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Validating blood microsampling for per- and polyfluoroalkyl substances quantification in whole blood

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ABSTRACT

Microsampling allows the collection of blood samples using a method which is inexpensive, simple and minimally-invasive, without the need for specially-trained medical staff. Analysis of whole blood provides a more holistic understanding of per- and polyfluoroalkyl substances (PFAS) body burden. Capillary action microsamplers (Trajan hemaPEN®) allow the controlled collection of whole blood as dried blood spots (DBS) (four 2.74 μL ± 5 %). The quantification of 75 PFAS from DBS was evaluated by comparing five common extraction techniques. Spiked blood (5 ng/mL PFAS) was extracted by protein precipitation (centrifuged; filtered), acid-base liquid-liquid extraction, trypsin protease digestion, and weak anion exchange (WAX) solid-phase extraction with analysis by high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Filtered protein precipitation was the most effective extraction method, recovering 72 of the 75 PFAS within 70 to 130 % with method reporting limit (MRL) for PFOS of 0.17 ng/L and ranging between 0.05 ng/mL and 0.34 ng/mL for all other PFAS. The optimised method was applied to human blood samples to examine Inter- ($n = 7$) and intra-day ($n = 5$) PFAS blood levels in one individual. Sixteen PFAS were detected with an overall Σ_{16}PFAS mean = 6.3 (range = 5.7–7.0) ng/mL and perfluorooctane sulfonate (branched and linear isomers, ΣPFOS) = 3.3 (2.8–3.7) ng/mL being the dominant PFAS present. To the authors knowledge, this minimally invasive self-sampling protocol is the most extensive method for PFAS in blood reported and could be a useful tool for large scale human biomonitoring studies.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) were first manufactured in the 1940s and subsequently contaminated the global environment [1], including omnipresence in humans. Organic fluorine was first detected in human blood in 1968 [2] and observed to increase substantially over the following decades [3]. The most commonly studied PFAS, perfluorooctanoic acid (PFOA; $C_8HF_{15}O_2$; CAS-RN 335-67-1) and perfluorooctanesulfonic acid (PFOS; $C_8HF_{17}O_3S$; CAS-RN 45298-90-6), are present in 99 % of the general population [4]. Methods have been developed for the determination of PFAS in blood, often focussed on serum with analysis via liquid-chromatography tandem mass spectrometry (LC-MS/MS) [5,6]. Most methods share common shortcomings associated with sample handling, method complexity or cost - whilst requiring highly invasive sampling via venous blood draw [7]. Microsampling and dried blood spot (DBS) technologies present promising improvements to both sample collection (cheaper and less invasive) and PFAS extraction [8,9].

The Organisation for Economic Co-operation and Development (OECD) defines PFAS as "fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom" [10] and over six million unique molecules fit this definition (as of 2023 via Pubchem PFAS tree). Estimates on the number of PFAS manufactured varies, the NORMAN Suspect List Exchange lists 6,400 PFAS [11]. The World Health Organisation's International Agency for Research on Cancer (IARC) lists PFOA as a possible carcinogen [12]. PFAS are linked to cancers of the kidney and testicles and are anticipated to be linked to breast cancer [13]. Additionally, PFAS are linked to thyroid and kidney disease [14], developmental effects and other health conditions [13]. Established health effects of PFAS exposure are primarily from studies of

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a few compounds (commonly PFOS and/or PFOA) [15]. Therefore health effects of the many other PFAS are unknown and require extensive analytical methods to aid in their determination.

PFAS are predominantly present within protein-rich tissues, compared to persistent organic pollutants (POPs) which tend to bioaccumulate in lipid-rich adipose tissue [16]. PFAS bind with blood serum proteins (serum albumin) [17], therefore blood [18] and serum [6] have been target matrices for analysis. Bioaccumulation factor (BAF) increases with fluoroalkyl chain length [19]. The primary human serum transport protein albumin has higher affinity for PFAS with aliphatic carbon length C6-C9, than shorter and longer aliphatic PFAS [20]. Resultingly, PFAS biological half-lives do not conform to the clear trend exhibited by BAF, for example linear PFSA serum half-life $C_4 < C_5 < C_7 < C_6 < C_8$ [21], with observations of odd-chain PFAS commonly at higher biological concentrations in wildlife than even-chain PFAS [22].

Agreeance in measured PFAS concentrations between paired DBS and venous whole blood samples have demonstrated DBS are a viable sampling matrix [8]. However, whole blood is a challenging matrix to store and often requires trained medical practitioners to perform the venepuncture. Freezing of blood samples, supresses a continuously changing matrix, as metabolic processes are inhibited at below normal physiological temperatures, however freezing can cause cellular components to lyse [23]. Serum is preferential for analysis as PFAS interact with serum proteins and because red blood cell content dilutes analyte concentrations. However, several PFAS are more concentrated in whole blood than in serum and plasma, often at a ratio of 5:1 [18]. DBS technology offers a simple alternative to whole blood samples without the challenges of storing it [7]. DBS have been employed for blood PFAS analysis previously with sample volumes of 50–75 µL [24,25]. These studies have been limited to the monitoring of few PFAS, commonly including PFOS and PFOA [24,25]. Recent methods benefit from both the sampling of low volumes (30 µL) and sub-ng/mL (sub part-per-billion) detection limits of larger PFAS cohorts [8]. Capillary microsampling devices ensure the collection of a controlled volume, addressing the shortcomings of traditional blood spot techniques (smearing and inconsistent sample volumes) and the potential for oversampling presented by volumetric adsorptive microsampling (VAMS^{TM}) devices (used for PFAS quantification from 10 µL of blood [26]). The Trajan hemaPEN® used in this study collects four 2.74 µL ± 5 % blood samples simultaneously via precision bore glass capillaries [27]. The capillary collection prevents over- or under-sampling enabling volumetric precision and integrated desiccant rapidly dries the blood onto pre-punched paper discs (Whatman 903 or PerkinElmer 226).

Blood PFAS levels vary with age, sex, residency and employment, as these factors influence PFAS exposure [28]. Blood PFAS concentrations are easily considered under two categories, background and exposed concentrations. Background concentrations refer to individuals and populations exposed to PFAS due to their environmental omnipresence. These populations are studied internationally through programs such as the U.S. National Health and Nutrition Examination Survey (NHANES) [29]. The production and widespread distribution of emerging and novel PFAS demands a widespread list of target analytes for such studies. Exposed concentrations refer to individuals and populations affected by either a singular or ongoing exposure event, such as consumption of contaminated drinking water and occupational exposures [30]. Select and severe cases, such as prolonged exposure experienced by fluorochemical plant workers in China (median concentrations: PFHxS = 764 ng/mL, PFOA = 427 ng/mL, and PFOS = 1725 ng/mL) [31], can result in blood PFAS levels up to one-thousand times greater than background concentrations [32]. Although these categorisations are simple, they readily complicate as each country (and often states) possess their own unique chemical profile, owing to the volume of chemicals produced, localised chemical regulations and commercial availability [33].

In this study, we investigated several methodologies for the extraction of 75 PFAS from DBS collected using the hemaPEN® microsampling device. The methodologies are contrasted against one another for accuracy of analyte recovery, precision and reproducibility, and suitability for high-throughput application. The best performing methodology under these three categories was then applied to a further range of spiked samples and samples collected of the research cohort ($n = 12$).

2. Materials and methods

2.1. Chemicals and materials

Ultrapure water was obtained from reverse osmosis water coupled with Milli-Q Reference A+ system (18.2 Ω, <5 ppm TOC, Merck, New South Wales, Australia). Hypergrade methanol (MeOH) (>99.9 %), hypergrade acetonitrile (ACN) (>99.9 %), hypergrade 2-propanol (IPA) (>99.9 %), hypergrade toluene (>99.9 %), hypergrade ethyl acetate (>99.9 %), formic acid (>99 %), ammonium hydroxide solution (28 % in H_2O, >99.99 %), glacial acetic acid (>99.99 %), sodium acetate (>99.0 %) and ammonium acetate (631-61-8) (>99.99 %) were purchased from Sigma-Aldrich (New South Wales, Australia). Hydrochloric acid (HCl) (32 % in H_2O, >99.9 %) was purchased from Thermo Fisher (Victoria, Australia).

Empty solid phase extraction (SPE) cartridges with two filter frits (pre-inserted) were obtained from Agilent Technologies (Delaware, USA). Weak anion exchange cartridges (OASIS WAX, 1 cc, 30 mg) were obtained from Waters Corporation (New South Wales, Australia).

Defibrinated horse blood was purchased from Southern Biological (Alphington, Australia). Chicken serum of New Zealand origin and trypsin (2.5 %) were purchased from Thermo Fisher (Victoria, Australia).

Seventy-five PFAS (including three technical mixes of linear and branched isomers) included 14 perfluorocarboxylic acids (PFCAs), 14 perfluorosulfonic acids and one chlorinated perfluorosulfonic acid (PFSAs), 17 perfluoroalkanesulfonyl fluorides (PASFs), three fluorotelomercarboxylic acids (FTCAs), three fluorotelomer unsaturated carboxylic acids (FTUCAs), three fluorotelomersulfonic acids (FTSAs), four disubstituted fluorotelomer phosphate diesters (diPAPs), three perfluoroalkanephosphinic acids (PFPiAs), eight perfluoroalkyl ether substances (PFESs), and five cationic/zwitterionic aqueous film forming foam PFAS (AFFF) were purchased as single-component solutions (50 µg/mL) from Wellington Laboratories (Ontario, Canada) (Table S1). Additionally, three n:2 FTCAs three perfluorophosphonic acids (PFPAs), two chlorinated-PFPAs and two perfluorophosphonic acids PAPs were considered for the method, however it was deemed these analytes required their own methods with unique parameters such as basic mobile phase conditions, and therefore were removed from the analytical method. These standards were then combined to form a stock solution such that each analyte was present at 500 ng/mL concentration in methanol, this was then diluted to 100 and 10 ng/mL in methanol. Twenty-three mass-labelled PFAS internal standards were purchased as single-component solutions (50 µg/mL) from Wellington Laboratories (Ontario, Canada) (Table S1). These mass-labelled standards were then combined to form a stock solution such that each internal standard was present at 100 ng/mL concentration in methanol, this was then diluted to 5 and 1 ng/mL in methanol.

The horse blood was then spiked with the PFAS analyte stock solution, such that the final concentrations were 50, 5 and 0.5 ng/mL in whole blood. This was done by adding the stock to centrifuge tubes and evaporating the methanol under low flow nitrogen. Then whole blood was added, vortexed for thirty minutes and allowed to rest at 4 °C for twenty-four hours. This process is the same employed in previous validation studies [6], ensuring solvent does not interact with the blood matrix prematurely and giving ample time for the homogenous dispersion of analyte throughout the blood.

2.2. Extraction methodologies

Extraction methods described briefly (Fig. 1):

Five extraction methodologies for 75 PFAS across 10 classes from DBS collected by the hemaPEN® were evaluated. The methodologies were compared for their accuracy and precision to establish their extraction efficiency and viability at a whole blood analyte concentration. The extraction methods investigated:

Method 1 - Protein precipitation (centrifuged) with different organic solvent volume (1a) 100 µL and (1b) 1000 µL: Addition of organic solvent (methanol) to whole blood or serum to denature present proteins, proteins then 'crash' out of solution and can be centrifuged into a 'pellet' of precipitate. This process of protein precipitation is commonly performed with a ratio of organic solvent to sample between 50–80 % [5,6]. To an Eppendorf tube, 4 × 2.74 µL DBS, 100 µL internal standard (ISTD) at 1 ng/mL in MeOH (and a further 900 µL of MeOH for (1b)) were added. Samples were vortexed for 30 min, and then centrifuged (2000g, 10 min). The supernatant was then transferred to a high recovery vial with a 15 µL taper.

Method 2 - Protein precipitation (filtered): Again proteins precipitate out of solution through the addition of organic solvents, however separation is achieved via filtration. 1 mL of ACN were used to precondition a 1 mL SPE filter cartridge containing two filter frits and no sorbent, then 4 × 2.74 µL DBS and 100 µL 1 ng/mL ISTD in MeOH were added. To each sample, 250 µL MeOH, 250 µL ACN, 250 µL IPA and lastly a further 250 µL of MeOH were added sequentially after the previous solvent has all flowed through the cartridge. Eluent was collected in a high recovery vial and any residual eluent was collected via positive pressure (Agilent – positive pressure manifold 48 processor – nitrogen gas).

Method 3 - Acid-base (liquid-liquid) extraction: The presence of strongly acidic conditions in the aqueous phase will protonate all PFAS into their neutral form (with the exception of cationic and zwitterionic PFAS), upon protonation the PFAS will preferentially partition into the organic phase relative to their octanol-water partition coefficients. To an Eppendorf tube, 4 × 2.74 µL DBS, 20 µL of 5 ng/mL ISTD in MeOH, 500 µL 0.1 M HCl and 500 µL ethyl acetate were added. Samples were vortexed for 30 min, and then centrifuged (2000g, 10 min). The organic layer was then transferred to a high recovery vial.

