea pups also displayed a greater variation in individual PFAS profiles compared to *A. p. doriferus*.

The *A. p. doriferus* pup (19-07), identified as an outlier for statistical modelling, had detectable concentrations of all PFAS (excepting PFBS) that were one to two orders of magnitude greater than the concentrations in all other individuals. Total summed PFAS concentration for this pup was also the highest in the study, irrespective of age/site ($\sum$PFAS = 2432 ng/g ww), and PFOS was the greatest contributor to total PFAS (88%) in this individual (Fig. 2).

Table S2 reports PFAS concentrations and Fig. 3 displays the $\sum$PFAS for all sample locations and age cohorts. The greatest $\sum$PFAS concentrations and observed values were seen in *A. p. doriferus*. Samples from all three *N. cinerea* colonies had similar $\sum$PFAS (Fig. 3) and observed ranges (Table S2). Results from *N. cinerea* pups at Seal Bay also displayed greater PFAS concentrations than identified in the *N. cinerea* bull (Tables 1 and S2). Excluding *A. p. doriferus* pup 19-07, two of the highest summed total concentrations were in juvenile *A. p. doriferus* ($\sum$PFAS = 172 ng/g ww and 68.9 ng/g ww). The *A. forsteri* pup had a $\sum$PFAS concentration of 30.19 ng/g ww which was greater than most *N. cinerea* pups but lower than *A. p. doriferus* pups. Similar contributions to total PFAS were seen in the *A. forsteri* pup as seen in *N. cinerea* individuals, with PFNA and PFOA contributing a large proportion (33%) to total PFAS (Table S2).

3.2. Multivariate analysis of PFAS concentrations in N. cinerea and A. p. doriferus pups

The first two components (PC1 and PC2) of the combined species PCA accounted for 70.1% of the total variability in PFAS concentrations (Fig. 4). Principle component 1 (PC1) primarily reflects concentrations of PFDA, PFUdA and PFOS; PC2 primarily reflects concentrations of PFOA, PFOSA and PFNA (Fig. 4a). The small angles between the loading vectors for PFOS, PFDA and PFUdA indicate strong correlations between these PFAS.

Based on redundancy analysis, species explained 60% of the total variance in PFAS concentrations across the sample ($F_{1,45}$ = 69.3, p = 0.005) and clear interspecies differences in PFAS loadings are evident (Fig. 4a). Individual *A. p. doriferus* sample scores clustered around

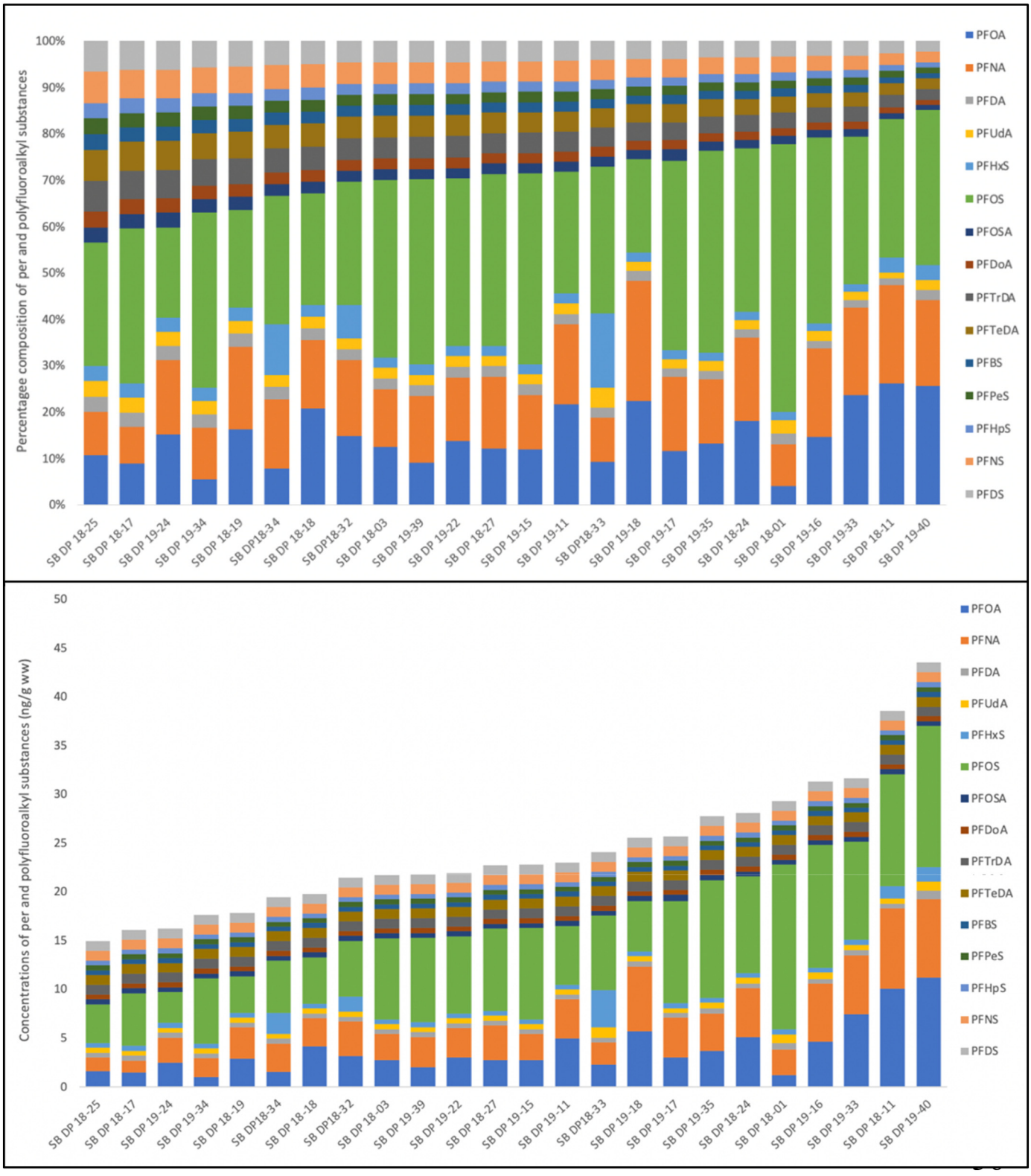

Fig. 1. 100% stacked bar graph displaying percentage composition of PFAS (top) and absolute concentrations with percentage composition of PFAS (bottom), in the liver of Australian sea lion (*N. cinerea*) pups (n = 28) sampled at Seal Bay. The descending order of compounds displayed in the legend correspond with the ascending order of contributions in each column from 0%, with PFOA as the first compound. The x-axis denotes individual pup identification.

positive PFOS/PFOSA loadings, while individual *N. cinerea* sample scores clustered around positive PFOA loadings. On a species-specific level, PC1 and PC2 accounted for 76.6% and 64.0% respectively of the total variance in *A. p. doriferus* and *N. cinerea* (Fig. 5). Qualitative comparisons indicated correlations between PFNA, PFDA and PFUdA and PFOS, PFOA and PFHxS in *A. p. doriferus* (Fig. 5a), and PFOA, PFNA and PFOS and PFHxS, PFUdA and PFDA in *N. cinerea* (Fig. 5b).

Spearman rank correlations (Table 3) reflect the qualitative PCA results. Strong positive correlations ($r_s > 0.70$) were seen for PFNA/PFDA, PFNA/PFOA, PFNA/PFOS, PFNA/PFUdA, PFOS/PFOA and PFUdA/PFDA in

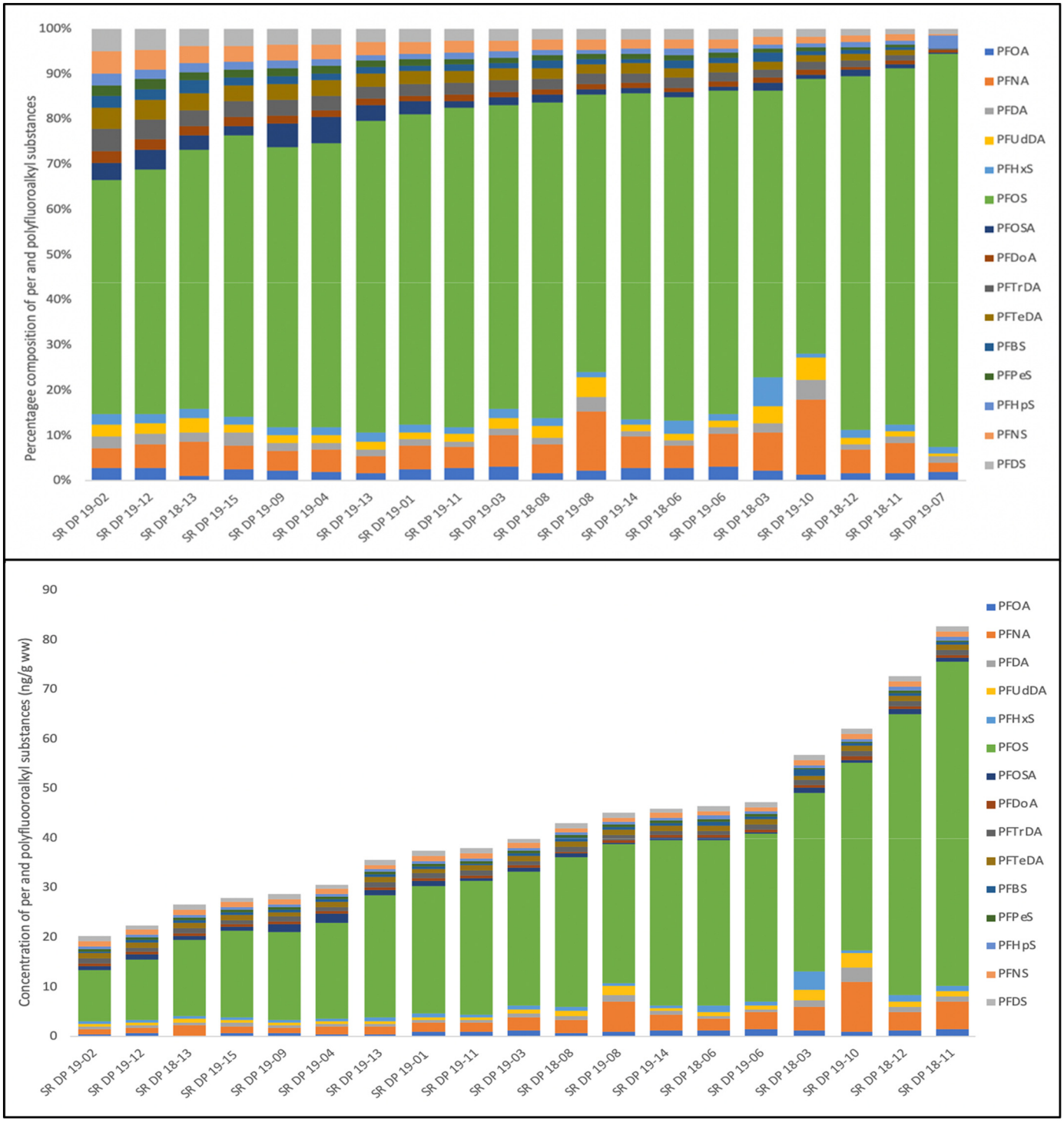

Fig. 2. 100% stacked bar graph displaying percentage composition of PFAS (top) and absolute concentrations with percentage composition of PFAS (bottom) in the liver of Australian fur seal (*A. p. doriferus*) pups (n = 20). The descending order of compounds displayed in the legend correspond with the ascending order of contributions in each column, with PFOA the first compound from 0%. SR DP 19-07 was identified as an outlier and excluded from the absolute concentration graph. The x-axis denotes individual pup identification.

A. p. doriferus. A very strong positive correlation ($r_s > 0.89$) was seen for PFNA/PFOA in *N. cinerea*.

4. Discussion

This study is the first to report PFAS concentrations in pinnipeds in Australian waters. Importantly, *A. p. doriferus* and *N. cinerea* pups have significantly different PFAS contribution profiles and concentrations, and some PFAS concentrations are of comparable levels seen in northern hemisphere marine species within all age groups, highlighting the ubiquity of these toxicants in the marine environment.

4.1. Interspecies comparison of PFAS concentrations

Significant interspecies differences were seen in PFAS concentrations indicating differing bioaccumulation patterns in these populations. However, similar to previous findings in other marine mammals and in polar bears (Gui et al., 2019; Houde et al., 2011; Lam et al.,

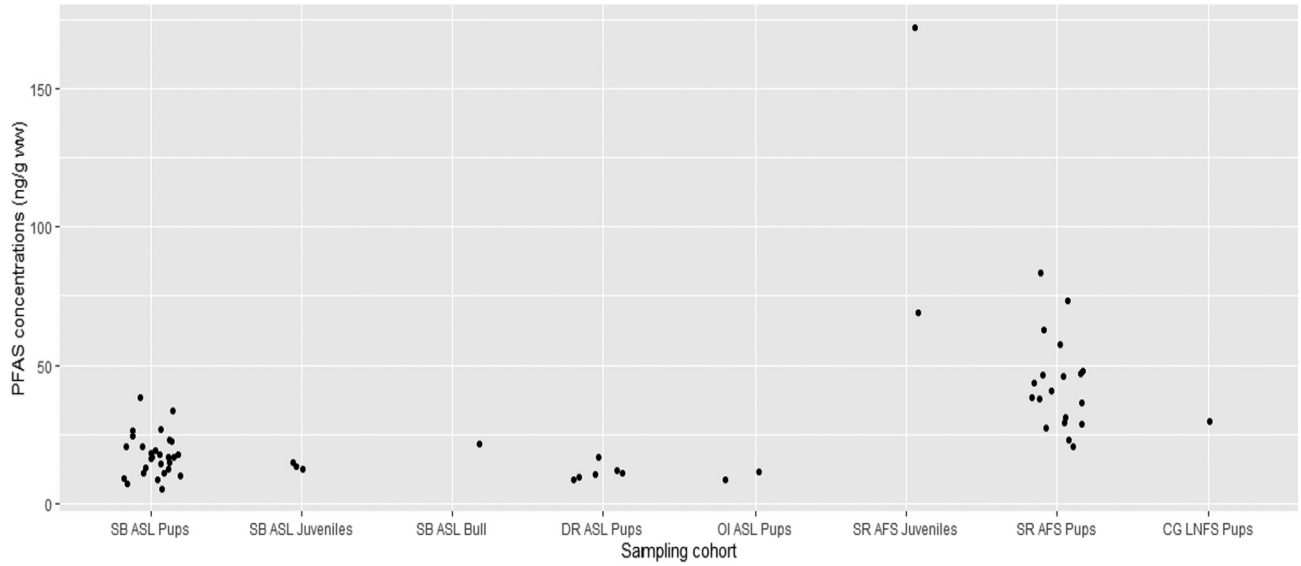

Fig. 3. Total per and polyfluoroalkyl substances (∑PFAS) concentrations for all individuals sampled in this study. From left to right: Australian sea lion (ASL; *N. cinerea*) pups sampled at Seal Bay (n = 28); ASL juveniles sampled at Seal Bay (n = 3); an ASL sea lion bull sampled at Seal Bay (n = 1); ASL pups sampled at Dangerous Reef (n = 6); ASL pups sampled at Olive Island (n = 2); Australian fur seal (AFS; *A. p. doriferus*) juveniles (n = 2) and pups (n = 19) sampled at Seal Rocks and a long-nosed fur seal pup (*A. forsteri*) sampled at Cape Gantheaume (n = 1). An outlier in the AFS pup group is not shown (∑PFAS = 2432 ng/g ww). SB = Seal Bay, DR = Dangerous Reef, OI = Olive Island, SR = Seal Rocks, CG = Cape Gantheaume.

2016; López-Berenguer et al., 2020; Schiavone et al., 2009; Shaw et al., 2009; Spaan et al., 2020), PFOS, PFOA and PFNA were the compounds seen at highest concentrations in this study. The concentrations of PFOS in *A. p. doriferus* pups (10.52–2119 ng/g ww) were greater than in *N. cinerea* (1.00–16.90 ng/g ww) and the *A. forsteri* pup (15.98 ng/g ww) and were comparable to concentrations reported in pinnipeds

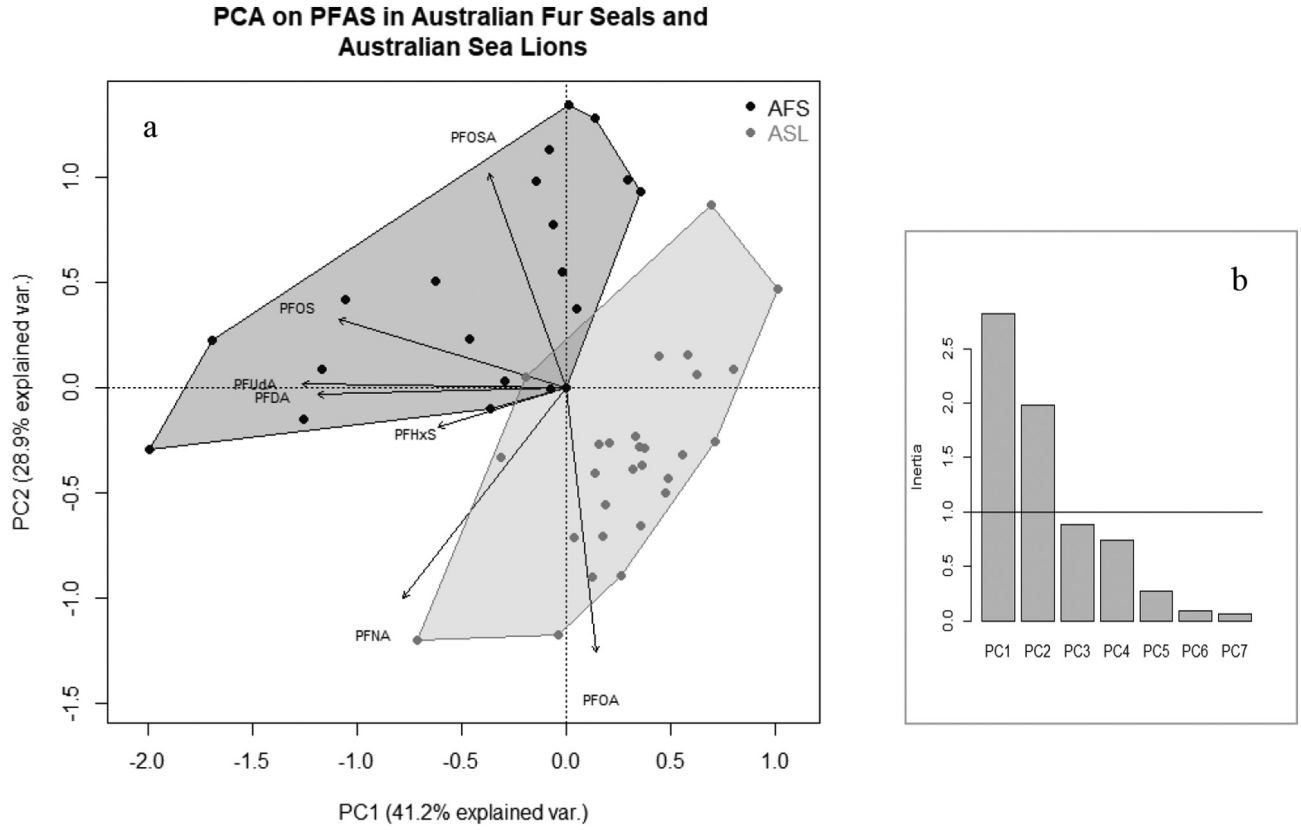

Fig. 4. a) Biplot of seven PFAS in Australian sea lion (*N. cinerea*) (light gray; n = 28) and Australian fur seal (*A. p. doriferus*) (dark gray; n = 19) in pup livers sampled at Seal Bay and Seal Rocks, respectively. PFAS loading vectors are displayed by arrows with the length and direction of the arrow indicating strength and increasing variance. Angles between arrows are representative of the strength of the correlation between PFAS compounds, with small angles indicating highly correlated compounds. The small dots (sample scores) represent individual liver samples. b) Scree plot displaying the PCA eigenvalues in descending order, which are values displaying how much variance is in the data. PCs with eigenvalues >1.0 were considered in the analysis. PFOA = perfluorooctanoic acid, PFNA = perfluorononanoic acid, PFDA = perfluorodecanoic acid, PFUdA = perfluoroundecanoic acid, PFHxS = perflurohexane sulfonate, PFOS = perfluorooctane sulfonate and PFOSA = perfluorooctane sulfonamide.

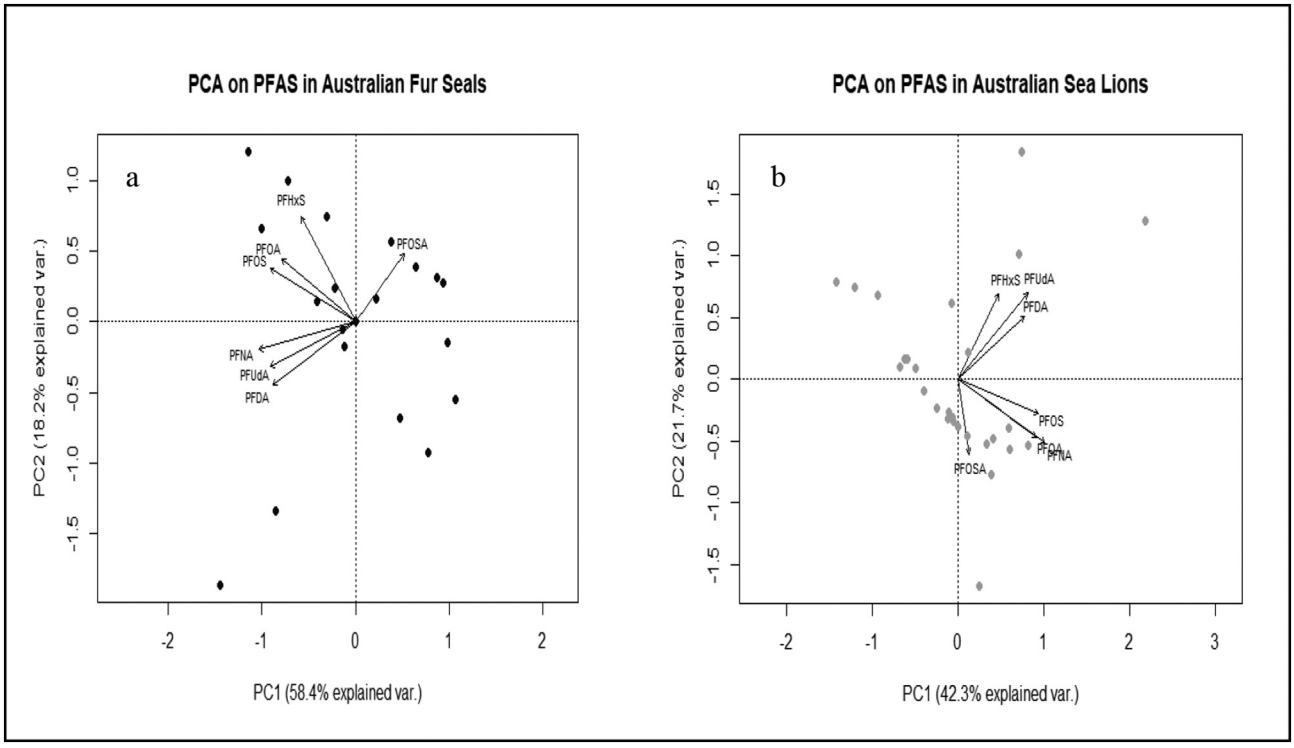

Fig. 5. Biplots representing the results of individual PCA on PFAS levels in a) *A. p. doriferus* from Seal Rocks and b) *N. cinerea* from Seal Bay. Refer to Fig. 4 caption for a description. PFOA = perfluorooctanoic acid, PFNA = perfluorononanoic acid, PFDA = perfluorodecanoic acid, PFUdA = perfluoroundecanoic acid, PFHxS = perflurohexane sulfonate, PFOS = perfluorooctane sulfonate, PFOSA = perfluorooctane sulfonamide.

and sea otters from the northern hemisphere (Kannan et al., 2006; Shaw et al., 2009). The large contribution of PFOS and PFOA to total PFAS concentrations in these species is likely due to a combination of several factors. Perfluorooctane sulfonate and PFOA have minimal degradation in the environment, they are end products of precursor compound degradation, are employed frequently in multiple commercial and industrial applications globally and are known to bioaccumulate in marine biota (Butt et al., 2010; Giesy and Kannan, 2001; Prevedouros et al., 2006).

The interspecies differences in PFAS concentrations determined in the study can likely be ascribed to a number of factors including differing point sources of contamination in the species' respective geographical foraging range, species-specific dietary preferences, species-specific toxicokinetics and the degree of maternal toxicant transfer to pups (for example, due to maternal parity) (Babut et al., 2017; Galatius et al., 2013; Keller et al., 2005; Spaan et al., 2020). PFAS were detected in all liver samples analysed and given that PFAS preferentially bioaccumulates in hepatic tissue (Van de Vijver et al., 2005), liver is an appropriate sample matrix for evaluating PFAS concentrations. Temporal variation is unlikely to be a contributing factor to interspecies differences observed in this study given that the samples were collected over a relatively short time period with respect to the toxicant half-lives.

Table 3
Spearman rank correlation for PFAS concentrations in liver samples of Australian sea lion (*N. cinerea*, n = 28) and Australian fur seal (*A. p. doriferus*, n = 19) pups at Seal Bay and Seal Rocks, respectively. For those compounds with significant correlations, the Spearman correlation coefficient (r_s) and p-value are shown.

		A. p. doriferus		*N. cinerea*	
PFAS 1	PFAS 2	r_s	p-Value	r_s	p-Value
PFDA	PFOSA	−0.5	0.03	–	–
PFNA	PFDA	0.82*	<0.01*	–	–
PFNA	PFOA	0.71*	<0.01*	0.90*	<0.01*
PFNA	PFOS	0.88*	<0.01*	0.64*	<0.01*
PFNA	PFOSA	−0.46	0.045	–	–
PFNA	PFUdA	0.89*	<0.01*	0.44	0.017
PFOA	PFDA	0.50	0.028	–	–
PFOA	PFHxS	0.66*	<0.01*	–	–
PFOA	PFOSA	0.47	0.04	–	–
PFOS	PFDA	0.66*	<0.01*	–	–
PFOS	PFHxS	0.62*	<0.01*	–	–
PFOS	PFOA	0.85*	<0.01*	0.47	0.01
PFOS	PFUdA	0.76*	<0.01*	0.57*	<0.01*
PFUdA	PFDA	0.78*	<0.01*	0.60*	<0.01*
PFUdA	PFOA	0.47	0.04	0.43	0.02

PFOA = perfluorooctanoic acid, PFNA = perfluorononanoic acid, PFDA = perfluorodecanoic acid, PFUdA = perfluoroundecanoic acid, PFHxS = perfluorohexane sulfonate, PFOS = perfluorooctane sulfonate, PFOSA = perfluorooctane sulfonamide.
* Indicates highly significant relationships where $r_s > 0.5$ and $p < 0.01$.

4.1.1. PFAS exposure in Australian pinnipeds

Proximity to point sources of PFAS contamination are a likely reason for the interspecies differences in PFAS bioaccumulation determined in this study. PFAS concentrations are higher in urbanised compared to remote locations, due to closer proximity to direct PFAS sources including defence bases, airports, industrial plants and degraded consumer products in landfill (Houde et al., 2006b). The large contribution of PFOS in *A. p. doriferus* pups could suggest an active source of this compound in their environment, with PFOS a major component in AFFFs which have been linked to substantial environmental contamination (de Solla et al., 2012). The proximity of Seal Rocks (32 nautical miles) to Melbourne, Australia's second largest city, as well as its proximity to a defence base (HMAS Cerberus, Victoria) could be important factors in the bioaccumulation of PFAS in *A. p. doriferus* pups and juveniles. The defence base in this area has previously been implicated in the possible discharge of contaminated ground water to the marine environment (Aurecon, 2018), resulting in a potential direct source of PFAS contamination from previous use of AFFFs on site, of which PFOS is the main component (de Solla et al., 2012). A further PFAS source in the region could be due to a catastrophic fuel fire at a gas plant on the east coast of Victoria in 1998, which took 52 h to extinguish using AFFF (Dawson and Brooks, 1999; Senversa, 2016). These AFFF foams were also utilised at this site for extensive firefighting training after the 1998 fire event,

and recent investigations at the site have identified PFAS concentrations in soil and groundwater above normal limits (EPA, 2017). A greater number of PFAS sources have been identified in the greater Melbourne region compared to in South Australia (Aurecon, 2018; Cowin, 2019; Senversa, 2017), which may explain the greater concentration of PFAS and greater number of individual PFAS detected in *A. p. doriferus* sampled in Victoria.

In *N. cinerea* and *A. forsteri*, greater contributions to total PFAS concentrations from carboxylates (PFOA and PFNA) were seen compared to *A. p. doriferus*. Perfluorooctanoic acid and PFNA are by-products of fluorochemical manufacturing (Ellis et al., 2004; Hoffman et al., 2011), they are found in AFFFs (Zhang et al., 2016), and are used in other commercial and industrial applications (Zhang et al., 2016). Ground water can be contaminated by PFOA from air emissions, which are deposited into soil through precipitation and can result in contamination of sites remote from the original source (Liu et al., 2017). As the production of fluorochemicals does not occur in Australia, the more likely source of these toxicants is the use of AFFFs at airports and defence bases located in Adelaide, South Australia (Department of Defence, 2002), approximately 70 nautical miles from Kangaroo Island. Zhang et al. (2016) suggests the source of legacy PFAS into the oceans, including the carboxylates PFOA and PFNA, is primarily through the contamination of rivers from AFFFs and waste from industrial applications, such as printing and waste management of landfill. Perfluorooctanoic acid can also result from the degradation of fluorotelomer alcohols (FTOHs) which are used in the production of PFAS (Huang et al., 2019), from wastewater treatment plants through passive volatilization, and from wastewater effluent (Chen et al., 2020; Ellis et al., 2004; Wallington et al., 2006). Ionic PFAS, detected in AFFFs, can readily degrade to PFOA through wastewater treatment with disinfectants and ozone and aerobic biotransformation (Mejia-Avendaño et al., 2016; Xiao et al., 2018). Atmospheric oxidation of FTOH has been identified as a reason for greater concentrations of PFOA in remote locations (Wallington et al., 2006). While the identification of specific PFAS point sources of contamination in each geographical location was beyond the scope of this study, the greater concentrations of PFOA in *N. cinerea* suggest that the marine food chain in South Australia has an increased exposure to this compound compared to Victoria. Spatial variability in PFAS concentrations has also been reported previously in pinnipeds, cetaceans and birds of prey from the northern hemisphere (Dietz et al., 2012; Gui et al., 2019; Routti et al., 2016; Spaan et al., 2020; Sun et al., 2019). Further investigation of PFAS concentrations at *A. p. doriferus* sites in more remote locations is critical to better understand these interspecies differences and to elucidate fine scale differences in contamination within the species' foraging range.