Method 4 - Trypsin protease digestion: Proteases such as trypsin disrupt protein structure through the cleavage of peptide bonds. Specifically, trypsin is of appeal, as it is a robust enzyme, capable of

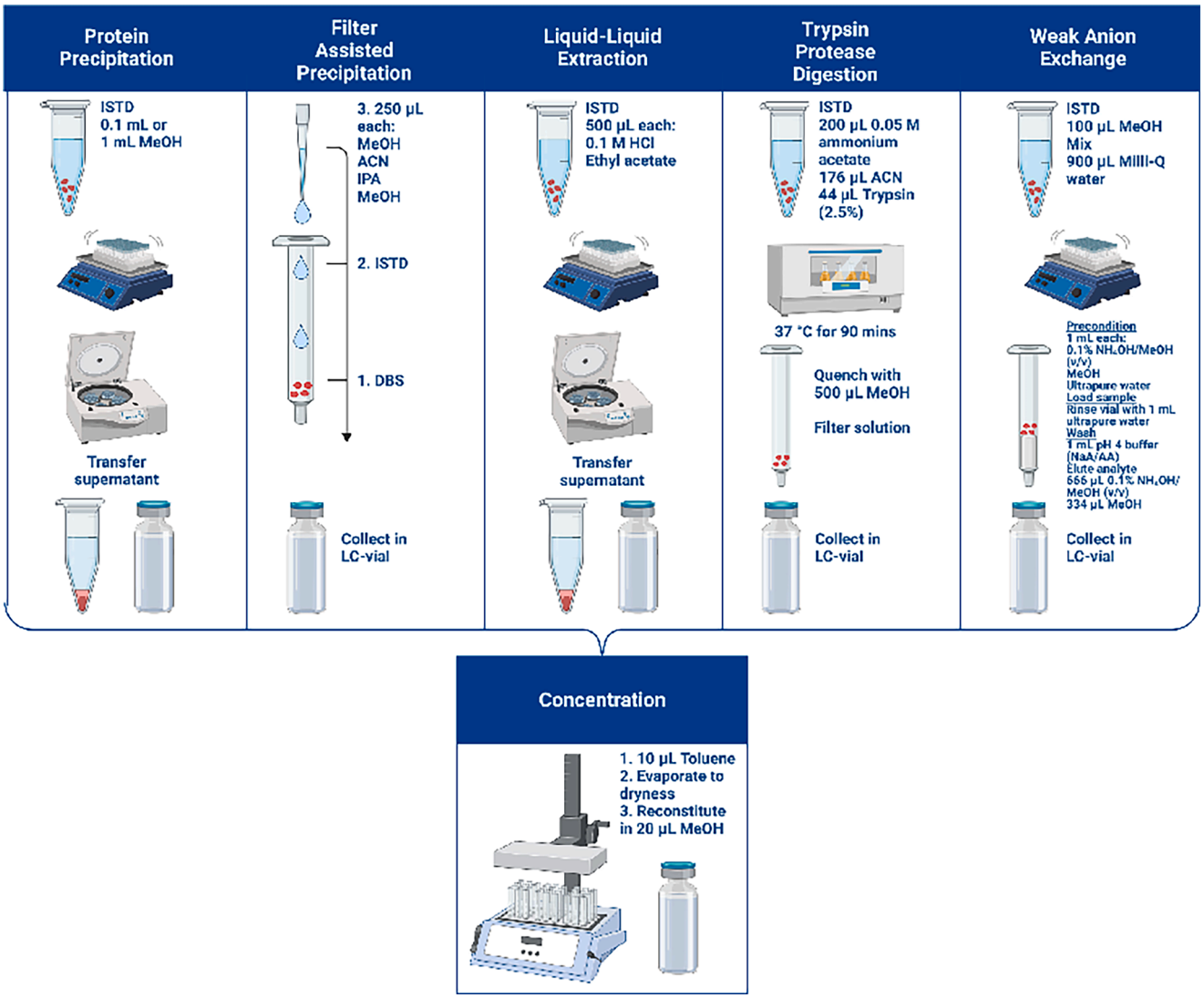

Fig. 1. Details of the five methodologies compared for the extraction of PFAS from DBS. AA, acetic acid; ACN, acetonitrile; HCl, hydrochloric acid; IPA, isopropyl alcohol (2-propanol); ISTD, internal standard; LC, liquid chomatography; MeOH, methanol; NaA, sodium acetate NH_4OH, ammonium hydroxide. Created with BioRender.com.

proteolysis in denaturing conditions of some organic solvents [34]. This enzymatic breakdown of proteins (cleavage of the peptide bond between the carboxyl group of arginine or lysine and the adjacent amino acids amino group, with the exception of proline [34]) coupled with protein precipitation post activity, may aid in the recovery of PFAS that favourably bind to proteins present in blood (such as the serum transport protein albumin) [20]. To an Eppendorf tube, 4 × 2.74 µL DBS, 20 µL of 5 ng/mL ISTD in MeOH, 200 µL 0.05 M ammonium acetate, 176 µL of ACN and 44 µL µ of trypsin (2.5 %) were added. Samples were then heated to 37 °C and mixed (180 rpm) for 90 mins via orbital shaking incubator (Ratek OM11). Following incubation, 500 µL of IPA were added, then samples were vortexed for 30 min, and then centrifuged (2000 *g*, 10 min). The supernatant was then transferred to a high recovery vial.

Method 5 - Weak anion exchange: Weak anion exchange (WAX) is a reversed-phase, water-wettable polymer sorbent developed by Waters Corporation (New South Wales, Australia), it is developed for the effective extraction of a wide range of PFAS analytes, including both short- and long-chain PFAS. To an Eppendorf tube, 4 × 2.74 µL DBS and 100 µL of 1 ng/mL ISTD in MeOH were added. Samples were vortexed for 30 min, then 900 µL of ultrapure water was added and the samples vortexed for a further 10 min. Oasis WAX (1 cc, 30 mg) cartridges were preconditioned with 1 mL of 0.1 % NH_4OH/MeOH (v/v), 1 mL of MeOH and 1 mL of ultrapure water (cartridges were prevented from drying out by retaining a small volume of water within the cartridge), samples were loaded post vortex onto the cartridge at flow rate of approximately 1-2 drops per second, sample Eppendorf tubes were then rinsed with 1 mL of ultrapure water (and loaded). The WAX sorbent was then washed with 1 mL of a pH 4 buffer consisting of sodium acetate and acetic acid. Lastly, analytes were eluted from the WAX sorbent via 666 µL 0.1 % NH_4OH/MeOH (v/v) and 334 µL MeOH into high recovery vials.

Extracts from all methods had 10 µL of toluene added and evaporated to dryness at room temperature under a gentle flow of nitrogen gas (0.13 L/min), and reconstituted in 20 µL of MeOH prior to LC-MS injection.

2.3. Sample collection

Dried blood spot (DBS) samples were collected from an individual within the research cohort. The individual voluntarily self-sampled by first wiping the side of their fingertip with an alcohol swab, allowing the solvent to evaporate and then finger-pricking using a Accu-Chek Softclix Lancing Device. The Accu-Chek devices and contact-activated lancets are preferential due to lower associated pain with the prick and control regarding the prick depth. A small portion of blood was then allowed to bubble out from the prick and was wiped away using a second alcohol swab, ensuring the removal of interstitial fluid, the solvent was then allowed 5-10 seconds to evaporate. Upon evaporation, a small droplet of blood was massaged to the surface of the pricked finger and sampled using the hemaPEN®. The hemaPEN® is a Class 1 registered in vitro diagnostic medical device (IVD) and included in the Australian Register of Therapeutic Goods for research use. Capillary action of the micro-sampling device ensured that four DBS were collected, each 2.74 µL ± 5 % in volume. The DBS were then allowed to dry for at least one hour prior to analyte extraction. The individual provided a single sample every day for a week from the same finger at 2 PM ($n = 7$), and five individual samples within a five-minute period (each from a different finger) on the eighth day.

2.4. Instrumental analysis

Sample analysis was performed on an Agilent 1290 Infinity II liquid chromatography system (LC) coupled with an Agilent 6495C tandem mass spectrometer (MS/MS) in negative electrospray ionisation (ESI-). The established LC-MS/MS method is an expansion of works by Coggan *et al.* [35] from 53 to 75 PFAS, with alterations to LC and MS/MS parameters.

Separation was achieved on a Zorbax eclipse plus RRHD C18 column (2.1 × 50 mm, 1.8 µm, Agilent Technologies, USA) with a C18 guard column attached (Fig. 2). Furthermore, the gradient elution consisted of 2 mM ammonium acetate in ultrapure water (A) and methanol (B) at 0.4 mL min^{-1} for a total runtime of 17 minutes. Source conditions for the mass spectrometer were: drying gas = 250 °C at 11 L min^{-1}, nebuliser pressure = 25 psi, sheath gas = 375 °C at 11 L min^{-1}, positive capillary and nozzle voltages = 3500 V and 1500 V, negative capillary and nozzle voltages = 2500 V and 1500 V, positive high and low pressure RF (iFunnel) = 150 V and 60 V, and negative high and low pressure RF (iFunnel) = 90 V and 60 V. The highest intensity m/z transition was used for quantification of each compound (complete MS/MS parameters are listed in Table S1). Linear calibration curves with 1/x weighting of internal standard corrected response were established for all compounds (with the exception of the FTSAs which were quadratic with 1/x weighting) from triplicate injections of 13 individual levels from 0.01 to 100 ng mL^{-1} ($R^2 > 0.99$), with accuracy ± 30 % in methanol.

2.5. Quality control/quality assurance (QA/QCs)

Internal standard recovery was determined by comparing the response of each sample to the average response of the calibration curve (Figure S2). The method reporting limit (MRL) was defined by the lowest calibration concentration with a signal-to-noise ratio (S/N) greater than 10:1 or three times the concentration in the blank calibration solution, whichever is greater. Samples with S/N between 3 and 10 were defined as less than the method reporting limit (< MRL) and responses with S/N less than 3:1 were considered non-detected (n.d.). The qualifier ratio (where two transitions were available) was set to ± 20 % of the median response ratio of the calibration curve. Acceptable retention times (RT) were defined as 5 % relative to the internal standard response of the calibration standards. If criteria for qualifier ratio or RT were not met, the sample was designated < MRL.

Both PFHxA (0.13 ng/mL) and L-PFOA (0.15 ng/mL) were detected in the horse blood, L-PFOS was also detected in the horse blood below the MRL. The average concentrations of PFAS detected in triplicate blank samples were subtracted from subsequent treatment groups to obtain a blank-corrected recovery.

2.6. Statistical analysis

Concentration data were acquired and quantified using Agilent MassHunter Workstation v10.1 and Quantitative Analysis v10.1 respectively. Descriptive statistics, statistical tests and data visualisation were completed using Microsoft Excel v16.47, Minitab v19.1.1 and R v4.3.1 with RStudio v2023.06.1 and tidyverse v1.3.2. Internal standard corrected recoveries were calculated as such.

$$\%Recovery(\%R) = \frac{Concentration_{Measured}}{Concentration_{Spiked}} \times 100$$

A two sample t-test ($\alpha = 0.05$) (sample variance dictated whether a homoscedastic or heteroscedastic test was employed, determined by an F-test) was undertaken upon each of the 75 PFAS replicate internal standard corrected recoveries for methods 1a and 1b to determine if the volume of organic solvent used in protein precipitation effect the accurate recovery of PFAS.

$$H_0 = \overline{\%R}_{1a} = \overline{\%R}_{1b}$$

Where, $\overline{\%R}$ is the average recovery of method replicates.

Furthermore, the replicate internal standard corrected recoveries of each PFAS for methods 1b, 2, 3 and 5, were first tested for equivalent variance by the means of multiple comparisons ($\alpha = 0.05$).

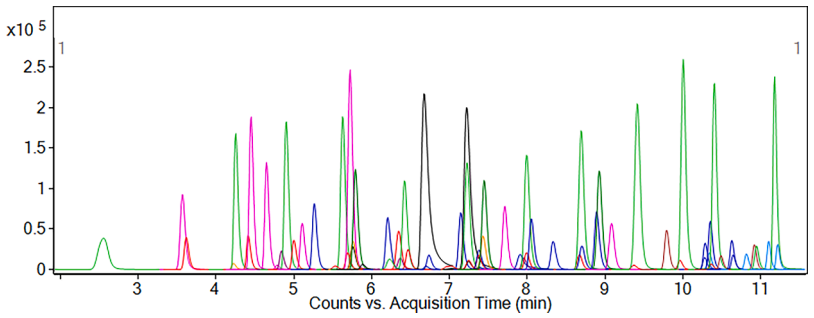

Fig. 2. Chromatogram of 75 PFAS simultaneous analytical method from 2 µL injection of 50 ng/mL standard. PFAS grouped by class: light green – PFCA, red – PFSA, dark blue – PASF, orange – FTCA, dark green – FTUCA, purple – FTSA, light blue – diPAP, brown – PFPiA, pink – PFEA, black – AFFF.

$$H_0 = s^2_{1b} = s^2_2 = s^2_3 = s^2_5$$

Where s^2 is variance of method recoveries. Should the replicates across methods demonstrate equivalent variance, then the average internal standard corrected recovery was compared by ANOVA with Tukey's post hoc pairwise test ($\alpha = 0.05$) to determine the accuracy of each method (where variance was inequivalent, Welch's test with Games-Howell post hoc was employed).

$$H_0 = \overline{\%R}_{1b} = \overline{\%R}_2 = \overline{\%R}_3 = \overline{\%R}_5$$

Where, $\overline{\%R}$ is the average recovery of method replicates. Method 4, was not included in statistical tests, due to consistently large variance across many PFAS and the inability to recover the majority of PFAS with an average within 70-130 % (37 of 75 PFAS).

Linear regression analysis ($\alpha = 0.05$) was undertaken on the daily sum PFAS concentration (such that x = sample day, and y = [$\sum$PFAS]), to investigate for changing blood PFAS content across the course of a week. Due to pre-established biological half-lives for the most commonly measured PFAS in human serum being greater than one year, the null hypothesis was that the slope coefficient was equal to zero.

$$H_0 \ : \ \beta = 0$$

3. Results and discussion

3.1. Comparison of methodologies

The internal standard corrected recovery of 75 PFAS was calculated from five horse DBS replicate samples spiked at 5 ng/mL ($n = 5$) for five different extraction methodologies with 23 mass-labelled PFAS internal standards at 9.09 ng/mL. Overall, recovery ranges (expressed to 95 % confidence) for the different extraction methodologies are as follows: Methods 1a – Protein precipitation (centrifuged) from 6.7 ± 31 % to 98 ± 8.8 %, 1b – Protein precipitation (centrifuged) from 5.2 ± 61 % to 119 ± 16 %, 2 – Protein precipitation (filtered) from 38 ± 15 % to 131 ± 14 %, 3 – Acid-base (liquid-liquid) extraction from 9.7 ± 60 % to 109 ± 15

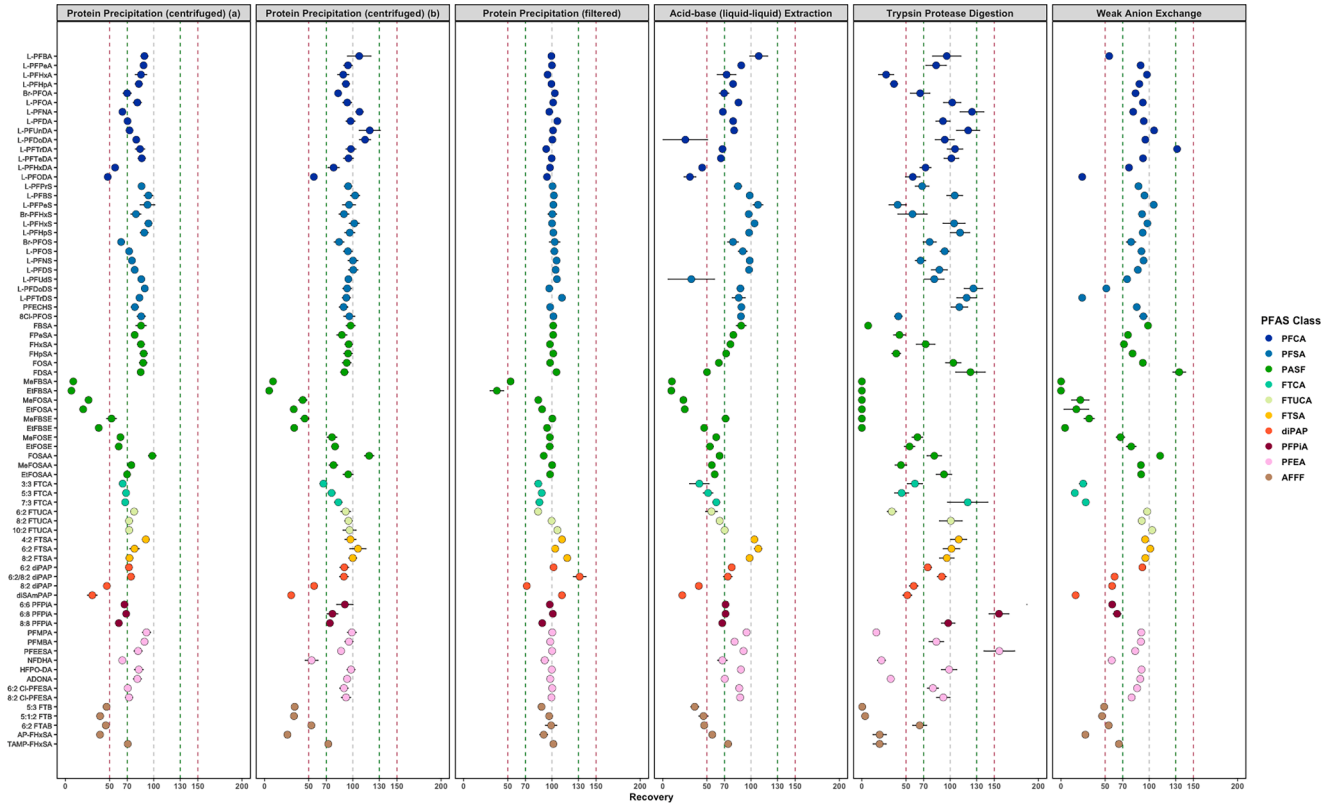

Fig. 3. Average internal standard corrected recoveries and 95 % confidence intervals of 75 PFAS (5 ng/mL) extracted by: protein precipitations, acid-base (liquid-liquid) extraction, protease digestion, and weak anion exchange. Green and red lines represent the 70-130 % and 50-150 % recovery ranges respectively.

%, 4 – Trypsin protease digestion from 0 % to 269 ± 29 %, and 5 – Weak anion exchange from 0 % to 250 ± 9.6 %.

The larger volume of organic solvent successfully extracted more PFAS (60 of 75 within 70-130 %) than the smaller volume (48 of 75) via method 1 (Fig. 3). Both volumes tested achieved relative standards deviations (RSDs) under 20 % for 71 of the 75 PFAS. Efficiency in the extraction of 40 of the 75 PFAS was statistically significantly different between the two differing volumes (Table 1), with 36 analytes recovering more efficiently (closer to 100 %) using larger volumes of organic solvent. PFAS extraction via protein precipitation is more effective using greater volumes of solvent, and henceforth method 1b is used for comparisons to other extraction methods. Historically, the viability of using greater ratios of sample to solvent has been limited by larger volumes of sample required for analysis. The costs of solvent and time in subsequent concentration steps, have led to the protein precipitation workflows commonplace today. The use of a 10.96 µL ± 5 % circumvents these costs and protein precipitation workflows with 1:100 ratios of sample to solvent are feasible.

Method 2 – Protein precipitation (filtered) recovered the most PFAS (72 of the 75 within 70-130 %) out of all the methods tested, with all 72 PFAS RSDs within 10 % (Fig. 3). Methods 3, 4 and 5 effectively recovered 42, 37 and 47 PFAS respectively, each with more variance than method 2. Method 4 was removed from statistical comparisons (Table 1) due to poor performance and large variance in PFAS recovery. The shortcomings of methods 3, 4 and 5 are summarised as follows; Methods 3 proved inept at extracting PFAS with greater octanol-water partition coefficients. Method 4 introduced undesired complexity for the handling of small samples for trace analysis, and for method 5 the volume of sample was less than advised for proprietary SPE sorbents. Method 2 recovers almost all of the 75 PFAS effectively with little variance, highlighting the benefit of simplified extraction techniques with minimal handling and transfer steps for the analysis of trace analytes from microsamples.

The U.S. EPA has set health advisory levels for PFOA, PFOS, perfluorobutane solfinate (PFBS) and hexafluoropropylene oxide dimer acid (HFPO-DA) [36]. Should blood analysis be undertaken solely on these four PFAS, both protein precipitation methods (methods 1b and 2) perform equally as best for the extraction of these compounds (Table 1), however all methods tested recover these four PFAS within 70-130 % at 5 ng/mL. Similarly, the European Food Safety Authority established a group threshold for the tolerable weekly intake of PFOA, PFNA, PFHxS and PFOS [37]. Again, should blood analysis be undertaken solely on these four PFAS, both protein precipitation methods (methods 1b and 2) extract these PFAS most efficiently. Furthermore, method 3 (acid-base (liquid-liquid) extraction) should not be employed for this analysis, as it does not effectively recover PFNA within 70-130 %.

The methods compared in this study greatly expand the list of target PFAS for analysis, with previous DBS, microsampling and serum methods targeting 25, 43 and 53 PFAS respectively [6,8,9]. Method 2 – Protein precipitation (filtered) successfully extracted a minimum of 29 more PFAS than preestablished methods. Despite the smaller sample volume used 10.96 µL ± 5 % (DBS 30 µL, microsampler 60 µL, serum 200 µL), MRLs were similar to previous methods (commonly within an order of magnitude) (Table S1). The DBS method by Poothong *et al.* [8] is a faster extraction, but requires additional LC apparatus for online SPE (in place of a concentration step) and matrix matched calibrations. The VAMS^{TM} method by Carignan *et al.* [9] employs a similar centrifuged protein precipitation method, highlighting that microsampling methods benefit from method simplicity and are cost effective. The serum method by Szabo *et al.* [6] uses enhanced matrix removal (EMR) proprietary SPE sorbent. This method is faster as no concentration step is required, however the collection of at least 0.5 mL of blood is necessary.

The comparisons of the methods undertaken confidently identify PFAS extraction via protein precipitation as the most accurate and consistent extraction methodology from DBS. Furthermore, attesting to this observation is the benefit of method simplicity when dealing with small sample and processing volumes, eliminating opportunities for analyte loss and contamination. Filtered protein precipitation uses minimal consumables, employs few manual handling steps, would be easily upscaled for high throughput and automation, and provides excellent recovery for the majority of PFAS. Filtered protein precipitation yielded consistently acceptable recoveries with acceptable precision across a range of PFAS, the effects of PFAS concentration and validation of the method on human DBS can be found below.

3.2. Extraction limitations

Both MeFBSA and EtFBSA were insufficiently recovered by all investigated methods. These PFAS are understood to have octanol-water partition coefficients (log(k_{OW})) (Calculated log(k_{OW}): MeFBSA – 3.59 and EtFBSA – 4.08) within the vast range of other PFAS investigated (Calculated log(k_{OW}): PFBA – 2.14 and PFODA – 11.51). MeFBSA is considered a semi-volatile neutral PFAS and has a net positive air-snow exchange, other PASFs (MeFOSA, EtFOSA, MeFOSE, EtFOSE and MeFBSE) all possess air-snow exchange values of approximately zero or slightly negative [38]. This indicates MeFBSA will preferably partition from snow into the air, and thus it is hypothesised that these analytes are extracted from the DBS matrix, but are then however volatilised and lost through the universal concentration step all methods underwent.

Like the PASFs discussed above, many cationic and zwitterionic PFAS (AFFF; 5:3 FTB, 5:1:2 FTB, 6:2 FTAB, AP-FHxSA, and TAMP-FHxSA) also poorly recovered across multiple methods. The poor recovery of these compounds is likely due to the pH sensitivity of these compounds and the differing complexities of their functional groups (when compared to the carboxylic and sulfonic acid mass-labelled ISTDs applied to them). Upon inspection of these compounds without the use of an ISTD, the general recovery of these PFAS did not improve (Table S3). Integration of these PFAS into methods optimised for the extraction of anionic PFAS require mass-labelled ISTDs that reflect their functional chemistry.

Similarly, PFODA and diSAmPAP both were poorly recovered (compared to their respective classes) by multiple extraction methods investigated. PFODA and diSAmPAP possess log(k_{OW}) greater than other PFAS analysed (Calculated log(k_{OW}): 11.51 and 12.60, respectively), including the ISTDs used for their quantification (Calculated log(k_{OW}): $^{13}C_2$-PFHxDA – 10.71 and $^{13}C_2$-6:2 diPAP – 9.42). Upon inspection of these compounds without the use of an ISTD, the general recovery of these PFAS did not improve. The extremely hydrophobic nature of these two analytes could be resulting in a greater affinity for the blood matrix, which some of the extraction methods explored are failing to overcome.

Lastly, utmost care and competence must be maintained throughout any extraction and analytical methodology. However, given the 50x concentration step undertaken after all the methods investigated, prevention of contamination must be a forefront focus. 6:2 FTSA was observed in blanks of all methods except method 2, ranging in concentration from <MRL to 0.13 ng/mL, it is understood that 6:2 FTSA is a widely observed background contaminant as described in EPA Method 8327.

3.3. Effects of concentration

As the best performing method across the 75 PFAS, method 2 – Protein precipitation (filtered), was employed at spiked concentrations of 0.5, 5, and 50 ng/mL PFAS in horse blood. Reflecting a range of baseline serum PFAS levels ranging from 0.24 – 7.26 ng/mL (perfluorodecanoic acid (PFDA) and ∑PFOS) in Australian populations [32], and exposed concentrations such as 44.4 ng/mL (range = <0.5 – 1, 400 ng/mL) serum PFOA content of Italian residents drinking contaminated water [39]. At 5 ng/mL method 2 recovered 72 of 75 PFAS within 70-130 % and a further two within 50-150 % (Table 2), with an average recovery across the 74 PFAS of 98 %. The average RSD of these PFAS recoveries was 3.3 % and all that were recovered effectively had an RSD

Table 1
Internal standard corrected recovery of the 75 PFAS across the extraction methods applied, with comparison via ANOVA/Welch's test with Tukey/Games Howell post. Depending upon data homoscedasticity, an ANOVA with Tukey post or Welch's test with Games-Howell post was undertaken per PFAS to investigate for differences between the effectiveness of extraction methods 1b, 2, 3 and 5.