4.1.2. Species-specific dietary preferences and toxicokinetics

Interspecies differences in PFAS concentrations may also be affected by dietary preferences, which are influenced by geographical foraging location and prey availability. Adult female *A. p. doriferus* are benthic foragers (Arnould and Costa, 2006), feeding on a predominantly fish and cephalopod diet on the shallow continental shelf off Bass Strait (Arnould et al., 2011; Kirkwood et al., 2008). Adult females from Seal Rocks were found to forage up to 315 km south of the colony (Arnould and Kirkwood, 2007) and their foraging behaviour and foraging range is influenced by seasonality and environmental factors (Arnould et al., 2011; Speakman et al., 2020). The diet of *N. cinerea* females is similar to that of *A. p. doriferus* females and includes cephalopods, plus a wide variety of benthic fish species and crustaceans (McIntosh et al., 2006; Peters et al., 2014), however the foraging ranges of these species do not overlap. Adult females from Seal Bay forage approximately 130 km south of Kangaroo Island along the continental shelf (Fowler et al., 2007), but unlike *A. p. doriferus*, foraging behaviour and range is not influenced by seasonality and lactation (Lowther et al., 2011). Marked individuality in *N. cinerea* foraging range and prey has also been reported for adult females in South Australia (Lowther et al., 2011), which could contribute to the greater variation in individual *N. cinerea* PFAS profiles identified in this study. Although *A. forsteri* is sympatric with *N. cinerea* and *A. p. doriferus* across both of their geographical ranges, this species has an epipelagic diet with adult females feeding primarily on pelagic fish (Hoskins et al., 2017; Page et al., 2005). These differences are relevant to PFAS bioaccumulation, given that benthic fish may have greater PFAS concentrations compared to pelagic species (Munoz et al., 2017). The extent of biomagnification of PFAS through water, sediment, and prey (Munoz et al., 2017) will also influence species-specific PFAS concentrations. Understanding PFAS dispersal from current sources is crucial to understanding these interspecies differences within the context of species-specific foraging locations in South Australia and Victoria.

Interspecies differences in PFAS profiles could also be attributed to differing toxicant metabolism (Galatius et al., 2013; Spaan et al., 2020). For example, toxicokinetic differences in humans and rats contributed to differences in placental and lactational transfer of PFAS (Pizzurro et al., 2019). In a study of mammals in the northern hemisphere, ringed seals and polar bears were reported to have a greater capacity to degrade PFAS precursors compared to killer whales (Gebbink et al., 2016).

While differing toxicokinetics cannot be excluded as contributing to interspecies differences identified in this study, we consider the effect of foraging range and prey preference to be of greater importance for the differences in PFAS accumulation in *A. p. doriferus* and *N. cinerea*.

4.2. Comparison of PFAS concentrations in Australian pinnipeds to other pinniped species

Only four previous papers document PFAS concentrations in southern hemisphere pinnipeds, including concentrations in the blood of southern elephant seals (Tao et al., 2006), skeletal muscle and liver of *A. gazella* (Bengtson-Nash et al., 2010; Schiavone et al., 2009), and in the liver of a single Weddell seal (*Leptonychotes weddellii*) (Routti et al., 2015) (Table S3). Compared to concentrations in *A. gazella* pups (Schiavone et al., 2009), liver PFOS concentrations in *N. cinerea* were similar, while concentrations in *A. p. doriferus* pups were three-fold higher. Concentrations of PFOS were also three and thirteen-fold higher, respectively, in *N. cinerea* and *A. p. doriferus* compared to the concentration in an adult *A. gazella* sampled at South Georgia, Antarctica (Bengtson-Nash et al., 2010). Greater exposure of PFAS in Australian pinnipeds compared to animals sampled in the Antarctic is not surprising given the potential for multiple PFAS sources along the Australian coast compared to PFAS sources in Antarctic fauna being limited to atmospheric and hydrospheric transport (Bengtson-Nash et al., 2010; Dreyer et al., 2009). A report of PFOS in bottlenose dolphins sampled in the Port River in Adelaide, South Australia, displayed high concentrations (510–5000 ng/g ww) in a similar region, however these animals are in close proximity to PFAS sources with limited flushing of waters (Gaylard, 2016).

When compared to northern hemisphere species, liver PFOS concentrations in *N. cinerea* and *A. p. doriferus* were one and two orders of magnitude lower than in harbour and ringed seals, and polar bears (Rigét et al., 2013; Shaw et al., 2009) (Table S3), however PFOS is the dominant compound as identified in previous studies (Gui et al., 2019; Hart et al., 2008; López-Berenguer et al., 2020; Sun et al., 2019). One explanation for higher PFOS concentrations in harbour seal pups (Shaw et al., 2009) is the short lactation period, estimated at 30 days, in this species (Muelbert and Bowen, 1993), as well as the fasting period post weaning which could increase PFAS concentrations in the liver (Aas et al., 2014). *Neophoca cinerea* and *A. p. doriferus* in comparison have much longer lactation periods (up to 18 months and 11 months respectively) (Arnould and Hindell, 2002; Lowther and Goldsworthy, 2011), with the pups in this study entirely maternally dependant at the time of sampling. However, individual *A. p. doriferus* pups had similar PFAS concentrations to those reported in harbour seal pups (Shaw et al., 2009) and sea otters (Kannan et al., 2006). The highest PFOS concentration detected in this

study in an *A. p. doriferus* pup was similar to concentrations reported in polar bears in Greenland (Rigét et al., 2013; Smithwick et al., 2005).

Perfluorooctanoic acid concentrations in *N. cinerea* were greater than concentrations reported for *A. gazella* pups from the Antarctic Peninsula (Schiavone et al., 2009), but similar to concentrations reported in harbour seal pups from the northwest Atlantic coast (Shaw et al., 2009). Despite the phase out of PFOS and its precursors in 2000–2002 by the 3M company (3M, 2000), and the decrease of PFOA production and environmental release (USEPA, 2018), a study of seawater and plankton collected in 2014 from the Northwest Atlantic Ocean identified ongoing release of legacy PFAS into the marine environment, typically via rivers (Zhang et al., 2019). This example highlights the need for monitoring of PFAS concentrations to better understand the risk of these toxicants for marine mammals, ecosystem health and potential human exposure, particularly when data is limited.

In this study, PFOSA concentrations in *A. p. doriferus* pups and juveniles were greater than previously reported in pinniped pups sampled in both the southern and northern hemispheres (Shaw et al., 2009; Schiavone et al., 2009), suggesting the persistence of precursors to short chain compounds like PFOS in the Australian marine ecosystem. The Australian Government has not yet ratified the listing of PFOS and PFOA despite signing the Stockholm Convention in 2004, an international agreement to protect human health from POPs (DAWE, 2019; HEPA, 2020). Although a PFAS National Environmental Management Plan has been recently established (HEPA, 2020), Australia still needs to accept and implement international standards, and there will inevitably be a transition period where these compounds remain in circulation with the potential for ongoing environmental contamination and bioaccumulation in native fauna.

4.3. Maternal transfer

The primary source of POPs, including PFAS, to pinnipeds is through diet (Houde et al., 2011). For neonatal pups solely reliant on maternal nutrition, PFAS concentrations are a reflection of maternal diet (Bytingsvik et al., 2012; Grønnestad et al., 2017). Pups in this study were 2–12 weeks of age and exclusively maternally dependant and land-based. Therefore, PFAS concentrations detected in the current study were acquired via placental and lactational transfer (the relative contribution of the latter dependent on pup age) and an important source of these toxicants for neonatal pinnipeds. In hooded seals (*Cystophora cristata*), placental transfer was the predominant source of PFAS transfer to pups and is considered to be a major excretory route of PFAS for adult females, followed by lactation (Grønnestad et al., 2017). In bottlenose dolphins, melon-headed whales, and harbour seals, foetuses and pups had greater PFOS concentrations compared to adult females (Hart et al., 2008; Houde et al., 2006a; Shaw et al., 2009). Consistent with findings in the harbour seal (Shaw et al., 2009), individual *N. cinerea* pups at Seal Bay had higher PFAS concentrations compared to an adult bull and juveniles sampled at this colony. In addition to parturition and lactation, urinary excretion and loss through the moult have been identified as potential routes of PFAS excretion (Kannan et al., 2001; Worley et al., 2017).

Primiparous pinniped females have the potential to transfer greater concentrations of PFAS to their offspring compared to multiparous females, given that primiparous females would have previously relied on alternative excretory routes. The high total PFAS concentration in the individual *A. p. doriferus* pup considered an outlier for the interspecies comparison (ΣPFAS = 2432 ng/g ww) could reflect maternal foraging within a toxicant hotspot, or alternatively the transfer of several years of bioaccumulation from a primiparous female. To better understand this mechanism of transfer, further investigation of the role of maternal parity on pup PFAS concentrations is warranted.

4.4. Relationship between individual PFAS in N. cinerea and A. p. doriferus

Differences in the relationships between individual PFAS for *N. cinerea* and *A. p. doriferus* suggests differing PFAS exposure and bioaccumulation patterns for these species (Gump et al., 2011; Mørck et al., 2015). Previous studies suggest that particular PFAS patterns can arise due to exposure to high concentrations of multiple compounds simultaneously, for example, from a similar point source within a region (Mørck et al., 2015; So et al., 2007). The strong relationship between PFOA and PFNA in *N. cinerea* pups suggests a common source for these substances, for example, atmospheric oxidation of FTOHs (Ellis et al., 2004), a recognised pathway for the formation of PFAS in remote regions (Hurley et al., 2004; Wallington et al., 2006).

Previous studies in marine mammals have identified significant correlations for PFOSA and PFOS (Galatius et al., 2013; Kannan et al., 2002b; Shaw et al., 2009). Perfluorooctane sulfonamide is a precursor to PFOS with transformation occurring in vertebrate liver microsomes (Xu et al., 2004). Previous studies have also identified low PFOSA and higher PFOS concentrations in neonatal and lactating female harbour porpoises (*Phocoena phocoena*) compared to other age cohorts suggesting greater biotransformation of PFOSA in neonates and lactating females (Galatius et al., 2011). In this study, PFOSA concentrations were similar to the LOR while PFOS concentrations were comparatively high in both species. These findings in all pups and juveniles in this study may indicate high rates of biotransformation of PFOSA to PFOS in the livers of *N. cinerea*, *A. p. doriferus* and in the *A. forsteri* pup.

Lastly, the finding of significant correlations for PFOS with a number of compounds (PFOA, PFNA, PFDA, PFHxS and PFUdA) detected in *A. p. doriferus* highlights the potential utility of PFOS as an indicator compound of PFAS contamination in this species (Powley et al., 2008).

4.5. Health implications of PFAS in Australian pinnipeds

Despite the widespread occurrence of PFAS, the impact of these toxicants on the health of wildlife is a key knowledge gap (Bourgeon et al., 2017; Fair et al., 2013; Ishibashi et al., 2008; Kannan et al., 2006), and this is the case for marine mammals. Reporting of PFAS concentrations in Australian pinnipeds is the first step toward understanding the health impacts of these toxicants on these species. However, in the absence of targeted investigations of potential cause and effect, and the lack of toxic reference values or predicted 'no effect' concentrations available for PFAS in pinnipeds, the health impact of PFAS in these species remains unknown. Lowest-observed-adverse-effect-levels (LOAEL) are references for safe concentrations of PFAS, based on dosage studies, with adjustments needed for animals and humans due to toxicokinetic differences (Cordner et al., 2019). These safe concentrations have been reported for a number of species including birds (Newsted et al., 2007), mice, monkeys and humans (Butenhoff et al., 2002; Dong et al., 2009; Lau et al., 2006; Lilienthal et al., 2017; MDH, 2012).

Examples of immunomodulation and potential associations with disease and mortality due to PFAS in marine mammals have been reported for example in bottlenose dolphins (Fair et al., 2013) and sea otters (Kannan et al., 2006), respectively. In sea otters, significantly greater PFAS concentrations were reported for individuals in an 'infectious disease' group compared to non-diseased and emaciated individuals (Kannan et al., 2006). Tentative critical concentrations (TCC) have also been calculated for hepatic toxicity in dolphins and porpoises, where PFOS concentrations were above the safe limit and adverse effects are likely in that population (Lam et al., 2016).

Further complicating the association between PFAS concentrations and health effects, is the lack of knowledge of the impact of interactions between different compounds on health status (Zeilmaker et al., 2018).

Although it was beyond the scope of this study to directly assess the relationship between PFAS concentrations and morbidity and mortality in Australian pinnipeds, the immunomodulating effects of these toxicants are of particular concern in *N. cinerea*, a species in which pups are severely impacted by endemic hookworm infection. With similar concentrations of some PFAS in pups from this study to those previously associated with morbidity in other species, targeted investigations into

the role of these toxicants if any, in morbidity and mortality, and on neonatal development are recommended.

4.6. Conclusion

This study reports PFAS concentrations in *A. p. doriferus*, *N. cinerea* and a single *A. forsteri* pup. The detection of concentrations of PFAS in neonatal pups demonstrates the important role of maternal transfer in the exposure of pups to anthropogenic toxicants. The differing accumulation patterns of PFAS in these species indicates species-specific exposure likely mediated by differing geographical foraging range and PFAS sources and highlight differences in the vulnerability of these species to toxicant effects. However, targeted investigations of the effects of these toxicants on health is critical to understanding the effect of PFAS on neonatal pinnipeds, and the role, if any, of these toxicants on population declines in these species.

Considering the role of pinnipeds as sentinels of marine ecosystem health and the potential adverse effects of PFAS on neonatal development, monitoring of temporal changes in concentrations is recommended. It is also critical to identify potential PFAS point sources for possible mitigation, particularly if future investigations reveal significant associations between PFAS concentrations and increased vulnerability of these species to adverse health impacts. The latter is particularly relevant to the endangered and declining *N. cinerea*.

Lastly, these results provide evidence of bioaccumulation of emerging anthropogenic compounds in upper trophic marine species in the Southern Ocean, with implications for wildlife, ecosystem and human health.

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CRediT authorship contribution statement

Shannon Taylor: Conceptualization, Data curation, Funding acquisition, Formal analysis, Investigation, Methodology, Writing – original draft. **Michael Terkildsen:** Data curation, Formal analysis, Writing – review & editing. **Gavin Stevenson:** Methodology, Resources, Writing – review & editing. **Jesuina de Araujo:** Methodology. **Chunhai Yu:** Methodology, Writing – review & editing. **Alan Yates:** Methodology, Supervision, Writing – review & editing. **Rebecca R. McIntosh:** Funding acquisition, Writing – review & editing. **Rachael Gray:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Trial of a novel experimental design to test depuration of PFASs from the edible tissues of Giant Mud Crab following exposure under natural conditions in the wild

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HIGHLIGHTS

- A new experimental design for assessing depuration of PFASs from crabs is evaluated.
- Giant Mud Crab showed depuration over comparatively long periods.
- There was evidence for differential PFAS concentrations between claws in individual crabs.

GRAPHICAL ABSTRACT

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ABSTRACT

Poly and perfluorinated alkyl substances (PFASs) are persistent organic pollutants (POPs) that are highly resistant to environmental degradation, and have been detected in a broad range of terrestrial and aquatic species. Portunid crabs have been shown to accumulate comparatively high concentrations of PFASs, but previous work examining depuration in crabs was inconclusive. Here, we trialled a novel experimental design to study depuration of PFASs from edible tissues of portunid crabs, using paired claw samples, and trial this design with Giant Mud Crab *Scylla serrata* exposed to the contaminant under natural conditions. We found evidence for depuration of perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS) and perfluorooctanoic acid (PFOA), but with depuration half-lives as high as 40 days (for PFOS). We also observed substantial variability in the data, including differences in PFAS concentrations between claws from the same individuals, potentially resulting from claw loss and re-growth prior to capture. These results have broad implications for assessing and minimising exposure risk in seafood species.

1. Introduction

Poly and perfluorinated alkyl substances (PFASs) have seen widespread use in industrial applications and consumer goods (Buck et al., 2011). These substances are also known to be persistent organic pollutants (POPs) that are highly resistant to environmental degradation

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(Inoue et al., 2012; Lindstrom et al., 2011), and have been detected in a broad range of terrestrial and aquatic species. Concerns about the human health effects of these contaminants have seen widespread efforts to characterise accumulation levels in biota, particularly in aquatic species that support fisheries (e.g. Berger et al., 2009; Chiesa et al., 2019; Chiesa et al., 2018a; Chiesa et al., 2018b; Christensen et al., 2017; Taylor and Johnson, 2016; Zhang et al., 2011). Research has demonstrated that bioaccumulation of PFASs does not follow conventional patterns observed for other POPs (Martin et al., 2013), and factors affecting uptake and depuration are unclear. This inhibits the use of modelling to predict concentrations in fish and crustaceans, and means that ongoing research is required to characterise the toxicokinetics of PFASs in species exposed in the environment which later enter the food supply.