PFAS	(1b) Protein Precipitation (centrifuged)			(2) Protein Precipitation (filtered)			(3) Acid-Base (Liquid-Liquid) Extraction			(5) Weak Anion Exchange			(1b), (2), (3), (5) ANOVA /Welch's Test	(1a) Protein Precipitation (centrifuged)		(1a), (1b) t-test	(4) Trypsin Protease Digestion	
	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2		Recovery (%)	σ^2		Recovery (%)	σ^2
L-PFBA	107	A	460	99	A	2	109	A	265	55	B	25	F = 105.27 p < 0.001*	89	43	t = 1.77 p = 0.14**	96	671
L-PFPeA	94	A / B	62	100	A	2	89	B	5	90	B	4	F = 39.64 p < 0.001*	88	11	t = 1.57 p = 0.15	84	348
L-PFHxA	89	A	103	95	A	5	72	A	290	97	A	2	F = 4.65 p = 0.037*	86	110	t = 0.54 p = 0.60	27	188
L-PFHpA	92	A / B	44	99	A	2	80	C	28	89	B	4	F = 17.19 p < 0.001	83	42	t = 2.17 p = 0.062	36	44
Br-PFOA	83	B	29	103	A	11	69	C	74	84	B	35	F = 25.78 p < 0.001	70	57	t = 3.25 p = 0.012	66	316
L-PFOA	94	A / B	65	101	A	1	86	B	9	93	B	7	F = 9.82 p = 0.001	81	54	t = 2.50 p = 0.037	102	248
L-PFNA	108	A	47	97	B	2	68	D	3	82	C	2	F = 111.36 p < 0.001	65	21	t = 11.73 p < 0.001	125	471
L-PFDA	97	B	68	106	A	12	80	C	5	94	B	3	F = 27.44 p < 0.001	70	18	t = 6.53 p < 0.001	92	167
L-PFUnDA	120	A	371	101	A	2	81	B	10	105	A	5	F = 64.95 p < 0.001*	73	32	t = 5.19 p = 0.003**	120	451
L-PFDoDA	114	A	105	101	A	1	26	B	1647	96	B	7	F = 11.54 p = 0.004*	80	16	t = 6.83 p < 0.001	94	301
L-PFTrDA	98	B	82	93	B	19	68	C	33	132	A	35	F = 81.28 p < 0.001	85	72	t = 2.40 p = 0.043	106	199
L-PFTeDA	95	A	80	100	A	10	66	B	9	93	A	3	F = 45.71 p < 0.001	86	44	t = 1.73 p = 0.12	101	179
L-PFHxDA	78	B	106	98	A	6	45	C	27	77	B	15	F = 62.76 p < 0.001	56	7	t = 4.63 p = 0.006**	72	102
L-PFODA	56	B	4	94	A	2	31	C	118	24	C	31	F = 516.57 p < 0.001*	48	34	t = 2.88 p = 0.021	58	178
L-PFPrS	95	A / B	51	101	A	3	85	B	45	88	B	0	F = 72.97 p < 0.001*	86	11	t = 2.42 p = 0.042	68	154
L-PFBS	103	A	69	102	A	1	99	A	5	95	A	4	F = 20.07 p < 0.001*	94	64	t = 1.66 p = 0.14	105	208
L-PFPeS	96	A	138	102	A	16	108	A	81	105	A	9	F = 1.65 p = 0.25*	93	181	t = 0.34 p = 0.74	40	249
Br-PFHxS	90	A	81	100	A	64	97	A	10	92	A	13	F = 2.83 p = 0.072	80	83	t = 1.75 p = 0.12	57	712
L-PFHxS	102	A	81	100	A	4	104	A	12	98	A	6	F = 1.25 p = 0.33	94	41	t = 1.55 p = 0.16	104	406
L-PFHpS	97	A	84	102	A	15	98	A	20	93	A	4	F = 2.24 p = 0.12	89	53	t = 1.39 p = 0.20	111	312
Br-PFOS	84	B	80	103	A	93	80	B	95	79	B	72	F = 7.41 p = 0.002	63	33	t = 4.50 p = 0.002	77	143
L-PFOS	94	A	61	103	A	34	91	A	65	91	A	35	F = 3.21 p = = 0.051	72	25	t = 5.35 p < 0.001	94	66
L-PFNS	100	A / B	81	105	A	3	99	A / B	19	94	B	11	F = 4.09 p = 0.025	75	20	t = 5.53 p < 0.001	66	87
L-PFDS	100	A	73	104	A	4	98	A	33	87	B	6	F = 9.35 p = 0.001	78	30	t = 4.87 p = 0.001	88	211

(*continued on next page*)

Table 1 (*continued*)

PFAS	(1b) Protein Precipitation (centrifuged)			(2) Protein Precipitation (filtered)			(3) Acid-Base (Liquid-Liquid) Extraction			(5) Weak Anion Exchange			(1b), (2), (3), (5) ANOVA /Welch's Test	(1a) Protein Precipitation (centrifuged)		(1a), (1b) t-test	(4) Trypsin Protease Digestion	
	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2		Recovery (%)	σ^2		Recovery (%)	σ^2
L-PFUdS	95	B	27	106	A	7	32	A / B / C	1797	75	C	22	F = 50.30 p < 0.001*	86	33	t = 2.60 p = 0.032	82	324
L-PFDoDS	94	A	63	97	A	11	88	A	40	51	B	23	F = 64.91 p < 0.001	90	23	t = 0.88 p = 0.40	126	286
L-PFTrDS	93	B	54	111	A	9	86	B	142	24	C	11	F = 132.49 p < 0.001	84	5	t = 2.57 p = 0.050**	119	317
PFECHS	90	A / B	62	98	A	6	89	B	23	86	B	3	F = 5.75 p = 0.007	79	43	t = 2.38 p = 0.045	110	230
8Cl-PFOS	96	A / B	101	102	A	1	89	B	26	93	A / B	46	F = 11.08 p = 0.005*	86	56	t = 1.82 p = 0.11	41	49
FBSA	98	A	68	101	A	32	89	A	80	99	A	23	F = 2.88 p = 0.068	86	90	t = 2.12 p = 0.067	7	2
FPeSA	88	A / B	85	101	A	1	80	B	26	76	B	6	F = 157.20 p < 0.001*	78	21	t = 1.98 p = 0.083	43	113
FHxSA	95	A	49	98	A	17	77	B	15	71	B	31	F = 31.43 p < 0.001	85	41	t = 2.32 p = 0.049	72	290
FHpSA	95	A	63	101	A	16	72	C	6	81	B	17	F = 34.91 p < 0.001	89	45	t = 1.29 p = 0.23	39	62
FOSA	93	A	62	98	A	18	64	B	2	93	A	9	F = 54.35 p < 0.001	88	47	t = 1.09 p = 0.31	104	194
FDSA	90	C	48	105	B	24	50	D	14	134	A	137	F = 109.69 p < 0.001	85	5	t = 1.59 p = 0.15	123	728
MeFBSA	10	B	8	53	A	17	10	B	25	0	C	-	F = 244.76 p < 0.001*	9	4	t = 0.46 p = 0.66	0	-
EtFBSA	5	B	10	38	A	152	10	B	33	0	B	-	F = 28.77 p < 0.001	7	3	t = 0.92 p = 0.38	0	-
MeFOSA	43	B	59	85	A	7	23	C	9	22	B / C	254	F = 346.47 p < 0.001*	26	4	t = 4.80 p = 0.005**	0	-
EtFOSA	33	B	25	89	A	3	25	B	23	17	B	510	F = 362.20 p < 0.001*	20	3	t = 5.39 p < 0.001	0	-
MeFBSE	46	C	58	100	A	1	71	B	40	32	C	88	F = 172.97 p < 0.001*	52	80	t = 1.27 p = 0.24	0	-
EtFBSE	34	C	4	94	A	18	47	B	23	5	D	5	F = 562.78 p < 0.001	38	19	t = 1.92 p = 0.091	0	-
MeFOSE	76	B	78	98	A	22	61	C	4	67	B / C	59	F = 32.03 p < 0.001	62	10	t = 3.36 p = 0.010	63	96
EtFOSE	80	B	41	97	A	36	53	C	38	80	B	78	F = 34.39 p < 0.001	60	7	t = 6.32 p < 0.001	54	90
FOSAA	119	A	73	91	B	10	64	C	6	112	A	13	F = 118.19 p < 0.001	98	48	t = 4.10 p = 0.003	82	175
MeFOSAA	78	B	60	100	A	3	56	C	3	91	B	8	F = 578.18 p < 0.001*	75	23	t = 0.84 p = 0.43	44	112
EtFOSAA	95	A / B	83	98	A	1	59	C	8	91	B	2	F = 227.55 p < 0.001*	70	21	t = 5.48 p < 0.001	93	198
3:3 FTCA	67	A	40	85	A	34	41	B	316	25	B	43	F = 32.11 p < 0.001	65	12	t = 0.57 p = 0.58	60	189
5:3 FTCA	76	B	45	88	A	12	51	C	77	16	D	8	F = 415.61 p < 0.001*	69	4	t = 2.34 p = 0.066**	45	165
7:3 FTCA	84	A	48	86	A	40	61	B	42	28	C	21	F = 95.33 p < 0.001	68	17	t = 4.40 p = 0.002	120	1356

(*continued on next page*)

Table 1 (*continued*)

PFAS	(1b) Protein Precipitation (centrifuged)			(2) Protein Precipitation (filtered)			(3) Acid-Base (Liquid-Liquid) Extraction			(5) Weak Anion Exchange			(1b), (2), (3), (5) ANOVA /Welch's Test	(1a) Protein Precipitation (centrifuged)		(1a), (1b) t-test	(4) Trypsin Protease Digestion	
	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2	Recovery (%)	CLD	σ^2		Recovery (%)	σ^2		Recovery (%)	σ^2
6:2 FTUCA	92	A / B	78	84	B	3	55	C	114	98	A	25	F = 21.05 p = 0.001*	78	38	t = 2.94 p = 0.019	34	68
8:2 FTUCA	95	A	48	100	A	12	65	B	17	92	A	8	F = 58.91 p < 0.001	72	36	t = 5.62 p < 0.001	101	430
10:2 FTUCA	96	A	143	106	A	1	70	B	22	103	A	33	F = 80.75 p < 0.001*	72	28	t = 4.13 p = 0.003	269	5912
4:2 FTSA	97	A / B / C	101	111	A	2	104	C	9	95	B	1	F = 113.49 p < 0.001*	91	35	t = 1.21 p = 0.26	110	210
6:2 FTSA	106	A	219	104	A	15	108	A	44	101	A	26	F = 1.04 p = 0.42*	78	72	t = 3.61 p = 0.007	101	228
8:2 FTSA	100	B	51	117	A	10	98	B	20	96	B	1	F = 62.76 p < 0.001*	73	40	t = 6.43 p < 0.001	96	176
6:2 diPAP	90	A / B / C	63	102	A	24	78	C	6	92	B	2	F = 44.86 p < 0.001*	72	31	t = 4.23 p = 0.003	75	49
6:2/8:2 diPAP	90	B	62	131	A	135	74	C	61	61	C	21	F = 67.53 p < 0.001	74	33	t = 3.59 p = 0.007	91	68
8:2 diPAP	56	B	28	71	A	9	41	C	11	58	B	16	F = 50.21 p < 0.001	47	11	t = 3.31 p = 0.011	59	60
diSAmPAP	30	B	2	111	A	30	22	C	14	17	C	7	F = 758.67 p < 0.001	30	77	t = 0.05 p = 0.96**	52	67
6:6 PFPi	91	A	224	98	A	10	71	B	10	58	B	10	F = 26.02 p < 0.001	67	25	t = 3.42 p = 0.009	213	1804
6:8 PFPi	77	B	98	101	A	3	71	B	3	64	B	51	F = 254.51 p < 0.001*	69	19	t = 1.68 p = 0.13	155	327
8:8 PFPi	74	C	34	89	B	15	67	C	27	250	A	576	F = 89.07 p < 0.001*	60	32	t = 3.72 p = 0.006	98	153
PFMPA	99	A / B	63	100	A	3	95	A / B	7	91	B	7	F = 4.43 p = 0.019	92	54	t = 1.46 p = 0.18	16	11
PFMBA	96	A / B	55	98	A	8	81	C	32	91	B / C	2	F = 13.61 p = 0.002*	90	15	t = 1.63 p = 0.14	84	178
PFEESA	87	B / C	34	100	A	3	91	C	3	84	B	1	F = 24.24 p < 0.001	82	55	t = 1.04 p = 0.33	156	791
NFDHA	53	B	138	92	A	45	68	B	75	58	B	32	F = 20.47 p < 0.001	64	15	t = 2.00 p = 0.080	22	51
HFPO-DA	98	A / B	52	100	A	3	88	C	15	91	B / C	1	F = 8.31 p = 0.001	83	63	t = 3.09 p = 0.015	99	197
ADONA	94	A / B	36	98	A	5	70	C	16	90	B	1	F = 52.33 p < 0.001	81	51	t = 2.90 p = 0.020	33	2
6:2 Cl-PFESA	90	A / B	58	100	A	1	87	B	25	86	B	6	F = 50.91 p < 0.001*	71	21	t = 4.87 p = 0.001	80	100
8:2 Cl-PFESA	92	A / B	71	99	A	2	88	B / C	12	80	C	15	F = 13.22 p < 0.001	72	44	t = 4.22 p = 0.003	92	152
5:3 FTB	34	C	18	88	A	23	36	C	51	49	B	22	F = 110.55 p < 0.001	47	12	t = 5.10 p = 0.001	0	0
5:1:2 FTB	33	C	20	97	A	22	46	B	71	47	B	38	F = 104.57 p < 0.001	39	30	t = 1.89 p = 0.095	4	7
6:2 FTAB	53	B	12	99	A	108	47	B	18	54	B	6	F = 31.18 p < 0.001*	46	20	t = 2.86 p = 0.021	65	162
AP-FHxSA	26	C	7	91	A	50	56	B	10	28	C	11	F = 239.21 p < 0.001	39	15	t = 6.28 p < 0.001	20	150
TAMP-FHxSA	72	B / C	11	102	A	13	74	B	35	66	C	19	F = 64.91 p < 0.001	71	16	t = 0.66 p = 0.53	20	144

less than 20 %. When the concentration of analyte was decreased to 0.5 ng/mL, the number of PFAS recovered within 70-130 % decreased by one to 71, with an average RSD of 10 %. Four PFAS had RSDs greater than 20 %; Br-PFOS (RSD 28 %) is the mixture of multiple isomers included in Wellington's technical grade PFOS standard, and thus it's true spiked concentration was 0.14 ng/mL (not 0.5 ng/mL). 3:3 FTCA, 5:3 FTB and 5:1:2 FTB (RSDs 22 %, 21 % and 25 %, respectively) exhibited greater recovery variance at lower concentrations, likely due to effects previously discussed having a more pronounced effect at ultra-trace levels. When the concentration of analyte was increased to 50 ng/mL, the number of PFAS recovered within 70-130 % decreased by one to 71, with an average RSD of 3.7 %. No PFAS had a recovery RSD greater than 20 % at 50 ng/mL.

Overall the filtered protein precipitation extraction method performed well for concentrations spanning three orders of magnitude, demonstrating confident and consistent recoveries of PFAS at sub-part-per-billion concentrations.

3.4. Validation on human dried blood spots

An individual was sampled daily for seven days and then five times on the eighth day. Their blood was extracted by filtered protein precipitation and analysed for 75 PFAS via LC-MS/MS. 16 PFAS were detected, belonging to four PFAS classes. All PFCAs from C4-C12 (except C5) were detected, as were PFSAs from C4-C8 (except C5). Additionally, 6:2 FTUCA and 6:2 Cl-PFESA were detected (Table S2). The dominant PFAS detected were both branched (Br) and linear (L) isomers of PFOS (Br-PFOS = 1.6 ng/mL (1.4-1.8 ng/mL) and L-PFOS = 1.6 ng/mL (1.4-1.9 ng/mL)), linear PFHxS = 0.87 ng/mL (0.78-0.99 ng/mL) and linear PFOA = 1.4 ng/mL (1.2-1.5 ng/mL). Considering the diluted concentration of these compounds in whole blood compared to serum (approximately 1:2 whole blood – serum PFHxS, PFOS and PFOA concentration) [18], the average concentrations of L-PFOS, ∑PFOS, L-PFHxS and L-PFOA, agree with Australian pooled serum data (L-PFOS = 3.52 ng/mL (SD = 1.06 ng/mL), ∑PFOS = 4.52 ng/mL (SD = 1.45 ng/mL), L-PFHxS = 1.87 ng/mL (SD = 0.96 ng/mL) and L-PFOA = 1.85 ng/mL (SD = 0.32 ng/mL) [32]. This agreement is further reinforced as results of these studies overtime have revealed trends of declining legacy PFAS blood concentrations (Queensland, Australia: PFOA – 2002 = 8.5 ng/mL, 2017 = 1.92 ng/mL, and PFOS – 2002 = 10.9 ng/mL, 2017 = 3.01 ng/mL. For Australian pooled biomonitoring data of the age range 0-15 and 5-15 years.) [32,40], the result of the voluntary phase-out of PFOS by 3M from 2000, and the 2010/2015 U.S. EPA PFOA stewardship program. The individuals blood appears to also follow this trend of declining blood PFAS levels (as $n = 1$ this is solely a logical observation with no statistical significance), however it is not as pronounced as in the 0-15 year age range, due to years of background exposure for adults and the appreciable biological half-lives of these PFAS.

The eighth day sampling indicated overall satisfactory reproducibility within the methodology, with ∑PFAS, Br-PFOS, L-PFOS, L-PFHxS and L-PFOA returning RSDs at or below 10 % across the five replicate samples. Furthermore, only Br-PFOS (11 %) and L-PFOS (11 %) possessed RSDs > 10 % across the seven daily samples. The daily samples were studied for short term trends in blood PFAS. Given the estimated serum half-lives of Br- and L-PFOS, L-PFHxS and L-PFOA range from 1.05 to 2.93 years [21], it is assumed that no detectable change in ∑PFAS will be observed. A linear regression was performed on the concentration of PFAS versus the day sampled (Fig. 4). The analysis failed to disprove the null hypothesis that the slope coefficient is equal to zero, therefore the concentration of blood PFAS overall remained consistent across the course of the week and observed inter-day variation was due to sampling (4 × 2.74 μL ± 5 %) and extraction. Overall, the results demonstrated the methodology to sufficiently extract PFAS from human whole blood in a reproducible manner. To the best of the authors knowledge, this is the most extensive blood PFAS method to date, requiring minimally invasive sampling, and presents a promising

* Welch's test (with Games-Howell post hoc) performed in place of ANOVA with Tukey post hoc or t-test, due to inequivalent variance between datasets
** Heteroscedastic t-test performed in place of homoscedastic t-test, due to inequivalent variance between datasets

Table 2
Internal standard correct recoveries of 75 PFAS at multiple concentrations (0.5, 5 and 50 ng/mL) for a filtered protein precipitation extraction methodology of PFAS from dried blood spots.

PFAS	Overall		0.5 ng/mL		5 ng/mL		50 ng/mL	
	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
PFCA								
PFBA	98	4.3	95	6.4	99	1.6	101	1.3
PFPeA	102	6.5	101	9.7	100	1.3	105	5.7
PFHxA	99	4.8	104	5.0	95	2.4	99	1.4
PFHpA	101	2.0	102	2.5	99	1.4	101	1.7
Br-PFOA	101	6.2	96	6.5	103	3.2	103	6.7
L-PFOA	101	1.9	99	1.6	101	1.2	103	1.0
PFNA	99	2.4	100	3.1	97	1.4	99	1.5
PFDA	105	3.6	104	5.1	106	3.2	104	1.8
PFUnDA	101	2.2	99	1.9	101	1.4	103	1.7
PFDoDA	101	1.4	101	1.8	101	1.1	100	1.5
PFTrDA	98	4.2	100	1.6	93	4.7	100	1.6
PFTeDA	101	2.5	101	2.5	100	3.2	102	1.6
PFHxDA	100	2.3	101	1.8	98	2.4	101	1.3
PFODA	97	4.5	96	5.4	94	1.5	101	2.1
PFSA								
L-PFPrS	104	6.3	105	9.5	101	1.7	105	5.0
L-PFBS	101	4.8	100	8.7	102	0.9	101	1.3
L-PFPeS	99	7.9	98	7.8	102	4.0	98	12
Br-PFHxS	118	28	156	21	100	7.9	98	4.4
L-PFHxS	102	4.1	104	5.5	100	2.1	103	3.5
L-PFHpS	102	3.4	103	4.3	102	3.8	101	2.6
Br-PFOS	109	18	118	28	103	9.4	105	3.7
L-PFOS	102	5.0	99	5.8	103	5.7	104	2.4
L-PFNS	105	1.8	105	2.8	105	1.5	105	1.4
L-PFDS	105	3.8	107	5.8	104	2.0	104	2.5
L-PFUdS	108	3.5	107	4.7	106	2.6	110	1.7
L-PFDoDS	102	5.2	105	4.7	97	3.4	103	4.0
L-PFTrDS	119	12	119	18	111	2.7	128	5.2
PFECHS	99	2.6	100	2.4	98	2.5	99	2.9
8Cl-PFOS	107	7.5	117	3.7	102	1.0	102	2.2
PASF								
FBSA	101	7.6	97	8.1	101	5.6	103	8.9
FPeSA	104	5.5	106	7.5	101	0.7	104	5.8
FHxSA	99	3.7	100	3.9	98	4.2	101	2.8
FHpSA	103	4.4	104	3.2	101	3.9	103	6.2
FOSA	101	4.1	105	3.3	98	4.3	101	1.2
FDSA	134	24	177	2.4	105	4.6	120	4.8
MeFBSA	60	29	79	19	53	7.8	47	11
EtFBSA	32	62	14	171	38	33	44	6.3
MeFOSA	84	6.4	80	8.0	85	3.2	88	4.4
EtFOSA	89	4.9	88	8.1	89	1.8	91	3.3
MeFBSE	98	6.7	93	10	100	0.8	101	3.7
EtFBSE	97	7.1	99	10	94	4.5	97	6.0
MeFOSE	101	5.7	105	7.1	98	4.8	100	0.7
EtFOSE	101	4.9	105	3.4	97	6.2	102	2.3
FOSAA	88	14	74	11	91	3.5	100	2.4
MeFOSAA	100	2.7	97	3.3	100	1.6	102	1.0
EtFOSAA	98	4.0	96	5.4	98	1.2	101	3.0
FTCA								
3:3 FTCA	79	14	78	22	85	6.9	75	6.3
5:3 FTCA	83	11	83	17	88	3.9	79	6.7
7:3 FTCA	78	14	68	18	86	7.4	82	5.8
FTUCA								
6:2 FTUCA	87	6.3	93	3.6	84	1.9	83	4.4
8:2 FTUCA	100	7.0	96	8.5	100	3.5	106	5.3
10:2 FTUCA	103	4.9	101	6.8	106	1.0	101	4.1
FTSA								
4:2 FTSA	110	5.9	114	8.1	111	1.3	106	4.8
6:2 FTSA	107	10	119	5.5	104	3.7	97	2.8
8:2 FTSA	111	8.4	110	12	117	2.7	105	4.2
diPAP								
6:2 diPAP	108	12	122	8.2	102	4.8	99	6.2
6:2/8:2 diPAP	131	7.2	128	3.2	131	8.9	133	9.1
8:2 diPAP	77	10	72	5.7	71	4.1	87	3.5
diSAmPAP	116	13	103	6.5	111	4.9	134	6.7
PFPiA								
6:6 PFPi	100	4.8	104	5.8	98	3.3	99	2.3
6:8 PFPi	100	6.5	101	11	101	1.6	98	3.5
8:8 PFPi	96	8.3	99	11	89	4.3	100	2.5
PFES								
PFMPA	101	1.8	102	1.9	100	1.7	101	1.4

(*continued on next page*)

Table 2 (*continued*)

PFAS	Overall		0.5 ng/mL		5 ng/mL		50 ng/mL	
	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
PFMBA	104	7.9	107	8.6	98	2.8	107	8.1
PFEESA	101	3.0	102	4.3	100	1.7	100	2.8
NFDHA	95	12	91	19	92	7.3	101	7.3
HFPO-DA	100	2.5	102	2.4	100	1.6	99	2.9
ADONA	100	3.9	103	5.4	98	2.2	100	2.5
6:2 Cl-PFESA	95	12	85	18	100	0.8	100	2.3
8:2 Cl-PFESA	106	8.7	117	6.6	99	1.4	102	2.0
C/Z PFAS								
5:3 FTB	89	4.7	92	3.2	88	5.5	86	4.0
5:1:2 FTB	88	15	77	21	97	4.9	91	6.3
6:2 FTAB	94	16	84	25	99	10	100	4.6
AP-FHxSA	94	7.2	95	7.2	91	7.8	96	7.0
TAMP-FHxSA	111	10	124	3.3	102	3.5	106	4.8

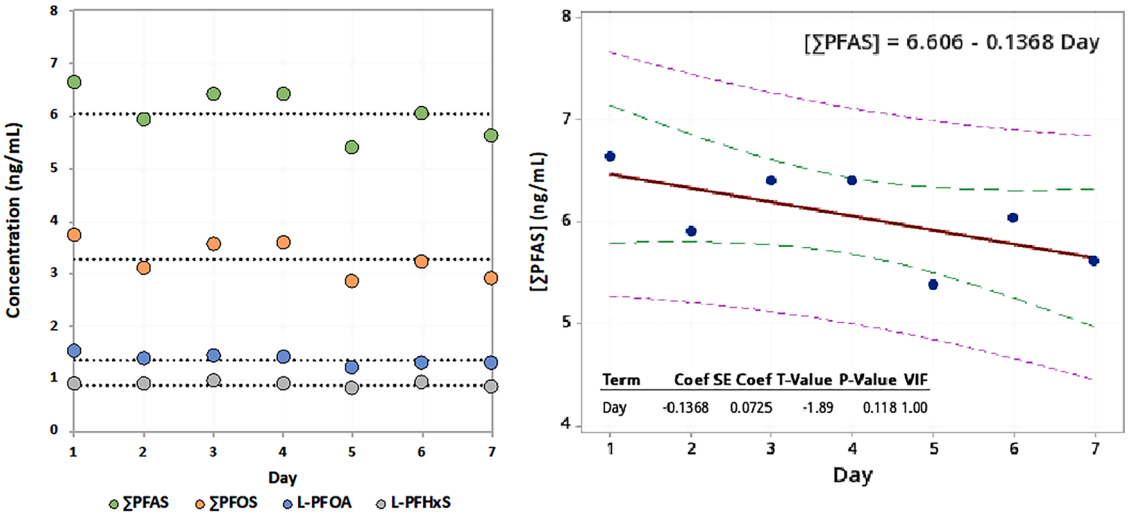

Fig. 4. Left: Plot of blood PFAS concentrations (ng/mL) (n = 1) sampled daily at the same time for a week. Horizontal dotted lines are the weekly average concentration. Right: Regression analysis of total blood PFAS concentration (ng/mL) versus sample day.

opportunity for large scale human biomonitoring applications.

4. Conclusion

A simple, reproducible and efficient microsampling methodology for extracting 72 PFAS from DBS was developed and validated using the hemaPEN®. When extracting trace level analytes from microsamples (4 × 2.74 μL), method simplicity and the minimisation of handling and transfer steps proved most beneficial to consistent analyte recovery and prevention of sample/blank contamination. Protein precipitation methods were found to be the most effective at extracting the majority of PFAS (72 of 75) at concentrations ranging from 0.5 to 50 ng/mL from DBS within acceptable US EPA recovery ranges. The established method could be readily scaled to the quantify larger concentrations, should exposure events warrant it. When applied to a human sample, replicate samples returned consistent results (<20 % RSD) with detected concentrations falling within pre-established Australian data. This methodology demonstrates the coupling of a finger-prick Microsampling technique, with a simple and robust extraction methodology, suitable for PFAS biomonitoring applications.

CRediT authorship contribution statement

Jordan M. Partington: Writing – original draft, Writing – review & editing, Methodology, Investigation, Formal analysis, Visualization. **Jaye Marchiandi:** Methodology, Writing – review & editing. **Drew Szabo:** Methodology, Writing – review & editing. **Andrew Gooley:** Conceptualization, Writing – review & editing. **Konstantinos Kouremenos:** Conceptualization, Writing – review & editing. **Fraser Smith:** Conceptualization, Writing – review & editing. **Bradley O. Clarke:** Project administration, Funding acquisition, Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

Human blood was voluntarily self-sampled with informed consent by a member of the research cohort, in compliance with The University of Melbourne Research Ethics and Biorisk Management Policy (MPF1341) and the Australian Government National Statement on Ethical Conduct in Human Research (2007). Ethics approval was gained through the University of Melbourne STEMM 1 Human Research Ethics Committee (2023-25725-44910-3).

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464522.