In contaminated coastal areas, portunid crabs have been shown to accumulate comparatively high concentrations of PFASs, particularly perfluorooctane sulfonate (PFOS) and other long chain perfluorocarbon compounds (Habibullah-Al-Mamun et al., 2017; Taylor et al., 2018; Wang et al., 2012). These species also represent a boutique seafood product with a high economic value, particularly for product that is sold live (Khan and Alam, 1992). Depuration of PFASs from market-sized seafood product that has been exposed to PFASs under natural conditions in contaminated estuaries may present an avenue for improving the marketability of crabs harvested from these locations. Little work has been done on the toxicokinetics of PFASs in marine crustaceans, and the sole previous study on this issue reported patterns that were unclear for a species of portunid crab (Giant Mud Crab *Scylla serrata*, Taylor et al., 2017). Specifically, variability in contaminant concentrations among exposed Giant Mud Crab meant that a depuration coefficient and half-life could not be estimated from experimental data. Here, we trialled a novel experimental design to study depuration in the edible tissues of portunid crabs, using paired samples. We report on the feasibility of the design, observed depuration rates for PFASs in the muscle tissue of Giant Mud Crab, and highlight factors that are important for future sampling of portunid crabs to characterise contaminant concentrations.

2. Methods

2.1. Experimental design and capture of animals

Following the work of Taylor et al. (2017), we developed an experimental design that involved testing of paired samples from individual crabs at the beginning and end of the depuration time period. Obtaining paired samples from animals is difficult, due to the amount of tissue nominally required for PFAS analysis. However, crabs represent a special case where tissue is present in autotomic appendages to the body (such as legs and chelipeds). Crab chelipeds are commonly autotomised during predator-prey and antagonistic interactions among animals, but are also excised manually during the course of normal aquaculture operations (e.g. Quinitio and Estepa, 2011; Rahman et al., 2020), and as part of normal fishing operations for species for which "crab-claw fisheries" exist (Gandy et al., 2016; Patterson et al., 2007). Crabs then subsequently regenerate their claws at the following moult (Rahman et al., 2020). The experimental design generated paired samples by removing one claw from experimental subjects directly at the beginning of the depuration (described below) and one at the specified depuration time point, which allowed for individual-specific depuration trajectories to be calculated. Determining individual depuration trajectories was a logical approach to overcome the previously identified issues arising from variation in contaminant concentrations among wild caught individuals.

The experiment was carried out at Port Stephens Fisheries Institute (New South Wales, Australia) from April–May 2017. Giant Mud Crab were collected from Tilligerry Creek ($n = 34$) which is contaminated with PFASs originating from a nearby point source, where aqueous film forming foams (AFFF) containing PFASs had been used for multiple decades. Additional reference samples were also collected from Wallis Lake ($n = 12$), a comparatively uncontaminated estuary (Fig. 1, Taylor et al., 2018). Reference animals were held under the same test conditions and acted as a control for any potential uptake of contaminant from unknown sources in the aquarium system. Animals were held in six independent tanks of 5000 L capacity, that were supplied with fresh oceanic seawater, with 3 separate replicate tanks for each treatment (contaminated, and reference animals).

Commercial sized Giant Mud Crab (>85 mm carapace length [CL]) were captured using traps which were baited with fresh Eastern Smooth Boxfish (*Anoplocapros inermis*), a marine species collected from offshore trawl catches. Traps were set overnight and retrieved the following morning. Two water samples were collected from each sampling location in 500 mL high-density polyethylene (HDPE) containers with polypropylene lids (supplied by the testing laboratory and batch checked as free from PFASs), and analysed for PFASs.

Following capture, Giant Mud Crab were placed in aerated 200 L tanks containing water from the collection area, and were driven back to the aquarium. At the aquarium, crabs had an individually coloured T-bar tag inserted into their posterior-dorsal musculature, to allow later identification of individual crabs for sampling at the specified depuration time points. Crabs were then cooled in an ice-seawater slurry, and all 46 crabs had one cheliped removed (which was later used to determine the "initial" PFAS concentration) by severing downward and in toward the body along the fracture plane located at the base of the merus and ishium (Gandy et al., 2016). Six crabs, including three from Tilligerry Creek and three reference samples, were euthanised by extended immersion in an ice-seawater slurry, and had both chelipeds removed at this time. These samples were used to determine the difference in PFAS concentrations in paired left and right claws sampled at the same time.

The remaining Giant Mud Crab were introduced into 5000 L tanks containing uncontaminated oceanic seawater (Table 1). Crabs were kept in cages (30 cm × 10 cm × 15 cm) within the 5000 L tanks over the course of the experiment, to prevent aggressive interactions, which may lead to further claw loss and mortality. The depuration period commenced when crabs were introduced to the aquarium system (with the aforementioned crabs reflecting the zero-hr time point), and animals were held for a maximum depuration period of 1128 h. Individual crabs (1 per tank) were sampled across 10 additional time points; 4.5, 9, 18, 36, 72, 144, 288, 456, 792 and 1128 h, as described below (reference crabs were collected at time points; 288 and 1128 h).

2.2. Animal husbandry and sample collection

Crabs were fed once daily on diced Pilchard (*Sardinops* spp., sourced from offshore oceanic habitats off south eastern Australia) at ~10% body weight per day, and water quality (dissolved oxygen, temperature, salinity, pH; measured using a Horiba U-52) checked twice daily. Tanks were cleaned after feeding and ~4000 L of water replaced with clean saltwater to prevent accumulation of any depurated PFASs and other nitrogenous wastes. At each sampling time point (hereafter referred to as "final" samples), an individual was selected from each tank, and euthanised by extended immersion in an ice-seawater slurry. The claw was then removed and muscle tissue dissected and stored in a snap-lock bag. Water samples were collected from all treatment tanks each week during the experiment, by immersing a batch-tested HDPE bottle in the middle of the water column of each tank. Water samples were tested for PFASs to ensure no contamination was occurring throughout the experiment.

2.3. Sample processing and analysis

Basic biometric information was collected from each crab, including carapace length (CL), sex, and moult stage (pre-moult, inter-moult, and post-moult, using Hay et al., 2005), to ensure biological data was

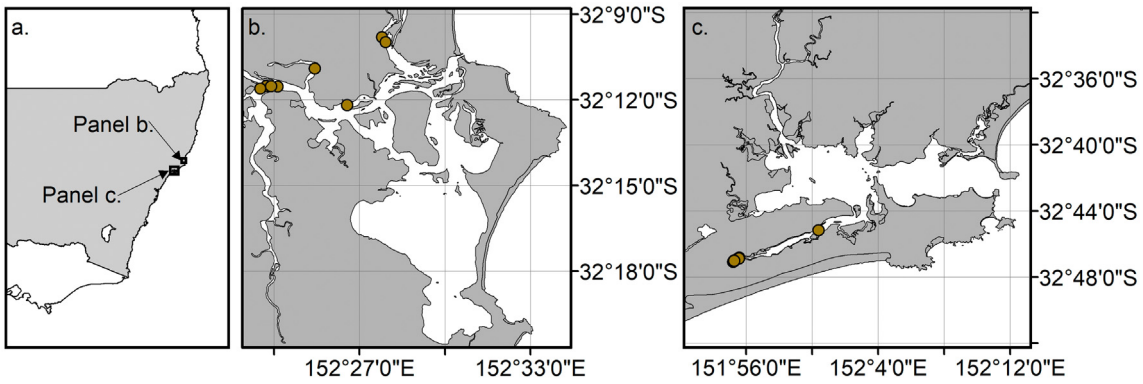

Fig. 1. Map of study area showing locations where Giant Mud Crab were captured in Wallis Lake (b., reference samples) and Port Stephens (c.).

available if required to assist interpretation of patterns in contaminant concentrations. Meat was removed from claws (the initial and final claw) for analysis, and analysed separately. Extraction and analysis of PFASs was based on reference method USEPA 537 (Shoemaker et al., 2008), using a high pressure liquid chromatography tandem mass spectrometer operating in scheduled multiple reaction monitoring mode, and is described in detail in Taylor et al. (2019). Tissue was digested in sodium hydroxide in methanol (MeOH), PFASs extracted using acetonitrile, and quality control standards were added between every 10 samples to monitor instrument variations (see Taylor et al., 2019). Sample analysis targeted the PFASs previously detected in Giant Mud Crab; PFOA, PFHxS and PFOS, and limits of detection (LOD) for these compounds were set as three times the blank value (these were 0.28 μg kg^{-1}, 0.33 μg kg^{-1} and 0.4 μg kg^{-1} respectively for muscle tissue, and 0.01 μg L^{-1} for all three compounds in seawater).

2.4. Data analysis

On the basis of previous investigations, PFOS and PFHxS were the main PFASs of interest. The patterns between initial and final concentrations of PFOS and PFHxS in each individual were first evaluated graphically, and depuration time points were subsequently combined into temporal groupings. The null hypothesis of no change in concentrations of PFASs following >36 h of depuration was evaluated separately for PFOS and PFHxS, by initially evaluating a then testing a null hypothesis of no relationship (i.e. $\beta = 0$) between [$PFAS_{initial}$] and [$PFAS_{final}$], and then testing a null hypothesis of parity (i.e. $\beta = 1$) between [$PFAS_{initial}$] and [$PFAS_{final}$] for depuration time points between 36 and 1128 h (using a Wald test; these models assumed an intercept of zero). To evaluate gross depuration rates in the species, [$PFAS_{final}$] data were pooled with previous data for Giant Mud Crab (Taylor et al., 2017) and a simple linear model used to evaluate the relationship between log([$PFAS_{final}$]) and depuration time point (h). Depuration half-lives were calculated using the formula $\frac{\log(2)}{\beta}$. For this analysis, values that were < LOD were considered to equal 0.5·*LOD*. All analyses were conducted in R v. 3.2.0 (R Core Team, 2020).

Table 1
Summary data for PFOS and PFHxS concentrations (for samples collected from Tilligerry Creek) in each depuration time group, for claws sampled at the initial time point, and the final time point, including the frequency of detection (Freq.) for the initial time point, mean, and range of concentrations.

PFAS	Time group	Freq. (%)	Initial (μg kg^{-1})		Final (μg kg^{-1})	
			Mean	Range	Mean	Range
PFOS	0 h	100	8.77	8.15–9.08	8.71	7.43–9.81
	≤ 36 h	100	5.87	3.50–9.20	6.72	3.40–11.00
	36–288 h	100	7.20	1.90–12.63	7.45	3.00–12.66
	>288 h	100	6.43	2.34–12.00	4.70	2.10–7.60
PFHxS	Initial	100	4.24	0.48–11.65	4.49	0.42–12.30
	≤ 36 h	83	2.58	0.33–9.20	3.29	0.40–15.00
	36–288 h	100	4.00	0.60–11.00	3.81	0.70–10.17
	>288 h	100	2.71	0.56–9.20	3.59	0.33–15.00

3. Results

3.1. General comments

Estuarine water at the Tilligerry Creek collection area contained PFOS (0.63 ± 0.17 μg L^{-1}), PFHxS (0.14 ± 0.02 μg L^{-1}), and PFOA (0.01 ± 0.00 μg L^{-1}). PFASs were below limits of detection for estuarine water collected at the Wallis Lake (reference) collection area, and no PFASs were detected in water samples from the tanks used to hold animals throughout the experiment. The high estuarine water concentrations at Tilligerry Creek were similar with previous reported concentrations in this area (Taylor et al., 2017). Surprisingly, PFOS was detected (1.2 μg kg^{-1}) in the initial samples of one set of animals collected from Wallis Lake (which were all added to the same tank), but these concentrations were much lower than animals from the contaminated study site, and had declined to <LOD before the first collection time point (288 h) for reference animals. There was no PFAS detected in the other reference animals at the commencement, or throughout the experiment. PFAS concentration data for samples from Tilligerry Creek are summarised in Table 1.

3.2. Depuration of PFASs

Initial time point (paired zero-hour) samples, which represented samples of each claw collected at the same time (i.e. without any depuration), showed some minor variability between claws within the same individual, however these were generally within the 30% threshold allowed for measurement precision (Fig. 2). For the data presented in Fig. 2, depuration would be evident in data points lying below the 1:1 line of parity, indicating [$PFAS_{final}$] < [$PFAS_{initial}$]. There was some evidence for this for the longer depuration trajectories (green circles in Fig. 2), and a linear model indicated that the slope of the relationship between [$PFOS_{final}$] and [$PFOS_{initial}$] was significantly different from zero ($\beta = 0.76$, $t = 7.60$, P < < 0.001). A Wald's test for the alternative null hypothesis of $\beta = 1$ (i.e. a linear model and *F*-test to evaluate the null hypothesis that [$PFOS_{initial}$] = [$PFOS_{final}$]) indicated that the slope of this relationship was also significantly lower than the slope of parity ($F_{1,14} = 5.69$, $P = 0.032$), suggesting depuration was occurring during the time period examined (based on paired samples). There was much greater variability in PFHxS concentrations among individuals (Fig. 2), and the slope was not found to be significantly different from zero ($\beta = 0.51$, $t = 1.92$, $P = 0.075$) nor significantly different from one (Wald test, $F_{1,14} = 3.47$, $P = 0.083$).

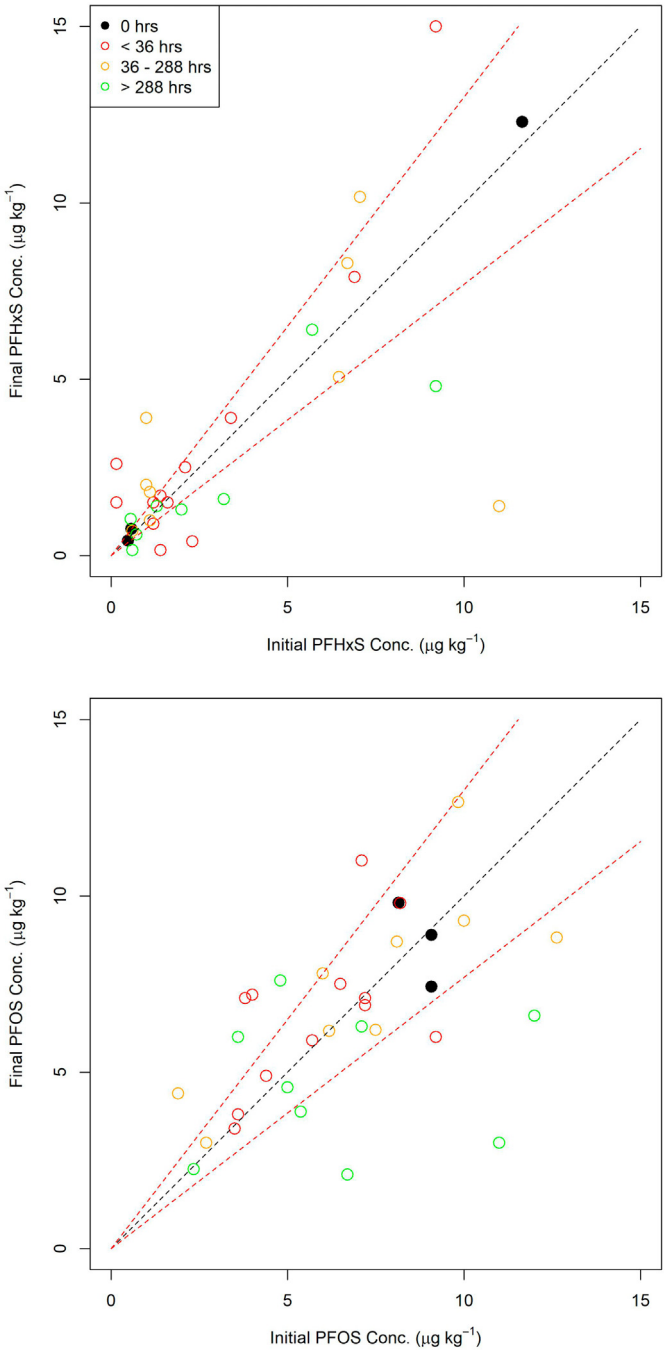

Fig. 2. Paired-individual PFHxS (upper panel) and PFOS (lower panel) concentrations in Giant Mud Crab claws, grouped for different depuration time points (coloured according to figure legend in the upper panel). The black dashed line represents the line of parity (where $[PFAS_{initial}] = [PFAS_{final}]$), and the dashed red lines represent the 30% relative percent difference (RPD) limits. Black filled circles represent paired samples for 0-h time points, reflecting the concentration in the right and left claw of an individual before any depuration had occurred.