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Article

Field-Based Distribution and Bioaccumulation Factors for Cyclic and Aliphatic Per- and Polyfluoroalkyl Substances (PFASs) in an Urban Sedentary Waterbird Population

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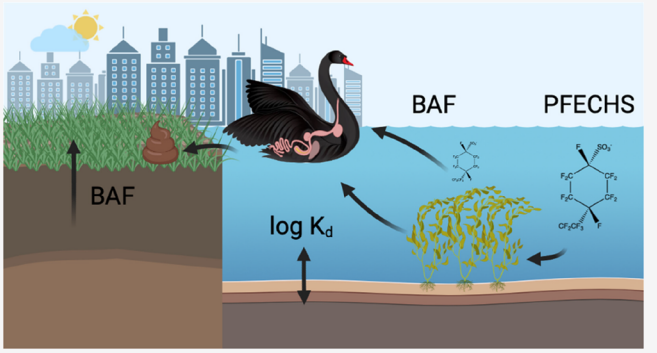
ABSTRACT: The field-based distribution and bioaccumulation factor (BAF) for per- and polyfluoroalkyl substances (PFASs) were determined in residential Black Swans (*Cygnus atratus*) from an urban lake (Melbourne, Australia). The concentrations of 46 aliphatic and cyclic PFASs were determined by HPLC-MS/MS in serum and excrement from swans, and water, sediment, aquatic macrophytes, soil, and grass samples in and around the lake. Elevated concentrations of $\sum_{46}$PFASs were detected in serum (120 ng mL^{-1}) and excrement (110 ng g^{-1} dw) were strongly related indicating a potential noninvasive sampling methodology. Environmental concentrations of PFASs were consistent with a highly impacted ecosystem and notably high concentrations of perfluoro-4-ethylcyclohexanesulfonate (PFECHS, 67584−42−3; $C_8HF_{15}SO_3$) were detected in water (27 ng L^{-1}) and swan serum (16 ng mL^{-1}). In the absence of credible putative alternative sources of PFECHS input to the lake, we propose that the use of high-performance motorsport vehicles is a likely source of contamination to this ecosystem. The BAF of perfluorocarboxylic acids increased with each additional CF_2 moiety from PFOA (15.7 L kg^{-1} ww) to PFDoDA (3615 L kg^{-1} ww). The BAF of PFECHS was estimated as 593 L kg^{-1} ww, which is lower compared with that of PFOS (1097 L kg^{-1} ww).



KEYWORDS: *Per- and polyfluoroalkyl substance (PFAS), perfluoro-4-ethylcyclohexanesulfonate (PFECHS, PFEtCHxS), black swan (Cygnus atratus), avian toxicology, noninvasive sampling*

INTRODUCTION

Characterizing the relationship between the concentration of per- and polyfluoroalkyl substances (PFASs) in the environment and the body burden in wildlife can be difficult in the field due to the spatial and temporal variability of species, sampling ability, and measurement of trace contaminant levels.[1] Long-term changes in emissions of PFASs to the environment further influence the variability for exposure to wildlife.[2] Typically, the field-based bioaccumulation factors (BAF) for PFASs in fish, marine mammals, and birds rely on steady-state assumptions of a species' spatial and temporal variability, but these are often confounded by large foraging ranges.[3] This results in uncertainty around field-based BAFs for birds with wide geographic ranges,[4] as these may be calculated from environmental samples that are not representative of actual exposure. Adding to this uncertainty is the low concentration of PFASs in environmental samples, especially in marine and remote areas,[5] leading to low detection frequency and absence of qualified data. In contrast, urban environments can be more concentrated with PFASs,[6] so the relationship between environmental contamination and body burden can be more accurately measured. The addition of advanced models of bioaccumulation that incorporate degradation of precursors,[7,8] coupled with using residential species with limited ranges in urban environments (with greater levels of PFAS contamination) provides a more robust evaluation of the transport and bioaccumulation pathways of PFASs.

PFASs are commonly detected environmental contaminants with known adverse health effects in humans[9] and wildlife.[10] They have been used in industrial and commercial products since the 1950s and are routinely detected in urban surface waters and sediment as a result of anthropogenic discharge.[11] The ratio of PFAS concentrations in water and sediment (distribution coefficient, K_d) are driven by hydrophobic interactions between the compounds and organic carbon in the sediment,[12] and electrostatic interactions resulting from ionic functional groups.[13] Perfluorooctanesulfonic acid (PFOS;

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Table 1. Summary of Mean Concentrations and Ranges of PFASs in Serum and Plasma, And Selected Tissues from Literature That Have Detected PFECHS in Avian Species[a]

bird	diet	tissue	*n*	PFECHS	PFHxS	PFHpS	PFOS	PFOA	PFNA	PFDA	PFUDA	PFDoDA	reference
Black swan	herbivore	serum	22	16	4.6	1.2	34	0.74	5.4	32	2.9	4.7	this study
(*Cygnus atratus*)				0.43−31	1.4−11	0.24−2.5	6.8−61	0.19−2.2	1.2−16	4.7−79	0.43−9.1	0.41−19	
Peregrine falcon	carnivore	plasma	57	0.12−1.27[b]	0.26−1.16[b]	NA	11.8−29.7[b]	0.42−1.20[b]	0.46−5.25[b]	1.39−2.49[b]	1.58−3.08[b]	1.18−2.53[b]	Sun et al.[88]
(*Falco peregrinus*)				0.03−3.96	0.09−4.89		4.97−124	0.11−2.38	0.83−21.8	0.45−9.65	0.78−9.84	0.24−4.05	
Common eider	piscivore	egg	5	0.21	0.90	0.36	17	0.22	1.4	0.74	1.3	0.81	de Wit et al.[35]
(*Somateria mollissima*)				0.17−0.25	0.89−0.91	0.29−0.43	12−22	0.21−0.22	1.1−1.7	0.49−0.99	0.84−1.7	0.58−1.0	
Common eider	picivore	liver	5	<0.4	<0.62	0.12	12	<0.03	1.4	0.86	0.98	0.46	de Wit et al.[35]
(*Somateria mollissima*)						0.082−0.15	5.7−19		0.68−2.2	0.32−1.4	<0.09−1.9	0.20−0.72	
Common guillemot	picivore	egg	5	2.9	1.2	2.5	390	0.34	4.8	7.5	19	6.1	de Wit et al.[35]
(*Uria aalge*)				2.7−3.1	1.2−1.2	2.2−2.8	370−400	0.32−0.36	4.3−5.2	7.2−7.9	18−20	6.0−6.2	
White-tailed eagle	carnivore	egg	5	2.5	1.1	3.0	170	0.84	17	9.2	13	4.6	de Wit et al.[35]
(*Haliaeetus albicilla*)				2.3−2.7	1.1−1.1	3.0−3.0	160−170	0.83−0.85	13−21	7.1−11	12−15	4.5−4.8	
Bald eagle	carnivore	egg	22	0.22	1.35	0.66	106	0.04	1.90	4.56	10.3	2.01	Wu et al.[89]
(*Haliaeetus leucocephalus*)				0.04−21.9	0.02−10.7	0.17−3.50	28.5−1338	nd−0.26	0.14−14.9	0.71−42.0	2.26−107	0.27−26.3	
Caspian tern	piscivore	egg	30	0.55−0.81[b]	0.49−0.66[b]	NA	387−1395[b]	0.3−1.38[a]	8.77−11.5[b]	27.9−69.4[b]	35.8−61.6[b]	10.6−22.3[b]	Su et al.[90]
(*Hydroprogne caspia*)				0.06−1.84	nd−4.61		114−4660	0.05−4.78	1.76−32.3	9.14−219	10.61−118	3.2−95.9	
Herring gull	omnivore	egg	50	0.15−0.28[b]	0.34−0.84[b]	NA	103−170[b]	0.22−0.35[b]	1.94−4.19[b]	4.57−6.62[b]	5.51−9.59[b]	2.32−4.09[b]	Su et al.[90]
(*Larus argentatus*)				nd−1.01	0.07−11.5		34.6−660	nd−0.87	0.33−8.02	1.20−12.7	1.74−15.2	0.87−8.64	
Double-crested cormorant	piscivore	plasma	66	<0.17		290−1112[bc] (NR)				75−171[bd] (NR)			De Silva et al.[91]
(*Phalacrocorax auritus*)													
Herring gull	omnivore	egg	100	0.115−1.03[b]	0.010−1.44[b]	NA	56.3−247[b]	0.018−0.32[b]	0.30−3.96[b]	2.25−6.39[b]	3.66−8.99[b]	1.56−3.94[b]	Letcher et al.[92]
(*Larus argentatus*)				nd−3.1	nd−11.5		14.1−660	nd−0.87	0.17−12.2	0.85−12.3	1.32−19.0	0.87−8.64	

[a]Serum = ng mL^{-1}, egg, and liver = ng g^{-1} ww. [b]Range of means from several colonies or years. [c]$\sum$PFSA; [d]$\sum$PFCA; NA = not analyzed; nd = not detected; NR = not reported.

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$C_8HF_{17}O_3S$) and perfluorooctanoic acid (PFOA; $C_8HF_{15}O_2$) have been historically discharged to the environment from aqueous-film-forming foams (AFFFs) and production of surface protection in textiles and carpets, among other sources.[14] Discharge from stormwater in residential areas is primarily driven by concentrations of PFASs in precipitation,[15] while in urban areas with commercial and industrial activity, concentrations of PFASs increase in nearby surface water bodies due to discharge of contaminated waste and soil.[16] The contamination of surface water bodies by PFASs can be further exacerbated by accidental discharges of aqueous-film-forming foam (AFFF),[17] PFAS-containing waste,[18] or other currently unknown sources unique to specific urban environments that may be present around the world, such as those from leisure activities, that is, motorsports and powerboating. Since the discovery of the global distribution of PFOS in the environment,[19] up to 40 replacement classes of PFASs can now be detected in the environment,[20,21] complicating efforts to characterize the fate and impact of substances with various chemistries.

One specific PFAS of interest is perfluoro-4-ethylcyclohexanesulfonate (PFECHS, 67584−42−3), a compound with potential endocrine-disrupting effects.[22] The sole use of this compound is currently documented in aviation-grade hydraulic fluid, formally under the trade name FC-98, for its ability to prevent evaporation, fire, and corrosion.[23] Since 2011, elevated concentrations (1−195 ng L^{-1}) of this compound have been reported in surface waters related to airports in Asia[24] and North America,[23,25,26] with one river containing potential discharge from no fewer than five airports upstream of the sampling site.[27] PFECHS has also been detected in precipitation in the high arctic, as a result of atmospheric transport and deposition,[28] although concentrations were orders of magnitude lower than airport-impacted waters (<0.0003−0.020 ng L^{-1}). There is limited evidence at this time to suggest that wastewater treatment plants (WWTPs) are sources of PFECHS, despite being known emitters of legacy PFASs.[29]

The increasing environmental contamination by PFASs means these compounds are commonly detected in many species. Birds are often used as biomonitors for the health of an ecosystem, specifically higher trophic order species, which are vulnerable to bioaccumulative toxicants in the environment. PFASs are detected in a range of birds around the world. Average serum concentrations of PFOS, the most abundant and commonly detected compound, range from <1 to 43 428 ng mL^{-1} (Supporting Information (SI) Table S6). Short-chain perfluoroalkylsulfonic acids (PFSAs; $C_{n<6}$) and perfluoroalkylcarboxylic acids (PFCAs; $C_{n<8}$) are not typically detected in avian species, while perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUDA) and perfluorododecanoic acid (PFDoDA) are detected in relatively higher concentrations (SI Table S6). Due to the high BAF for long-chain PFASs,[30] some of the highest reported environmental contamination in birds were reported in Antwerp, Belgium, an area of highly modified human development near a PFAS manufacturing plant, responsible for historic production of PFOS and PFOS-related substances.[31] The majority of studies that report the field-based bioaccumulation or biomagnification of PFASs are conducted on Arctic species, such as gulls and kittiwakes,[32] guillemots,[33] ducks,[34] and sea eagles.[35] PFECHS has been identified in the egg and liver from birds in the northern hemisphere, predominantly in higher-trophic order piscivorous species (Table 1). This indicates the potential for biomagnification of PFECHS from dietary exposure and the transfer to offspring. PFECHS is bioaccumulative in fish (log BAF = 2.5−3.0),[23] but as noted by de Wit et al.[35] there has been no direct evidence of bioaccumulation or biomagnification of this compound in birds. Again, previous studies on the environmental exposure of birds to PFASs have focused on highly migratory birds or species with wide geographical ranges, which can make it difficult to understand the relationship between local environmental contamination and body burden.

We report on the development of a field-based values for the transport and bioaccumulation of cyclic and aliphatic PFASs throughout an urban lake system and discuss a potential novel source of PFAS to the environment. Concentrations of 46 PFASs were determined in water, sediment, soil (abiotic), macrophyte, grass and a resident population of Black Swan (*Cygnus atratus*) serum and excrement (biotic) matrices. The bioaccumulation factor (BAF) was determined for PFECHS for the first time in avian species. The calculated values were validated by comparing the field-based distribution coefficients (K_d) and BAF with values of legacy PFASs, such as PFOS and PFOA. Concentrations of PFASs were compared between paired serum and excrement from Black Swan samples to explore the use of a noninvasive sampling technique for future biomonitoring research.

MATERIALS AND METHODS

Study Site and Species. The study was conducted at Albert Park Lake (37° 50′ 50″ S, 144° 58′ 14″ E), an artificial 45 ha water body in the central business district of the metropolitan city of Melbourne, Australia, the ninth most populous city in the Southern Hemisphere and second-most populous city in Australia, with a population of more than 5.1 million people. The roads encircling Albert Park Lake have been the site of motorsport events since 1953, including the current annual Formula One Australian Grand Prix (Figure 1).

Albert Park Lake is home to a resident population of Black Swans (*Cygnus atratus*, hereafter "swans"). A large number of swans on the lake (including all individuals that participated in this study) are tagged with numbered neck collars, allowing them to be individually recognized. At first capture, the age of adult individuals were conservatively estimated via plumage development and weight, at least one year or older from the date of first capture (SI Table S4). Since exact age was not known for most birds, age was not included as an explanatory variable in statistical analyses. Since 2006, a monthly census of the swans on the lake has been conducted, during which the identities of all tagged swans present on the lake are recorded. Additional sightings of tagged swans in other locations in the greater Melbourne area are contributed through a citizen science program which allows for upload of sightings into a web-based app (myswan.org.au).[36] Between June 2006 and December 2019, the individuals sampled in this study were observed 3145 times (range 1−522 observations per individual). Ninety-seven percent of these sightings (n = 3052) were at Albert Park Lake.[36] Thus, this population can be considered resident and there is a very high likelihood that substrate matrices in and around Albert Park Lake are their primary PFAS exposure sources. Swans are typically herbivorous, with their diet primarily consisting of aquatic macrophytes and benthic algae.[37] Albert Park Lake is a popular recreation site and the swans are sometimes fed human food products. However, the excrement of the swans sampled consisted primarily of undigested plant material (data not shown) and besides potential exposure to PFAS-containing

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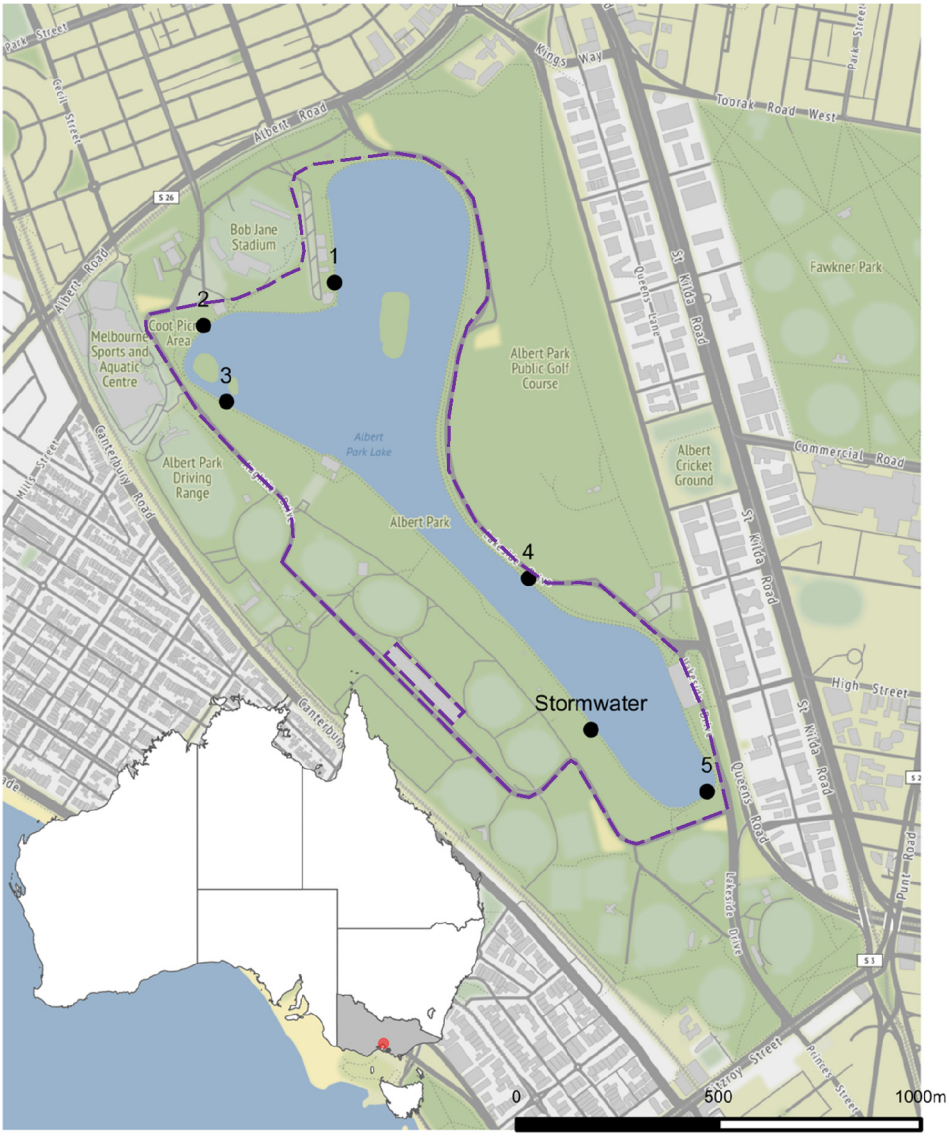

Figure 1. Map of the study area with sites 1−5 shown along with stormwater (SW) sampling location in Albert Park, Melbourne, with inset of Australia and relative location of Victoria (gray) and sampling location (red). The Albert Park Circuit and pit lane are highlighted in purple.

packaging,[38] human foods, such as bread, are not contaminated with PFAS.[39]

Sampling and Sample Preparation. Male (n = 10) and female (n = 12) adult swans were lured onto shore and captured by hand on the 4th and 10th December 2019, nonlethal sampling was conducted with the permission of the Australian Bird and Bat Banding Authority (Authority 1405), DWELP/Parks Victoria (10008176), and the University of Melbourne Ethics Register (1814554.1). For each bird, head-to-bill length was measured with digital callipers (±0.1 mm) and total body mass was measured by spring balance (±0.1 kg). Approximately 1 mL of blood was collected from restrained swans by medial metatarsal venipuncture using a 25 G needle into an 8 mL heparinised tubes (BD Vacutainer, PST II). The blood was stored on ice before being centrifuged (3392g, 15 min) and the serum was frozen at −20 °C until analysis. During handling, swans invariably defecate; paired samples of excrement (∼10 g ww) were also collected in 50 mL polypropylene (PP) centrifuge tubes and kept frozen at −20 °C until analysis.

Water, sediment, soil, aquatic macrophyte (*Cycnogeton* sp.), and grass (Gramineae) samples were collected from in and around the lake on the fourth December 2019. At each site (Figure 1), three surface water samples were collected into 50 mL PP centrifuge tubes at approximately 15 cm depth. The water was treated with sodium azide (∼500 mg) to prevent microbial activity. Adjacent to each water sampling site, sediment, soil aquatic macrophyte, and grass samples were collected into 50 mL PP centrifuge tubes in triplicate. Sediment and soil samples were not available at every site; for instance where the banks were reinforced with stones and where the pavement covered the soil. In each case, approximately 10 g of ww soil and sediment were scraped from the surface with the PP tube, which includes the interphase area. Water samples were kept refrigerated (4 °C), while soil and sediment samples were frozen (−20 °C) until extraction and analysis. Soil and sediment samples were freeze-dried for analysis, and fraction of organic carbon (f_{oc}) was determined in dried sediment samples by loss on ignition.

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Water Extraction. PFASs were extracted from water by solid-phase extraction (SPE), in a method adapted from Coggan et al.[40] Briefly, weak anion exchange cartridges (Oasis WAX, 150 mg) were preconditioned with 4 mL each of methanol, 0.1% ammonium hydroxide in methanol and ultrapure water. The samples (50 mL) were spiked with 1 ng of the mass-labeled internal standard mix and loaded on the cartridge that was eluted under a vacuum (~17 kPa) at a rate of approximately 2 mL min^{-1}. The cartridges were then treated with 4 mL of pH 4 buffer (15 mM sodium acetate/0.1 mM acetic acid) and allowed to dry under a vacuum. The sample collection tubes were washed with 2 mL of methanol and collected through the SPE cartridges into 15 mL PP centrifuge tubes. The cartridges were eluted with a further 4 mL of 0.1% ammonium hydroxide in methanol. The eluent was evaporated to dryness at 35 °C under a gentle stream of ultrapure nitrogen gas and then reconstituted to 200 μL. The eluent was then transferred to 250 μL PP autosampling vials for LC-MS/MS analysis.

Soil, Sediment, Plant, and Excrement Extraction. Samples of soil (n = 10), sediment (n = 10), grass (n = 7), macrophytes (n = 7), and excrement (n = 22) were homogenized by shaking with ceramic pellets (2000 rpm, 1 h). Approximately 1 g of sample was transferred to a 50 mL PP centrifuge tube. Each sample was spiked with 25 ng mass-labeled internal standard mix and 5 mL of 10 mM sodium hydroxide in methanol. Samples were then vortexed (2000 rpm) and sonicated for 30 min, respectively. Acetic acid (100%) was added to neutralize the extract and 50 mg each of octyldecylsilane (C18) and primary secondary amine (PSA) were added before centrifugation (3392g, 15 min). The supernatant was then filtered (0.45 μm, PES) and transferred to a 1 mL PP autosampling vial for analysis.

Serum Extraction. Serum samples were prepared for analysis by protein precipitation method, extracting PFASs with acetonitrile.[41] Serum (200 μL) was transferred to a 2 mL PP centrifuge tube and 2.5 ng of mass-labeled PFAS mix was then added to each serum sample. Each sample was then diluted to 500 μL with acetonitrile where a white precipitate was formed. Each sample was then briefly vortexed (30 s, ~2000 rpm) and centrifuged (2000g, 10 min) before the supernatant was transferred to a 1 mL PP autosampling vial for analysis.

LC-MS/MS Analysis. Forty-six PFASs were analyzed by LC-MS/MS, including 11 perfluoroalkyl carboxylic acids (PFCA), nine perfluoroalkyl sulfonic acids (PFSA), three perfluoroalkyl phosphinic acids (PFPiA), three fluorotelomercarboxylic acids (FTCA), four fluorotelomersulfonic acids (FTSA), nine perfluoroalkanesulfonyl fluorides (PASF), four disubstituted fluorotelomerphosphate diesters (diPAP), and three perfluoroalkylether acids (PFEAAs). Naming conventions for PFASs are according to Wang et al.[42] and a complete list of PFASs and CAS numbers are available in SI Table S1. The analysis was performed on an Agilent 1290 Infinity II liquid chromatography system coupled with an Agilent 6495C triple quadrupole mass spectrometer. Chromatographic separation of PFASs was achieved in a 11.5 min gradient elution (15 min acquisition) using a 50 mm C18 Zorbax Eclipse column (Agilent, Mulgrave, Victoria) using 2 mM ammonium acetate aqueous phase and 100% methanol organic phase. Chromatographic separation for PFECHS in a 4 min gradient elution (6 min acquisition) using a 100 mm PFP Poroshell column (Agilent, Mulgrave, Victoria) using 2 mM ammonium acetate aqueous phase and 100% methanol organic phase, where successful separation of PFECHS from its isomers, such as perfluoropropylcyclopentanesulfonate (PFPCPeS) was achieved.[24] A detailed summary of each analytical method, including successful separation of PFECHS (SI Figure S2) and its Level 1 HRMS confirmation, can be found in the Supporting Information. Concentrations for each matrix are reported as follows: water (ng L^{-1}), sediment, soil, grass, macrophyte and excrement (ng g^{-1} dw), and serum (ng mL^{-1}).

Quality Assurance and Quality Control (QAQC). Quality of data was verified by the addition of a method blank (MB) and laboratory control sample (LCS) for each matrix, the latter of which was spiked with 25 ng native PFAS mix. One LCS and MB was included with each batch of six water samples, 11 serum samples, 11 excrement samples, seven plant samples, and 10 soil and sediment samples. The average internal standard response from the samples and QAQC were compared to the average internal standard response from the calibration curve. A detailed report on QAQC results can be found in SI Figure S4. Briefly, internal standard recoveries for each mass-labeled compound were between 36% and 110%. The internal standard corrected recoveries for LCS were between 66% and 130%, except for perfluorobutane-1-sulfonamide (FBSA) (44%), perfluorooctane-1-sulfonamide (FOSA) (20%), 3:3 fluorotelomercarboxylic acid (3:3 FTCA) (8%), 5:3 FTCA (7%), and 7:3 FTCA (11%) in water, which were omitted from the analysis. The concentration of PFASs in the MB were below the method detection limit for each compound in each matrix, respectively.

Data Treatment, Statistical Analysis, and Modeling. Data were acquired and quantitated using Agilent MassHunter Workstation and Quantitative Analysis 10.1 respectively. The method reporting limit (MRL) was defined by the lowest calibration level for compounds with $S/N > 3{:}1$. Samples with $S/N < 3$ for primary transition were defined as nondetects (n.d.). Concentrations of PFASs in samples had to meet the following conditions for quantification: (1) S/N for primary transition >10:1, (2) ISTD recovery response between 50% and 150%, (3) concentration greater than the lowest calibration level, (4) retention time within 5% of highest calibration result, (5) qualifying ion ratio (where available) within 20% of highest calibration result. Results that did not meet one or more of these conditions were treated as < MRL.