When data from the current experiment were pooled with previous Giant Mud Crab depuration data (Taylor et al., 2017), log-linear modelling detected weak but significant negative relationships for PFOA ($F_{1,61} = 4.85$, P = 0.032, Fig. 3; 35% of data were > LOD) and PFOS ($F_{1,61} = 9.84$, $P = 0.002$, Fig. 3; 100% of data were > LOD) concentration and depuration time. There was no relationship between PFHxS concentration and time ($F_{1,61} = 2.22$, $P = 0.141$, Fig. 3; 81% of data were > LOD), however, when PFOS + PFHxS concentrations in each sample were summed there was a significant decrease ($F_{1,61} = 8.96$, $P = 0.004$, Fig. 3) in the combined concentration over time (a common practice in risk assessment in Australia is to sum PFOS and PFHxS for dietary assessment, following advice from FSANZ, 2017). Overall depuration half-lives were ~ 958 h, and ~ 896 h for PFOS and PFOS + PFHxS respectively (a half-life was not calculated for PFOA due to the high proportion of samples < LOD), and depuration of PFOS to below the current screening value (5.2 µg kg^{-1}) for PFOS in Australia (FSANZ, 2017) occurred after ~549 h (or ~ 23 days).

4. Discussion

This is the first study to provide some evidence for differences in concentrations of PFASs in the muscle tissue of different appendages in a marine crustacean. The fundamental premise of the experimental design being evaluated relied on initial PFAS concentrations in both chelipeds within an individual captured from the wild being relatively similar. Our results suggest that in some instances this may not have been the case (this is observed in Fig. 2, where concentrations exceed the upper red dashed line representing the 30% relative percent difference) – some *post*-depuration PFOS concentrations were almost twice the initial concentrations measured in the initial claw sample, despite depuration occurring before the final time point was sampled (indicating the differences between claws may have been even larger before depuration occurred). The differences in PFAS concentrations between chelipeds observed for some individuals was surprising, and added some complexity to the interpretation of outcomes from the experiment. However, there was still evidence for depuration of PFASs in Giant Mud Crab, occurring over extended time scales. While there are obvious implications for assessing exposure risk, both between-claw variation, and evidence of depuration in Giant Mud Crab, are novel findings that are of relevance to toxicokinetics of PFASs and assessment of crabs exposed through environmental emissions.

4.1. Depuration in Giant Mud Crab

Despite the confounding patterns described above, the data presented here provide reasonable evidence for depuration of PFASs in Giant Mud Crab. Cheliped PFOS concentrations were significantly lower following >36 h of depuration in paired samples, and statistically significant decreases in average [PFAS] over time were modelled. Depuration, however, was relatively slow compared with other aquatic animals in which depuration and elimination of PFASs have been studied (Martin et al., 2003; O'Connor et al., 2018; Taylor et al., 2017). As outlined in recent studies (O'Connor et al., 2018; Taylor et al., 2017), if depuration is rapid enough, holding live animals for a short period following capture may present an effective means of eliminating a majority of PFAS contaminants from edible tissues before the product is onsold. Unfortunately, while holding Giant Mud Crab for ~3 weeks may allow depuration to below the FSANZ (2017) trigger value prior to being sold at market, crabs would need to be held in water, as opposed to air, which is the medium most commonly used to hold this species between capture and sale of live animals. Further, holding periods for local markets do not normally exceed 2 days.

Depuration rates in Giant Mud Crab may be related to growth and moulting. Portunid crabs generally grow in a stepwise fashion, with incremental increases in size occurring through subsequent moult cycles, where the exoskeleton is shed and a larger shell is generated. In very large crabs, the intermoult period can be quite long. It is not possible to evaluate the influence of the moulting-growth cycle from the current study, since no crabs moulted during the experiment. If depuration is related to growth rate then depuration rates may be greater in smaller crabs, which have a higher growth rate and more regular moult frequency, which likely leads to a higher turnover of tissue, but comparatively long depuration periods in Giant Mud Crab mean that growth dilution could also occur in smaller crabs. Tissue turnover in the chelipeds

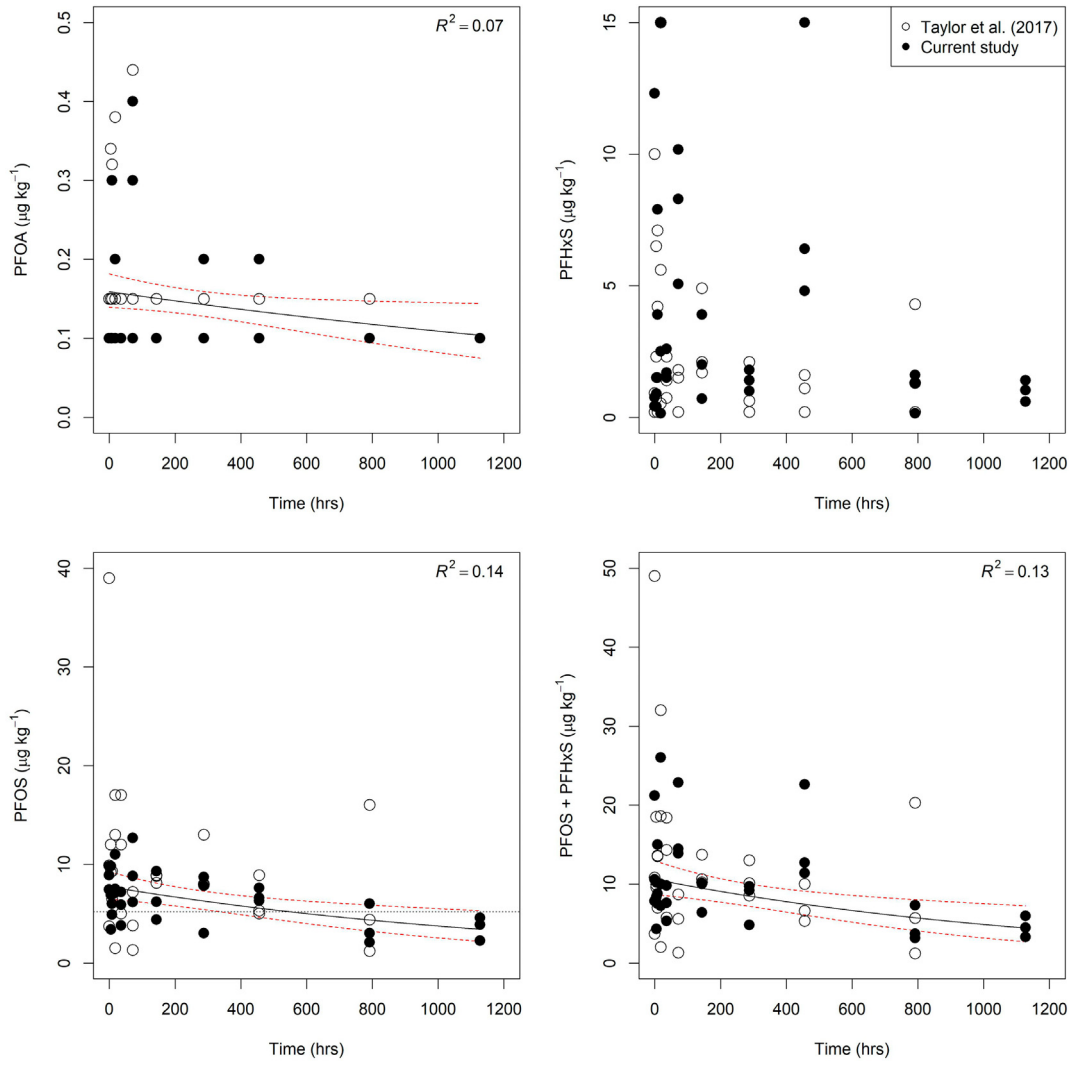

Fig. 3. Concentration data for PFOA (top left), PFHxS (top right), PFOS (bottom left) and PFOS + PFHxS (bottom right) in Giant Mud Crab, showing data from the depuration experiments reported in Taylor et al. (2017) and the current experiment ([$PFAS_{final}$] data; as indicated in figure legend). Fitted models (black lines) are shown where a significant slope parameter was estimated, and dashed red lines indicate 5% and 95% confidence intervals. The horizontal dotted line indicates the PFOS trigger value recommended by FSANZ (2017). Note that data are plotted on different *y*-axis scales in different figure panels.

may be comparatively slow (with the exception of regeneration following autotomy, discussed below) relative to the main body of the crab, as they are distal to the main body. Consequently, future depuration studies of portunid crabs may consider sampling body muscle tissue rather than chelipeds, although this could not follow the paired-sample design employed here. Laboratory experiments under controlled exposure conditions may provide useful information to better understand tissue distribution alongside accumulation and elimination.

4.2. Factors impacting variation, and technical considerations

One of the possible causes of the variation in concentration between claws is claw loss and regrowth prior to capture. Claw loss in portunid crabs under natural conditions is a relatively unstudied phenomenon. While portunid crabs are known to lose chelipeds through misadventure, interactions with aggressive conspecifics or predators (Mariappan et al., 2000) or interactions with fishing gear, the frequency at which limb loss occurs is generally unknown. The most comprehensive review on the subject has suggested injury was evident in 20–30% of decapods examined in wild populations (Juanes and Smith, 1995), and that chelipeds were the most commonly injured (or lost) body part in crabs. However, it was also noted that there was a high chance that studies might not detect recent autotomy, since regrowth of limbs in *Scylla* spp. has been shown to primarily occur within the first moult cycle following autotomy (see Rahman et al., 2020). Muscle tissue in newly regenerated claws may therefore reflect conditions encountered in recent weeks and months by crabs, which may lead to muscle tissue in newly regenerated limbs carrying different contaminant concentrations than older limbs. This could occur for a number of reasons, such as crabs moving between areas of higher and lower contamination when new limbs are forming, transient pulses of contaminant into the environment during regeneration, or differences in the biochemcial composition of the muscle through the moult cycle (e.g. Sugumar et al., 2013). While this is purely speculative, it does provide a potential explanation of the variation between claws described earlier, and could well contribute to the variability encountered in both Taylor et al. (2017) and the current study. Redistribution of PFASs around the crabs body following claw removal may also explain the patterns observed, however currently there is no evidence to suggest that this may occur in crabs, so also remains an area for further research.

There is a possibility that the loss of the crabs claw may have impacted the results of the experiment. For example, de-clawing of *Cancer pagurus* led to increased haemolymph glucose and lactate, although this was only detected up to 24 h following claw removal (Patterson et al., 2007). Gandy et al. (2016) also detected up to ~41% mortality following removal of a claw in Stone Crab *Menippe mercenaria*; there was no mortality of crabs during our experiment. Previous work has also noted potential animal welfare issues associated crab claw removal, particularly in the context of crab claw fisheries (Patterson et al., 2007). While there is considerable debate regarding the ability of invertebrates in general to sense pain (Sneddon, 2015), no studies have identified nociceptors or receptive fields in decapod crustaceans (Sneddon, 2018). Nonetheless, we took the extra step of cooling crabs before handling, to reduce stress and sensory perception. If future studies employ a similar experimental design, in addition to keeping sample numbers to the minimum required (as recommended by Patterson et al., 2007), they should consider employing induced autotomy which may have lesser short-term impacts on crab physiology. This should further reduce the chance that physiological artefacts may impact experimental outcomes.

Clearly, the differential tissue concentrations between crab claws reported here suggest that the distribution of PFASs both between tissues/organs, and within similar tissues across an animal's body plan, requires further study. For future studies examining contaminant concentrations in the edible tissues of crabs, compositing body muscle tissue with claw muscle tissue may provide more robust estimates of overall contaminant load, although this strategy could not be applied in the experimental design employed here. It is also important to note cheliped loss can lead to physiological and behavioural changes in crustaceans that may impact growth (Juanes and Smith, 1995), and the additional "regenerative load" (see Mariappan et al., 2000) following claw removal at the beginning of the experiment may have affected the rate of depuration. The similar patterns observed in data reported in both the current study and Taylor et al. (2017) suggest that if there was any impact of this, it is likely to be low.

4.3. Conclusions and future work

PFASs are emerging contaminants and there is still uncertainty surrounding their toxicokinetics in aquatic species; the current study adds several elements to the existing body of knowledge to support future work. Firstly, while the novel experimental design and use of paired initial-final depuration concentrations were somewhat confounded due to potential cheliped loss prior to collection, such a design may still prove useful where crabs are exposed under controlled conditions (such as conventional dose-depuration experiments), but additional factors (as noted above) should be considered. Secondly, depuration of PFASs in Giant Mud Crab does occur, albeit at lower rates than observed for other crustaceans (e.g. School Prawn). Finally, future studies quantifying contaminant concentrations in the edible tissues of exploited crab species should consider compositing all edible tissue within the animal to achieve a more accurate measure of contamination within the organism. This is less relevant when larger numbers of individuals are sampled, as a greater sample size will provide a more representative cross section of concentrations across normal and recently-regenerated claws. Assessing within-tissue partitioning of PFASs across an animal's body plan represents an important area for future research for crabs and other species.

CRediT authorship contribution statement

Matthew D. Taylor: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Project administration. **Daniel D. Johnson:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Sandra Nilsson:** Investigation, Writing - original draft, Writing - review & editing. **Chun-Yin Lin:** Investigation, Writing - original draft, Writing - review & editing. **Jennifer Braeunig:** Investigation, Writing - original draft, Writing - review & editing. **Jochen Mueller:** Supervision, Resources, Writing - original draft, Writing - review & editing. **Karl C. Bowles:** Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Short Communication

First reports of per- and poly-fluoroalkyl substances (PFASs) in Australian native and introduced freshwater fish and crustaceans

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Abstract. Per- and poly-fluoroalkyl substances (PFASs) are persistent organic pollutants that have been extensively used in commercial and industrial applications, such as aqueous film-forming foam (AFFF) formulations. Widespread use of AFFFs has led to an increasing number of reports documenting PFAS contamination around civilian and military airports. However, research on the presence and distribution of PFASs in Australia is lacking. This study presents the first report of PFASs in Australian native and introduced freshwater species, sampled from a watercourse adjacent to the regional airport and colocated fire training ground near Tamworth, New South Wales, Australia. Perfluorooctane sulfonate was the most abundant PFAS compound in biota samples from this area, and both introduced common carp *Cyprinus carpio* and native Murray cod *Maccullochella peelii* had average concentrations higher than the Australian trigger value of 5.2 μg kg^{-1}. Common yabby *Cherax destructor* and golden perch *Macquaria ambigua* carried low concentrations, and common yabby also had low concentrations of perfluorohexane sulfonate. Differences in foraging habits provided some potential explanations of the differences observed among species. There is a clear and pressing need to better understand potential toxicological and reproductive effects of PFASs on Australian freshwater species.

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Introduction

Per- and poly-fluoroalkyl substances (PFASs) are emerging contaminants that are of increasing interest to toxicologists and ecotoxicologists alike (Houde *et al.* 2011). This group of compounds is highly resistant to environmental degradation, and in the 2000s some PFASs were listed under the Stockholm Convention on Persistent Organic Pollutants (Wang *et al.* 2009; Blum *et al.* 2015). Historically, these substances have been extensively used in a diverse range of commercial, manufacturing and industrial applications (Paul *et al.* 2009). One such application was in aqueous film-forming foam (AFFF) formulations, which were developed as an effective means to combat hydrocarbon-fuel fires (Filipovic *et al.* 2015). The dominant PFAS compound in these formulations was often perfluorooctane sulfonate (PFOS; Place and Field 2012) and, as a consequence of the widespread use of AFFFs over previous decades, there is an increasing number of reports documenting PFOS (as well as other PFASs) contamination in soil, sediment, groundwater and surface water in the areas surrounding civilian and military airports across the world.

Houde *et al.* (2011) highlighted that research on the presence and distribution of perfluorinated compounds in Australia was lacking. In the period since, several studies that characterise PFASs in Australian ecosystems have emerged (e.g. Baduel *et al.* 2014; Thompson *et al.* 2011*a*, 2011*b*), and in recent years contamination surrounding airports and military bases has been of increasing concern (URS Australia 2015; Taylor and Johnson 2016; Bräunig *et al.* 2017). In particular, consumption of fish and aquatic crustaceans captured in areas adjacent to these facilities has been identified as a potential exposure pathway for subsets of the population who may regularly catch and eat these products from contaminated locations (AECOM 2016). However, there are very few published studies of PFAS concentrations in the edible tissues (e.g. fish fillets) of exploited freshwater or marine fish and crustaceans with which to assess risk (Ye *et al.* 2008). Although recent investigations published in the scientific literature have provided some data on PFAS concentrations in the edible tissues of exploited aquatic biota in Australia (Thompson *et al.* 2011*b*; Taylor and Johnson 2016; Taylor *et al.* 2017), there are no such reports yet published on exploited freshwater species. This study presents the first report in the literature describing PFASs present in the edible tissues of Australian native and introduced freshwater species.

Materials and methods

Study area and contamination

Late in 2016, contamination of groundwater and surface water was identified in the vicinity of Tamworth Regional Airport (Fig. 1), in the New England region of New South Wales (NSW), Australia. Tamworth is a major regional centre in north-western NSW and is situated on the Peel River, a waterway in the Namoi catchment of the Murray–Darling Basin. Tamworth Regional Airport contains several firefighting training and storage areas that have been subject to past use of AFFF products. Surface and groundwater sampling indicated that a PFAS contamination plume was present, travelling in a north-eastern direction from the airport, towards the Wallamore Anabranch of the Peel River and the Peel River itself (Fig. 1). Limited surface and groundwater sampling revealed concentrations that exceeded trigger values for drinking water at the time (specified by enHealth 2016), but there was a general cline of decreasing PFAS concentrations in groundwater with an increasing distance from the airport.

Sampling

Animal handling was permitted under NSW Department of Primary Industries (DPI) Animal Care and Ethics Permit 14-11, and collection of animals was performed under NSW DPI Section 37 Permit number P01/0059.

Sampling was targeted at four species that occur in the area that are relevant to local recreational fishers (note that commercial harvest of fish from inland waterways is not permitted in NSW). Species included the native species common yabby *Cherax destructor*, golden perch *Macquaria ambigua* and Murray cod *Maccullochella peelii* and the introduced species common carp *Cyprinus carpio*. Sampling was undertaken in waterbodies surrounding Tamworth Airport, including Boltons Creek (which was targeted for collection of common yabby) and nearby regions of the Peel River that could be navigated by boat (which was targeted for the large-bodied fish species golden perch, Murray cod and common carp; Fig. 1). The Wallamore Anabranch, although likely to represent a location where the plume interacts with the waters of the river, is unlikely to regularly support populations of large-bodied fish species such as those targeted in the present study. In addition, this region could not be readily accessed, so was not included in the sampling effort. Other native species, such as freshwater catfish *Tandanus tandanus*, also occur in the area but are not targeted by anglers so were not sampled for testing.

Common yabby were sampled using opera house traps baited with food scraps. Traps were deployed along Bolton's Creek between the airport and the Peel River on 15–17 February 2017, and sampling yielded 42 individuals. Trapping was also conducted in the Peel River downstream of Bolton's Creek, but no common yabby were captured. Fish samples were collected using a Smith-Root Model 2.5-kVA generator-powered pulsator electrofisher (Vancouver, WA, USA) mounted on a small 3.7-m outboard-powered punt, on 28 February–1 March 2017. Sampling was initially conducted at the closest location to the Wallamore Anabrach that could be safely navigated by boat at the time of sampling (Site 1), and then progressively moved downstream from that point (Site 2; Fig. 1), with all available and accessible habitat types sampled using intermittent electrofishing. Five

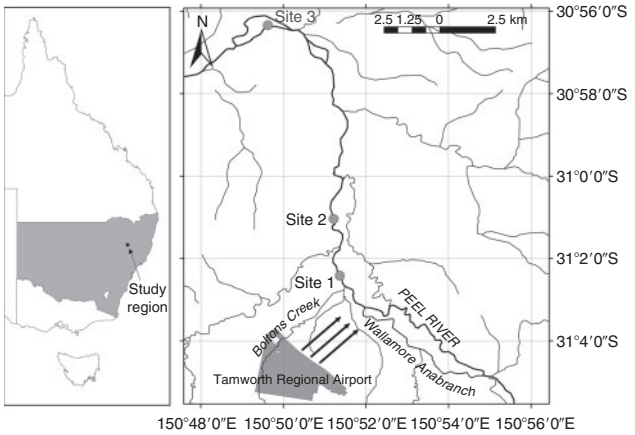

Fig. 1. Map of the study area showing the location of the study region in eastern Australia (left panel, New South Wales shaded grey), Tamworth Regional Airport (grey polygon in right panel), and the locations sampled in this study including Sites 1–3 and Boltons Creek (right panel). The Peel River and Wallamore Anabranch are labelled, and minor watercourses in the area are also indicated. Bold arrows indicate the general direction of the contamination plume from the airport.

Murray cod and two golden perch were also captured at a third site, located further downriver (Site 3; Fig. 1). Up to 2500 s of electrofishing effort was conducted at each site. All samples were killed in an ice slurry upon capture and frozen until processing and compositing were undertaken in the laboratory.

Sample processing

All samples were analysed as composites, and thus reflected average concentrations of several individuals across the spatial area sampled (see Manning *et al.* 2017). Four composites were analysed for each species, and each contained between 5 and 10 animals per composite (Table 1). Animals were sequentially assigned to composite samples in a random fashion, until equal numbers of animals were assigned to the composite set (up to a maximum of 10 individuals per composite set). Animals were then prepared for analysis, by initially weighing and measuring total length (or carapace length for crustaceans). A fillet from one side of the fish's body, or the whole of the abdominal tissue from common yabby, was extracted and added to the composite. Composite samples were homogenised using a knife mill or hand-held homogeniser and stored in Falcon polypropylene tubes (Corning, NY, USA) at −20°C until extraction and analysis.

Chemical analysis

Samples were analysed using solid phase extraction and liquid chromatography–tandem mass spectrometry (LC/MS/MS) based upon the USEPA-821-R-11-007 method (Shoemaker *et al.* 2008). Samples were analysed across two laboratories, with common carp, golden perch and Murray cod analysed at NSW Office of Environment and Heritage Environmental Forensics Laboratory (Lidcombe, NSW, Australia; hereafter referred to as OEH) and common yabby analysed at the Australian Government National Measurement Institute (North Ryde, NSW, Australia; hereafter referred to as NMI). Samples were analysed for an expanded suite of 10 (OEH analyses) or 13 (NMI analyses) PFAS compounds.

Table 1. Concentrations of per- and poly-fluoroalkyl substances in samples collected from waterways surrounding Tamworth Regional Airport

Number, number of individuals in composites, with the number of composites analysed in parentheses; Average, arithmetic mean (only calculated where Frequency was ≥50%; samples where concentrations were less than the limit of reporting (LOR) were handled as described in the Results); Frequency, frequency-of-occurrence in composites; Recovery, percentage surrogate recovery. 8:2 FTS, C_2H_4-perfluorodecane sulfonate; 6:2 FTS, C_2H_4-perfluorooctane sulfonate; PFBS, perfluorobutane sulfonate; PFDA, perfluorodecanoic acid; PFHpA, perfluoroheptanoic acid; PFHxS, perfluorohexane sulfonate; PFHxA, perfluorohexanoic acid; PFNA, perfluorononanoic acid; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; PFPeA, perfluoropentanoic acid; PFUdA, perfluoroundecanoic acid; PFDoA , perfluorododecanoic acid; NA, compound not analysed for this species. No internal standards were available for PFUdA

Common name	Species name	Number		Analyte (μg kg^{-1} wet weight)												
				8:2 FTS	6:2 FTS	PFBS	PFDA	PFHpA	PFHxS	PFHxA	PFNA	PFOS	PFOA	PFPeA	PFUdA	PFDoA
Murray cod	*Maccullochella peelii*	5 (4)	Average	–	–	–	–	–	–	–	–	5.85	–	NA	NA	NA
			LOR (μg kg^{-1})	3	2	0.3	0.4	0.3–0.4	0.5–0.7	0.3	0.3	0.3	0.3	NA	NA	NA
			Frequency (%)	0	0	0	0	0	0	0	0	100	0	NA	NA	NA
			Recovery (%)	234–299	329–361	98–112	68–84	80–95	98–112	80–95	87–105	99–107	64–78	NA	NA	NA
Golden perch	*Macquaria ambigua*	5 (4)	Average	–	–	–	–	–	–	–	–	2.13	–	NA	NA	NA
			LOR (μg kg^{-1})	3	2	0.3	0.4	0.4	0.5–0.6	0.3	0.3	0.3	0.3	NA	NA	NA
			Frequency (%)	0	0	0	0	0	0	0	0	100	0	NA	NA	NA
			Recovery (%)	154–302	307–387	85–107	51–75	84–93	85–107	84–93	87–95	43–86	94–103	NA	NA	NA
Common carp	*Cyprinus carpio*	5 (4)	Average	–	–	–	0.50	–	–	–	–	12.23	–	NA	NA	NA
			LOR (μg kg^{-1})	3	2	0.3	0.4	0.4	0.6	0.3	0.3	0.3	0.3	NA	NA	NA
			Frequency (%)	0	0	0	100	0	0	0	0	100	0	NA	NA	NA
			Recovery (%)	184–340	258–382	75–110	63–76	69–98	75–110	69–98	72–100	39–92	80–110	NA	NA	NA
Common yabby	*Cherax destructor*	10 (4)	Average	–	–	–	–	–	0.87	–	–	1.40	–	–	–	–
			LOR (μg kg^{-1})	0.5	0.5	0.5	0.5	0.5	0.5	0.3	0.5	0.5	0.3	0.3	0.5	0.5
			Frequency (%)	0	0	0	0	0	50	0	0	75	0	0	0	0
			Recovery (%)	218–261	105–132	60–75	63–76	79–89	60–75	79–89	133–166	83–95	95–109	273–343	–	318–401

Samples had known amounts of ^{13}C isotopically labelled analogues of the target analytes (Wellington Laboratories, Guelph, ON, Canada) added to act as internal standards for identification and quantification of the target analytes. PFAS compounds were extracted through saponification by tumbling with alkaline methanol. The resultant extract was centrifuged (10 min, 20°C, 200 rpm) to remove solids and the concentrated supernatant was then purified by solid phase extraction. Additional ^{13}C isotopically labelled standards were then added to the sample to serve as an injection standard to determine internal standard recoveries. Samples were analysed on an Agilent Technologies (Palo Alto, CA, USA) 1100 HPLC, AB Sciex (Concord, ON, Canada) 4000 Qtrap MS/MS HPLC–triple quadrupole mass spectrometer–computerised data system (NMI analyses) or a Shimadzu (Kyoto, Japan) 8050 ultrahigh pressure LC/MS/MS system (OEH analyses). Multiple reaction monitoring (MRM) of two characteristic transitions was performed and identification confirmed when target ions were detected in both the MRMs within established retention time windows. The limits of reporting (LORs) were determined for each compound in each sample based on noise (5× noise) and laboratory blank levels (3× blank levels), and varied between samples and machines as a result of instrument performance and the level of sample contamination. Quantification of analytes was based on the use of the labelled surrogates and linear calibration standards and was reported as the total of all isomers. Analyte concentrations were corrected for surrogate recoveries to overcome matrix suppression or enhancement, and results are reported on a wet weight basis. Validation of the method included analysis of a fish standard reference material provided by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA; SRM 1946), which had an assigned reference value for PFOS, and resulted in measurements within 10% of the assigned value. Quality assurance and quality control monitoring indicated that the relative percentage difference of repeated measurements was usually <20% and always <30%.

Data analysis

Summary statistics are reported for all PFASs detected in samples, and a single-factor analysis of variance (ANOVA) and Tukey's *post hoc* test were used to compare concentrations of dominant PFAS (defined as PFAS compounds present in >75% of samples) among species. All analyses were undertaken in R, ver. 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria, see http://www.R-project.org/, accessed 16 April 2017).

Results

PFAS compounds had varying LORs and surrogate recoveries (Table 1). High surrogate recoveries were obtained for fluorotelomer compounds, perfluorononanoic acid, perfluoropentanoic acid and perfluorododecanoic acid (Table 1); however, results for these samples were all below the LOR, so this is of minor concern. PFOS was the dominant PFAS compound detected, and was present in all composite samples except one common yabby composite. ANOVA indicated that concentrations were significantly different among species ($\log_{10}$-transformed dependent variable; $F_{3,12} = 14.44$, $P < 0.001$; note that in this analysis the PFOS concentration was set to 0.5 × LOR for the single common yabby composite that had a PFOS concentration less than the LOR; Table 1). *Post hoc* analyses revealed that PFOS concentrations in common yabby were significantly lower than in Murray cod and common carp, and PFOS concentrations in golden perch were significantly lower than in common carp (Fig. 2). There was a minor level of diversity in the PFAS compounds detected among species, with PFDA detected at low concentrations in common carp, and perfluorohexane sulfonate (PFHxS) detected at low concentrations in common yabby (Table 1). No other PFAS compounds (including perfluorooctanoic acid) were detected at concentrations >LOR in any species. Average PFOS concentrations in both common carp and Murray cod were greater than the current Food Standards Australia New Zealand trigger value of 5.2 μg kg^{-1} (Food Standards Australia New Zealand 2017; Fig. 2), and although not significantly different, PFOS concentrations in common carp appeared to be more than twice those in Murray cod (Table 1; Fig. 2). PFOS concentrations in the golden perch and common yabby were below the trigger value (even when common yabby PFHxS was added to PFOS concentrations, as suggested by Food Standards Australia New Zealand 2017).

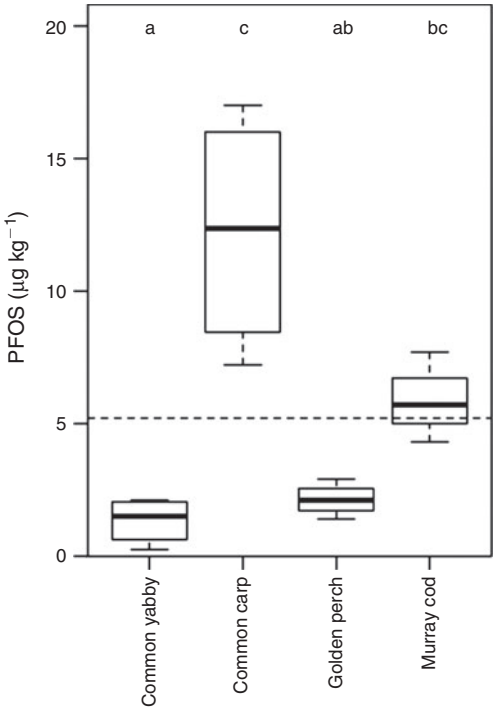

Fig. 2. Box and whisker plots showing the median (horizontal line), range (whiskers) and interquartile range (boxes) perfluorooctane sulfonate (PFOS) concentrations across the species analysed. Different letters denote significant differences in PFOS concentrations between species. The dashed horizontal line indicates the trigger value suggested by Food Standards Australia New Zealand (2017).

Discussion

Because this is the first report of contamination of Australian native freshwater biota with PFASs, there are no available data to compare concentrations with native species captured from

other locations, be they contaminated or uncontaminated. Conversely, PFAS contamination in common carp has been studied extensively in the literature, and, although this is an introduced species in Australia, this has been suggested as a useful sentinel species for comparison of baseline contamination because of its worldwide distribution (Ye *et al.* 2008). Although PFOS concentrations in common carp were the highest detected in the current sample set, they are relatively low compared with studies in other freshwater systems outside Australia. For example, in an area of the Upper Mississippi River (MN, USA) adjacent to a 3M manufacturing facility, PFOS concentrations in common carp were as high as 90 μg kg^{-1} (Ye *et al.* 2008; that study also detected low concentrations of PFDA in the muscle tissue of common carp). In common carp sampled from Baiyangdian Lake, China, dry weight PFOS concentrations were as high as 69.9 μg kg^{-1} (Zhou *et al.* 2012). However, both these studies analysed individual fish samples as opposed to composite samples, which may explain the differences with the maximum value reported in the present study (the maximum PFOS concentration in common carp from the Peel River was 17 μg kg^{-1}).

Broad interspecific differences in PFOS concentrations were observed, and this may be explained, in part, by the differing foraging habits displayed by the species examined. Common carp generally feed by sieving of the sediment to extract detrital and other benthic fauna, and this is a feeding behaviour that is relatively uncommon in Australian native species (Koehn 2004). Both Murray cod and golden perch are predatory fish (as opposed to detritivorous), feeding on fish and crustaceans, and Murray cod may be considered to be an apex predator in the Murray–Darling Basin (Ebner 2006). Dietary analysis has shown that Murray cod is much more piscivorous (fish represent 90% of prey by volume) than golden perch (fish represent 16% of prey by volume, with the remaining 84% being decapod crustaceans, mainly *Macrobrachium*), and common carp are by far the most common teleost prey of Murray cod (Ebner 2006). The higher PFOS concentrations in common carp likely arise from the species' ingestion of sediment during feeding (long-chain perfluorosulfonates are readily sorbed to sediment particles; Higgins and Luthy 2007), and this exposure pathway is supported by the conclusions of previous research on freshwater cyprinids (e.g. Babut *et al.* 2017). The dietary relationships for Murray cod point to the possibility that exposure may occur through consumption of contaminated common carp, and this may explain the moderate PFOS concentrations in this species relative to golden perch and common yabby. There is limited information on the diet and feeding habits of common yabby. Laboratory studies suggest zooplankton are important in the species diet (Meakin *et al.* 2008), although up to 64% of the diet may be supported from allochthonous (terrestrial) sources (Reid *et al.* 2008), which may explain the low levels of contamination in this species.

Previous investigations of PFAS contamination in Australia have largely focused on assessing health risks through consumption of estuary-caught seafood, particularly for professional fishers and their families who may source seafood from a relatively localised area (e.g. Taylor and Johnson 2016; Taylor *et al.* 2017). Although there is no commercial harvest of fish or crustaceans from inland waterways in NSW, these waterways support significant recreational fisheries. Hölzer *et al.* (2011) showed that recreational harvest of fish from a PFAS-contaminated lake (Lake Möhne, Germany) had the potential to contribute to blood plasma PFOS concentrations for anglers who regularly consumed fish from this location; median concentrations for most fish species in that lake were >30 μg kg^{-1} (with maximum concentrations as high as 150 μg kg^{-1}). Although PFOS concentrations for some species in the present study exceeded the Australian Government trigger value, Murray cod are subject to seasonal closures (from September to December each year), and common carp are not usually targeted for consumption by anglers in NSW. Common yabby, Murray cod and golden perch are subject to bag limits and possession limits, and the latter two species are also subject to size limits (including a 55–75-cm slot limit for Murray cod). Many freshwater anglers targeting iconic native species such as Murray cod practice catch-and-release rather than consuming their catch (see West *et al.* 2016). All these factors may act to limit human health risks associated with exposure through fish caught from this area of the Peel River, but anecdotal information suggests that some members of the local community may consume common carp (C. Watson, pers. comm.). Although it is unlikely that anglers would harvest enough fish from the affected area of the Peel River to reach the tolerable daily intake for PFOS every day of the year, these data have been used to provide precautionary advice on fish consumption for relevant members of the local community.

Recent documents released by the Australian Government Department of Defence have identified a large number of sites across Australia that have past use of AFFFs, many of which are adjacent to inland rivers and streams (Department of Defence 2017). The preliminary results presented herein will assist in the assessment and management of initial responses to new contamination events, as well as providing a basis for sampling programs in support of the assessment of any potential human health risks. However, although these preliminary data are informative, more work is required to improve our knowledge of PFAS contamination in Australian freshwater species. Important areas to target include further characterisation of exposure pathways, and potentially spatially oriented sampling to identify concentration patterns in biota along major river systems subject to contamination (particularly in migratory species). In addition, studies from elsewhere indicate toxicity of PFASs in fish and crustaceans, and effects on reproduction (e.g. Ji *et al.* 2008; Han and Fang 2010; Wang *et al.* 2011). The Canadian Government provides a concentration benchmark (based on toxicological effects) for freshwater fish health of 8300 μg kg^{-1} muscle tissue (Environment and Climate Change Canada 2017), but suggests a much lower environmental quality guideline for wildlife species (such as fish) when they are preyed upon by mammals and birds (4.6 and 8.2 μg kg^{-1} respectively). The effects of these compounds on the health and reproduction of Australian native species are completely unknown, but given these thresholds, the concentrations measured in the Peel River may have implications for avian or mammalian (such as rakali *Hydromys chrysogaster*) predators that consume fish. This represents an important area for future research for these species and their predators, especially those of conservation concern.

Conflicts of interest

The author declares that he has no conflicts of interest.

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Baseline

Preliminary investigation of perfluoroalkyl substances in exploited fishes of two contaminated estuaries

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ABSTRACT

Perfluoroalkyl substances (PFASs) are being increasingly detected in a range of aquatic and terrestrial ecosystems, often resulting from the use of legacy fire-fighting foams. This study conducted an initial investigation of the concentrations of PFASs in the commercially and recreationally exploited species Dusky Flathead, Mud Crab, School Prawn, Sea Mullet, Yellowfin Bream, Eastern King Prawn and Sand Whiting, across two contaminated estuaries. All samples contained perfluoro-*n*-octane sulfonate (PFOS) except four Yellowfin Bream samples (two from each estuary). Perfluoro-*n*-octanoic acid (PFOA) was detected only in School Prawn samples from Fullerton Cove, while perfluoro-*n*-hexane sulfonate (PFHxS) was detected in prawn muscle and in fish liver samples from both estuaries. This study presents one of the first surveys of PFAS in a range of edible saltwater fish and crustaceans in Australia, and these baseline levels of contamination will prove useful for informing future surveys of these emerging contaminants.

Perfluorinated chemicals or perfluoroalkyl substances (PFASs) are emerging contaminants of international concern (Murray et al., 2010), and their presence is being increasingly detected in a range of aquatic and terrestrial ecosystems. While some recent reviews suggest that Australia is unlikely to be affected by transport of the contaminants from northern hemisphere sources, local sources for such pollutants still exist (Thompson et al., 2011b). Several unpublished preliminary investigations in Australia have identified these substances in soil, water, and biota, and much of this contamination has arisen from the use of legacy fire-fighting foams. The historic use of these substances, particularly around airports and other fire-fighting training facilities, mean that such facilities represent a potentially significant local source of this persistent pollutant.

Knowledge of baseline perfluorinated contaminant levels in Australia is lacking, especially in commercial fishes and crustaceans. Some exploratory work has detected PFASs in and around major Australian cities (Gallen et al., 2014; Thompson et al., 2011a,b), but to our knowledge there are few published local studies that have detected these contaminants in marine biota (Baduel et al., 2014; Thompson et al., 2011b). Identifying pollutant sources and the passage of pollutants through ecological systems is essential to understanding potential exposure pathways, and managing any ecological and health effects.

A PFAS contamination issue has recently come to light surrounding a regional airport at Williamtown, New South Wales, Australia. This facility is both a domestic airport and a major air force base, and used legacy fire-fighting foams containing PFASs for several decades into the early 2000s. Initial investigations revealed PFAS contamination within the airport itself and subsequent work identified that the contaminant was present in the network of drains surrounding the facility (URS Australia Pty Ltd, 2015). Williamtown and the surrounding area is bordered by two large estuaries, the Hunter River (to the south) and Port Stephens (to the north), and surface and ground water from the airport drain into both estuaries through Tilligerry Creek (Port Stephens) and Fullerton Cove (Hunter River, Fig. 1). This study conducted an initial investigation of the concentrations of PFASs in a number of commercially and recreationally important species of fish and crustaceans in both estuaries. The Hunter River and Port Stephens are two large adjacent estuaries on the mid-north coast of New South Wales, Australia (Fig. 1). The Hunter River is a mature, wave-dominated barrier estuary, with abundant mangrove and saltmarsh habitat, whereas Port Stephens is a tide-dominated drowned valley estuary, containing extensive mangrove, saltmarsh and seagrass habitats. Port Stephens has a smaller catchment (4950 km^2) and larger waterway area (126 km^2), whereas Hunter River has a much larger catchment (22,000 km^2) and smaller waterway area (30 km^2) (Roy et al., 2001). The catchments of both estuaries are largely agricultural and forested; however, the lower reaches of the Hunter River have significant industrial areas. Both estuaries support substantial commercial fisheries, and the two point-sources of

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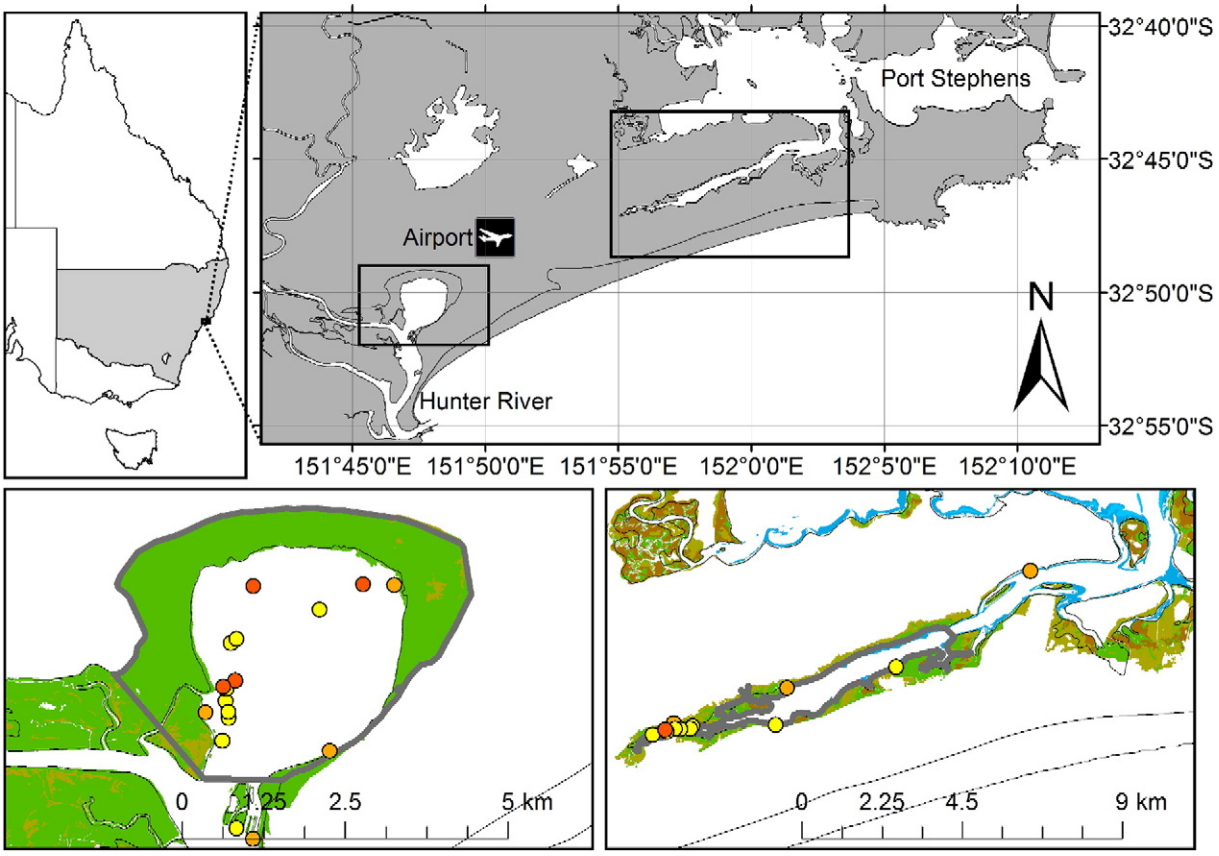

Fig. 1. Map of study area indicating the location of Port Stephens and Hunter River, and sampling locations for crabs (yellow circles), prawns (red circles) and fish (orange circles) in Fullerton Cove (lower left panel) and Tilligerry Creek (lower right panel). The dark grey outline in the lower panels indicates the estuarine contamination zone and fishing closure areas. Brown shading indicates saltmarsh habitat, green shading indicates mangrove, and blue shading indicates seagrass. The airport which is the source of the contamination is shown relative to the two estuaries in the upper right panel.

contamination are adjacent to some of the most heavily fished areas in these estuaries, particularly for crustaceans.

Samples were collected in Fullerton Cove and Tilligerry Creek between 10 September 2015 and 1 October 2015. Commercially sized animals were captured from various locations close to the point-source of contamination within each estuary (Fig. 1), using both contracted commercial fishing vessels (with government staff on board) or fishery-independent trapping and trawling. Fish were targeted nocturnally using ≈3 in. mesh nets, whereas prawns were captured using an otter trawl (6 m mouth, 1 in. mesh) and crabs were targeted using baited traps (traps were baited using fish harvested from offshore areas).

Following capture, animals were placed on ice and dissected in the fisheries research laboratory at Port Stephens Fisheries Institute. Whole animals were weighed and total length (TL) or carapace length (CL) was measured. For fish, a ≈ 30 g portion of fish muscle was dissected for analysis from each individual and the skin was removed. Some fish livers were also removed for analysis. For crabs, 30 g of meat was dissected from each individual from the chelipeds and the abdominal segment. For prawns, 9–40 animals captured from the same tow were shelled (but not deveined) and composited to yield a mass of ≈30 g of prawn meat. Following preparation, samples were kept refrigerated and transported directly to National Measurement Institute (NMI) for analysis.

Analysis was conducted using isotopic dilution, based on reference method USEPA 537. Samples were prepared for analysis by homogenisation using a knife mill or hand-held homogeniser and stored in 50 mL Falcon® polypropylene tubes (Corning) at −20 °C. Samples had known amounts of ^{13}C isotopically labelled analogues of the target analytes (Wellington Laboratories, Canada) added and were extracted with saponification by tumbling with alkaline Methanol. The extract was centrifuged, and the supernatant concentrated then purified by solid phase extraction. A ^{13}C isotopically labelled standard was added to the sample to serve as a recovery standard. Qualitative/quantitative analysis for PFASs was performed using an Agilent 1100 HPLC, ABSciex 4000 Qtrap MS/MS high performance liquid chromatograph/triple quadrupole mass spectrometer/computerised data system (LC/MS/MS). Multiple reaction monitoring (MRM) of two characteristic transitions was performed, with identification confirmed when target ions

Table 1
Relative Percent Difference (RPD) from duplicate analyses (of Tilligerry Creek samples), to evaluate reproducibility of analyte concentrations. Only perfluoro-*n*-hexane sulfonate (PFHxS) and perfluoro-*n*-octane sulfonate (PFOS) were detected in these samples.

Species name	Common name	Analysis 1		Analysis 2		Relative Percent Difference	
		PFHxS (mg kg^{-1})	PFOS (mg kg^{-1})	PFHxS (mg kg^{-1})	PFOS (mg kg^{-1})	PFHxS (%)	PFOS (%)
Sillago ciliata	Sand Whiting	<0.00050	0.00075	<0.00050	0.00087	–	14
Acanthopagrus australis	Yellowfin Bream	<0.00050	0.00047	<0.00050	0.00036	–	23
Penaeus plebejus	Eastern King Prawn	0.00160	0.03600	0.00140	0.03500	3	3

Table 2
Representative limit of reporting (LOR) and % recovery for perfluoroalkyl substances measured in aquatic samples collected from Fullerton Cove and Tilligerry Creek. Crab samples were analysed for PFOA, PFOS, and branched-chain isomers only. LORs occasionally varied due to matrix interference issues, but were standardised on a batch basis. No internal standards were available for PFHpA and PFBS.

Sample		Analyte[a]											
		PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFBS	PFHxS	PFOS	6:2 FTS	8:2 FTS
Crab	LOR (mg kg^{-1})			0.00030							0.00030	0.00050	0.00050
	% recovery			82 (70–93)							87 (73–100)	117 (98–134)	104 (79–122)
Fish muscle	LOR (mg kg^{-1})	0.00050	0.00050	0.00030	0.00010	0.00050	0.00050	0.00050	0.00010	0.00050	0.00030	0.00050	0.00050
	% recovery	68 (15–122)		94 (65–146)	61 (28–115)	60 (30–111)	67 (27–114)	93 (28–152)		66 (71–155)	82 (49–130)	79 (40–144)	77 (47–141)
Prawn	LOR (mg kg^{-1})	0.00500	0.00095	0.00032	0.00019	0.00050	0.00050	0.00050	0.00020	0.00050	0.00032	0.00050	0.00050
	% recovery	38 (29–55)		72 (56–99)	72 (53–107)	68 (55–105)	79 (63–110)	89 (69–115)		66 (57–89)	78 (59–110)	82 (69–121)	97 (72–133)
Fish liver	LOR (mg kg^{-1})	0.00500	0.00425	0.00047	0.00012	0.00050	0.00050	0.00050	0.00020	0.00050	0.00047	0.00050	0.00050
	% recovery	44 (9–54)		87 (79–93)	80 (69–88)	88 (80–99)	88 (73–109)	85 (67–113)		71 (28–95)	100 (86–118)	182 (69–261)	165 (139–220)

[a] Acronyms refer to perfluoro-*n*-hexanoic acid, (PFHxA), perfluoro-*n*-heptanoic acid (PFHpA), perfluoro-*n*-octanoic acid (PFOA), perfluoro-*n*-nonanoic acid (PFNA), perfluoro-*n*-decanoic acid (PFDA), perfluoro-*n*-undecanoic acid (PFUdA), perfluoro-*n*-dodecanoic acid (PFDoA), perfluoro-*n*-butane sulfonate (PFBS), perfluoro-*n*-hexane sulfonate (PFHxS), perfluoro-*n*-octane sulfonate (PFOS), C_2H_4-perfluoro-octane sulfonate (6:2 FTS), C_2H_4-perfluoro-decane sulfonate (8:2 FTS).

were detected in both the monitored MRMs within established retention time windows. The limits of reporting (LORs) were determined for each compound in each sample based on noise and laboratory blank levels, and varied between samples as a result of instrument performance and the level of sample contamination. Quantification of linear and branched isomers of all analytes was based on the use of the ^{13}C labelled surrogates and linear calibration standards, and the total of all isomers reported. Analyte concentrations were corrected for recovery of ^{13}C isotopically labelled surrogates to overcome matrix suppression/enhancement, and results were reported on a wet weight

Table 3
Concentration (and Standard Deviation, SD) of PFASs in commercially sized fish, prawns and crabs captured in Fullerton Cove and Tilligerry Creek. All samples were muscle tissue, except where liver tissue is indicated. Where means were greater than the limit of reporting (LOR), frequency of detection (FOD) in samples were all 100% with the exception of PFOA in Fullerton Cove School Prawn (62% FOD), PFOS in Fullerton Cove Yellowfin Bream (75% FOD), and PFOS in Tilligerry Creek Yellowfin Bream (71% FOD).

Estuary	Common name	Species name (n, size range)[#]		PFHxA (mg kg^{-1})	PFHpA (mg kg^{-1})	PFOA (mg kg^{-1})	PFNA (mg kg^{-1})	PFDA (mg kg^{-1})	PFUdA (mg kg^{-1})	PFDoA (mg kg^{-1})	PFBS (mg kg^{-1})	PFHxS (mg kg^{-1})	PFOS (mg kg^{-1})	6:2FTS (mg kg^{-1})	8:2FTS (mg kg^{-1})
Fullerton Cove	Dusky Flathead	*Platycephalus fuscus*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	<0.0005	0.0079	<0.0005	<0.0005
		($n = 4$, 36–72 cm)	SD	–	–	–	–	–	–	–	–	–	0.0075	–	–
	Mud Crab[a]	*Scylla serrata*	Mean	–	–	<0.0003	–	–	–	–	–	–	0.0021	<0.0005	<0.0005
		($n = 9$, 6–13 cm)	SD	–	–	–	–	–	–	–	–	–	0.0008	–	–
	School Prawn	*Metapenaeus macleayi*	Mean	<0.0050	<0.0005	0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0002	0.0029	0.0172	<0.0005	<0.0005
		($n = 8$)	SD	–	–	0.00003	–	–	–	–	–	0.0014	0.0056	–	–
	Sea Mullet	*Mugil cephalus*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	<0.0005	0.0034	<0.0005	<0.0005
		($n = 2$, 37–50 cm)	SD	–	–	–	–	–	–	–	–	–	0.0020	–	–
	Yellowfin Bream	*Acanthopagrus australis*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	<0.0005	0.0011	<0.0005	<0.0005
		($n = 8$, 27–39 cm)	SD	–	–	–	–	–	–	–	–	–	0.0007	–	–
Fullerton Cove - liver	Yellowfin Bream	*Acanthopagrus australis*	Mean	<0.0050	<0.0050	<0.0005	<0.0002	<0.0005	<0.0005	<0.0005	<0.0002	0.0018	0.0090	<0.0005	<0.0005
		($n = 3$, 27–39 cm)	SD	–	–	–	–	–	–	–	–	0.0011	0.0043	–	–
Tilligerry Creek	Dusky Flathead	*Platycephalus fuscus*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	<0.0005	0.0081	<0.0005	<0.0005
		($n = 8$, 39–76 cm)	SD	–	–	–	–	–	–	–	–	–	0.0051	–	–
	Eastern King Prawn	*Penaeus plebejus*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	0.0024	0.0420	<0.0005	<0.0005
		($n = 2$)	SD	–	–	–	–	–	–	–	–	0.0011	0.0085	–	–
	Mud Crab[a]	*Scylla serrata*	Mean	–	–	<0.0003	–	–	–	–	–	–	0.0042	<0.0005	<0.0005
		($n = 8$, 9–13 cm)	SD	–	–	–	–	–	–	–	–	–	0.0032	–	–
	Sand Whiting	*Sillago ciliata*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	<0.0005	0.0013	<0.0005	<0.0005
		($n = 8$, 33–42 cm)	SD	–	–	–	–	–	–	–	–	–	0.0012	–	–
	Yellowfin Bream	*Acanthopagrus australis*	Mean	<0.0005	<0.0005	<0.0003	<0.0001	<0.0005	<0.0005	<0.0005	<0.0001	<0.0005	0.0005	<0.0005	<0.0005
		($n = 7$, 19–30 cm)	SD	–	–	–	–	–	–	–	–	–	0.0002	–	–
Tilligerry Creek - liver	Dusky Flathead	*Platycephalus fuscus*	Mean	<0.0050	<0.0050	<0.0005	<0.0001	<0.0005	<0.0005	<0.0005	<0.0002	0.0036	0.1350	<0.0005	<0.0005
		($n = 3$, 39–76 cm)	SD	–	–	–	–	–	–	–	–	0.0027	0.1026	–	–

[#] n is the number of samples, sizes indicated are total length (TL) for fish and carapace length (CL) for crabs. No sizes are indicated for prawns as they were analysed as composites.
[a] Mud Crab were not analysed for the full suite of PFASs.

basis. Validation of the method included analysis of a fish standard reference material provided by the National Institute of Standards and Technology (SRM 1946, NIST, USA) which had an assigned reference value for PFOS, and resulted in measurements within 10% of the assigned value. Three samples were analysed in duplicate and the Relative Percent Difference (RPD) of the analyte concentrations were within reasonable levels for both perfluoro-*n*-hexane sulfonate and perfluoro-*n*-octane sulfonate (no other analytes were present at concentrations greater than the limit of reporting in these samples; Table 1).

The limit of reporting (LOR) and % recovery of ^{13}C labelled standards varied between analytes and different species/tissue types (Table 2 lists both the full names and acronyms of each analyte). Good recovery levels were achieved for the two main analytes of interest (PFOA and PFOS), being 72–94% and 78–100% respectively. The only analyte that had consistently poor recovery across species/tissues was perfluoro-*n*-hexanoic acid (PFHxA).

A total of seventy samples were screened for PFAS levels (Table 3), which were captured throughout Fullerton Cove and Tilligerry Creek (Fig. 1). All samples contained PFOS except four Yellowfin Bream samples (two from each estuary). PFOA was detected only in School Prawn samples from Fullerton Cove, and PFHxS was detected in prawn muscle and in fish liver samples from both estuaries (Table 3). Single-factor analysis of variance was used to evaluate differences in the levels of $\log_{10}$-transfromed PFOS concentrations among several factors (all analyses were done in R v.3.2.0, R Development Core Team, 2016). There was no significant difference in PFOS concentration between samples from Fullerton Cove and Tilligerry Creek for Yellowfin Bream ($F_{1,9} = 3.338$, $P = 0.101$), Dusky Flathead ($F_{1,10} = 0.075$, $P = 0.790$) or Mud Crab ($F_{1,15} = 3.075$, $P = 0.099$; note that other species were not captured in both estuaries). There was a significant difference between species (pooled across estuaries, but excluding Eastern King Prawns and Sea Mullet due to low replication; $F_{4,51} = 12.036$, $P \ll 0.001$). Post-hoc comparisons indicated that PFOS concentrations in School Prawn > Dusky Flathead > Mud Crab > Sand Whiting = Yellowfin Bream (Tukeys-HSD; $P < 0.05$). Also, concentrations of PFOS were greater in liver tissue relative to muscle tissue for both Yellowfin Bream ($F_{1,7} = 25.060$, $P = 0.002$) and Dusky Flathead ($F_{1,9} = 29.180$, $P < 0.001$), and the concentrations in Dusky Flathead liver were an order of magnitude greater than any other species or tissue ($\mu = 0.135$ mg kg^{-1}). The PFOS values for School Prawn, Eastern King Prawn and Dusky Flathead appeared highly variable between individuals or prawn composites (Fig. 2).

Analysis of fish obtained from other locations in New South Wales (through Sydney Fish Markets) in September 2015 was conducted at the same time as the sampling described in the current study, to provide some indication of background levels of PFAS in muscle tissue across New South Wales (Williamtown Contamination Expert Panel, 2015). These results revealed all PFAS were below the LOR for Dusky Flathead (n = 1), Yellowfin Bream (n = 1), Sand Whiting (n = 1), Eastern King Prawn (n = 1), School Prawn (n = 1) and Mud Crab (n = 2); however, Sea Mullet (n = 1) returned a PFOS concentration of 0.00037 mg kg^{-1}. The PFOS levels in Sea Mullet reported in the current study ($\mu = 0.0034$ mg kg^{-1}) were within the range of those reported in this species in Sydney Harbour (0.0008–0.0049 mg kg^{-1}) by Thompson et al. (2011b). High PFOS concentrations in liver tissue were also detected for Sea Mullet (0.0440–0.1070 mg kg^{-1}) by Thompson et al. (2011b), and for a number of stingray species (0.002–0.117 mg kg^{-1}) from the Brisbane River, Queensland (Baduel et al., 2014). The species-specific patterns in contaminant levels and variability likely relate to a range of factors, including trophic relationships, energetic requirements, site fidelity and movement rates, and also animal growth rates. In addition, overall animal size, and moult stage (for crustaceans) likely affect contaminant levels (Baduel et al., 2014), and the toxicokinetics of the contaminants may be expected to differ between species (Kudo and Kawashima, 2003).

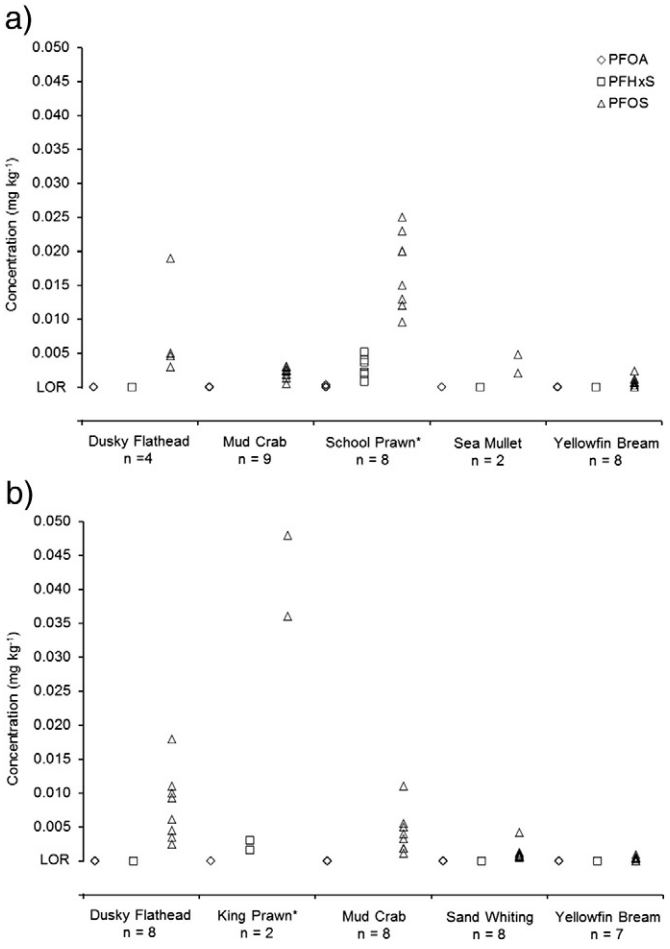

Fig. 2. Variability of muscle tissue perfluoro-*n*-octanoic acid (PFOA), perfluoro-*n*-hexane sulfonate (PFHxS) and perfluoro-*n*-octane sulfonate (PFOS) concentrations between animals captured within Fullerton Cove (a) and Tilligerry Creek (b). An asterisk (*) indicates that these animals had to be analysed as composites, due to biomass requirements for analyses.

This manuscript presents one of the first surveys of PFAS in a range of commercially exploited saltwater fish and crustaceans in Australia. These data reveal that local point-source contamination may have an appreciable effect on the concentrations of these substances in the muscle and liver tissue of a range of prawn, crab and fish species, however a broader survey is required to properly evaluate this. Establishing these baseline levels of contamination in a range of edible aquatic species surrounding a contamination zone is an important step in informing future surveys of these emerging contaminants, and also for comparing levels across time (as these substances are largely being phased out of use). An expanded sampling program will place contamination from this particular source in a wider context, as the ubiquitous presence of these substances in consumer goods may mean that there is a diffuse background of PFAS across other estuaries. Such broad-scale investigation is required to fully appreciate the context of any human health implications, and also levels of exposure to these contaminants through a range of different pathways.

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