Descriptive statistics, statistical analyses and data visualization were performed with R v4.0.2[43] and RStudio (1.2.5019, Boston, MA) with tidyverse v1.3.0.9000,[44] ggplot2 v3.2.2,[45] ggmap v3.0.0[46] and NADA v1.6−1.1[47] packages. Descriptive statistics for compounds detected in >50% samples from each matrix were calculated by nonparametric Kaplan−Meier (KM) survival estimates for mean, standard error and 95% confidence interval.[48] All values are reported to two significant figures. Differences in concentrations of the $\sum_{46}$PFAS in abiotic matrices between sites were determined by analysis of variance (ANOVA) and post hoc Tukey pairwise analysis was performed on significant results. Differences in concentrations of the $\sum_{46}$PFAS in serum and excrement between male and female swans were determined by Welsh's t test. The linear relationship between paired serum and excrement samples from each bird were analyzed by generalized liner model for compounds with 100% detection frequency. Compounds with detection frequencies >50% (censored data) were analyzed by maximum likelihood estimate (Tobit) analysis for linear regression. The average distribution coefficient between water and sediment (K_d) and the soil organic carbon−water distribution coefficient (K_{oc}) from four sites (n = 4) was calculated as described by Sima and Jaffé:[15]

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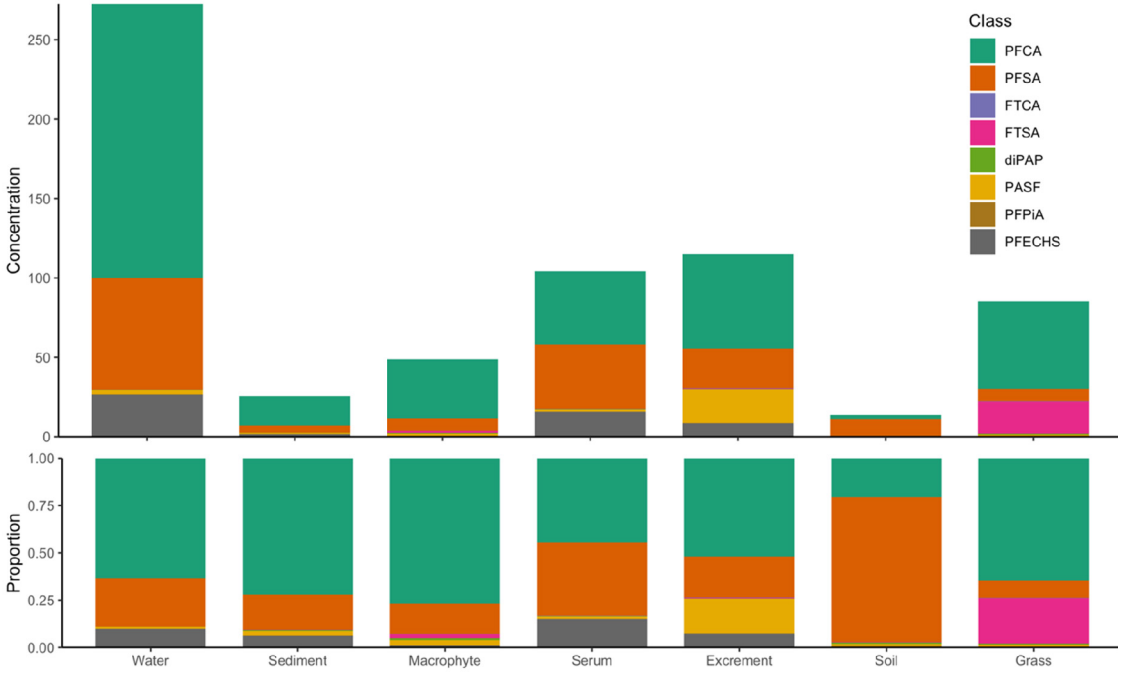

Figure 2. Plot of average concentrations and proportions of seven classes of PFASs and PFECHS from water (ng L^{-1}), sediment (ng g^{-1} dw), macrophyte (ng g^{-1} dw), swan serum (ng mL^{-1}), swan excrement, soil and grass (ng g^{-1} dw) from Albert Park, Melbourne, Australia in 2019.

$$K_d = \frac{S_e}{C_w}$$

$$K_{oc} = \frac{K_d}{f_{oc}}$$

Where S_e equals the mean concentration of each compound in sediment (ng g^{-1} dw), C_w equals the mean concentration of each compound in water (ng mL^{-1}) and f_{oc} is the fraction of organic carbon, assuming homogeneity of the sediment between sites and linearity of the isotherm.

The field-based bioaccumulation factor (BAF) was determined by the relationship between mean concentraions of PFASs in soil and grass (mg kg^{-1} dw), water and macrophyte (g mL^{-1} dw) and water and swan (L kg^{-1} ww) for compounds that were detected in >50% of samples, respectively. The error in the calculated K_d, K_{OC}, and BAF are calculated with Gaussian Propogation Error based on the mean and standard deviation of concentrations of PFASs in water, sediment and serum, respectively. The average BAF was calculated as described by Arnot and Gobas [3]:

$$BAF = \frac{C_{plant/animal}}{C_{water/soil}}$$

Body condition (the energy capital accumulated by the bird as a result of feeding), was determined by calculating the scaled mass index (SMI) for each individual.[49] The Thorpe-Lleonart model was applied to the mass-length data from a large data set from measurements of male and female Black Swans from 2006 to 2020 (unpublished data):

$$\text{SMI: } \hat{M}_i = M_i\left[\frac{L_0}{L_i}\right]^b$$

Where M_i and L_i are equal to the observed body mass and head-to-bill length of individual i respectively, L_0 is equal to the mean head-to-bill length of the population, and b is equal to the standardized major axis (SMA) regression exponent. L_0 and b were calculated for male (L_0 = 142.7 mm; b = 3.494) and female (L_0 = 132.8 mm; b = 3.823) swans, respectively. The scaled mass index for each swan can be found in SI Table S4.

RESULTS AND DISCUSSION

Summary of Overall PFAS Concentrations. Thirty-five of the 46 PFASs tested for in this study were detected in at least one matrix (water, sediment, macrophytes, serum, excrement, soil, or grass). Average concentrations of $\sum_{46}$PFAS were 252 ng L^{-1} in water, 25 ng g^{-1} dw and 14 ng g^{-1} dw in sediment and soil, 51 ng g^{-1} dw and 85 ng g^{-1} dw in macrophytes and grass, 123 ng mL^{-1} dw and 113 ng g^{-1} dw in swan serum and excrement (Figure 2). A complete summary of mean PFAS concentrations and ranges for each matrix can be found in SI Table S5. PFCAs and PFSAs were the most frequently detected (100%) and abundant compound classes in the majority of samples from all matrices. PFECHS was detected in all aquatic matrices and the serum and excrement of swans, while 4:2 fluorotelomersulfonic acid (4:2 FTSA) was detected >50% in grass and FBSA was detected >50% in water and swan excrement (see summary statistics presented in SI Table S5). Three PFEAAs (6:2 Cl-PFESA, 8:2 Cl-PFESA, and ADONA) were not detected in any matrix sampled in the study area. Compounds from FTCA, FTSA, PASF, and diPAP groups with frequency of detections <50% in each matrix will not be discussed further (SI Data Tables S7 − S13).

PFAS Concentrations in Abiotic Matrices. Compounds frequently detected in both water and sediment from Albert Park Lake were PFOA, PFNA, PFDA, PFUDA, PFDoDA, perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), and PFOS, each having detection frequencies >50%. PFOA had the highest mean concentration in water (47 ± 3.1 ng L^{-1}), followed by PFOS (31 ± 9.6 ng L^{-1}) and PFHxS (12 ± 0.56 ng L^{-1}). The mean concentrations of PFOA, PFOS,

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and PFHxS in this study exceed the concentrations found in the catchment from the closest major waterway (~1.5 km), where concentrations were 1.7−2.2 ng L^{-1}, 5.1−13.9 ng L^{-1}, and 3.0−7.0 ng L^{-1}, respectively.[50] Furthermore, average concentrations of PFOS exceed the Australian guideline for 99% species conservation (0.23 ng L^{-1}) and the maximum concentration of PFOS (151 ng L^{-1}) exceeded the 95% species conservation guideline of 130 ng L^{-1} [51].

The compounds with the highest mean concentrations in sediment were PFDoDA (3.8 ± 1.2 ng g^{-1} dw), PFDA (3.5 ± 1.0 ng g^{-1} dw), PFOA (2.9 ± 1.4 ng g^{-1} dw), and PFOS (2.9 ± 1.0 ng g^{-1} dw), respectively. The profile of increased concentrations of PFCAs and PFSAs with greater CF_2 moieties is well described other studies[52] and is influenced by the water−sediment distribution characteristics described below. Concentrations of PFOS in sediment from Albert Park Lake are less than sediment typically impacted by legacy AFFF discharge (<0.2−1660 ng g^{-1} dw)[53] and are more comparable with estuarine sediment from urban areas of Australia (2.1 ± 2.0 ng g^{-1} dw).[54]

PFECHS was detected in 100% of water samples and in 40% of sediment samples with concentrations ranging between 1.3−44 ng L^{-1} and <0.35−7.2 ng g^{-1} dw, respectively. The concentration of PFECHS in this lake is higher than average concentrations found in the Laurentian Great Lakes region (0.2−5.7 ng L^{-1}),[23] and is similar to the ranges reported from surface waters adjacent to airports in China (1.0−324 ng L^{-1})[24] and Canada (4.3 ± 1.4 ng L^{-1}).[26] Elevated concentrations of PFECHS recently discovered in surface waters in Melbourne have been linked to the uncontrolled discharge of PFAS-containing chemical wastes (<0.05−77 ng/L^{-1}).[18]

PFASs were not commonly detected in soil, with only one PFCA and two PFSAs detected in >50% of soil samples from four sites surrounding Albert Park lake. The most frequently detected compounds were PFOS with a mean concentration of 9.6 ± 1.5 ng g^{-1} dw, while concentrations of PFHxS and PFDA were lower at 0.9 ± 0.2 ng g^{-1} dw and 1.2 ± 0.3 ng g^{-1} dw, respectively. The mean concentration of PFOS in soil were within the range of the Australian ecological guideline for indirect exposure of 10 ng g^{-1} for the dietary exposure of secondary consumers.[51] Concentrations of PFDA, PFHxS, and PFOS from Albert Park are consistent with soils from agricultural, residential and industrial areas (0.179−1.07 ng g^{-1} dw, n.d.−0.276 ng g^{-1} dw, and 8.6−10.4 ng g^{-1} dw, respectively) that are not impacted by AFFF discharge.[55] PFECHS was only detected in 10% of soil samples from Albert Park Lake ranging from <0.3 to 0.4 ng g^{-1} dw. To our knowledge, PFECHS concentration has not been reported in the soil from any field-based study.

Here we report some of the strongest positive correlations between concentrations of PFASs in surface waters ever observed in the field (SI Figure S7), indicating that either (1) there are two distinct sources of PFASs to the surface water environment at Albert Park, or (2) the water−sediment distribution is impacting the concentration of PFAS in water. The first group of compounds include PFBA, PFPeA, PFHxA, PFOA, PFBS, PFPeS, PFHxS, FBSA, and PFECHS, all of which were strongly correlated with each other (Spearman's $R > 0.70$, $p < 0.05$). The second group of compounds include PFNA, PFDA, PFUDA, PFDoDA, and PFOS, and were also strongly correlated (Spearman's $R > 0.80$, $p < 0.05$). There was no clear correlation between compounds in each grouping, indicating there was no relationship between concentrations of these compounds. While the source of PFECHS to Albert Park is currently unknown, sources from aircraft, pulse stormwater events, or infiltration from contaminated groundwater is unlikely because the catchment area is zoned entirely for residential and mixed commercial areas (SI) that do not contain heavy or specialized industry.[16,56] A likely source of PFECHS is runoff from roads around the perimeter of the lake which are traversed by high-performance race cars during the annual Australian Grand Prix. Information on the nature and type of synthetic lubricants that are used by competing manufacturers, the number of cars participating on the circuit, or the number of accidents at Albert Park Circuit is not available in the public domain. However, given that weight minimization, heat control and braking are critical demands of motorsport engineering, teams can gain significant competitive advantage by using sophisticated, ultralight hydraulic systems such as miniature servovalves that have been developed for aircraft, missiles and spacecraft and which rely on high-temperature, flame-resistant aircraft hydraulic fluids. Thus, the source of this particularly unique PFAS should be investigated further here and other locations throughout the world hosting high-performance racing events, for cars and other specialist vehicles and boats.

Field-based Distribution Coefficients. The field-based log distribution coefficients (log K_d) and the log organic-carbon corrected distribution coefficients (log K_{oc}) were calculated from the Kaplan−Meier mean concentrations of compounds that were detected in >50% of both water and sediment samples including PFECHS (Table 2). The log K_d for PFCAs increased with the number of CF_2 moieties, from PFOA (1.79 mL g^{-1}) to PFDoDA (3.47 mL g^{-1}). The average difference in log K_d from PFOA to PFDoDA increased by an average of 0.4 mL g^{-1} per CF_2 moiety which is consistent with previous literature which reports an increase in laboratory-derived log K_d by 0.5−0.6 mL g^{-1} per CF_2 moiety.[12] The log K_d for PFOS and PFECHS were 1.97 and 1.83 mL g^{-1}, respectively, demonstrating that the cyclic PFECHS tends to partition slightly more strongly to water, compared to its acyclic C_8 (PFOS) counterpart. The distribution coefficient for both PFOS and PFECHS was previously reported downstream from the Beijing Capital International Airport at 2.84 and 1.74 mL g^{-1} respectively.[24] The difference in log K_d between PFOS and PFECHS in this study (0.14 mL g^{-1}) is not similar to the previous report of 1.1 mL g^{-1} by Wang et al.[24] this may be due to differences in the f_{oc} (11%) or pH (9) between the sites (SI Table S2). Overall, the water−sediment distribution of PFASs comparable with organic carbon normalized K_{oc} from field-based studies where log K_{oc} of PFOA and PFOS in sediments ranged between 1.3−4.5 and

Table 2. Summary of Log_{10}-Transformed Distribution Coefficient (Log K_d) and Organic Carbon Normalized Distribution (Log K_{oc}) between Water and Sediment at Four Sites at Albert Park Lake, Melbourne, Australia 2019

compound	log K_d (L kg^{-1} dw)	log K_{oc} (L kg^{-1} dw)
PFOA	1.79 ± 0.08	2.75 ± 0.34
PFNA	2.00 ± 0.23	2.96 ± 0.31
PFDA	2.16 ± 0.03	3.12 ± 0.40
PFUDA	3.05 ± 0.12	4.00 ± 0.48
PFDoDA	3.47 ± 0.07	4.42 ± 0.47
PFOS	1.97 ± 0.04	2.93 ± 0.36
PFECHS[a]	1.83 ± 0.07	2.80 ± 0.34

[a]Calculation of K_d and K_{oc} included for PFECHS 40% detection in sediment.

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2.5−4.7 units, respectively.[57] In each case, the calculation of the field-based water−sediment distribution is advantageous in urban environments where elevated concentrations of PFASs are likely to occur. It has been noted by Zareitalabad et al.[57] that field measurements of K_d are typically higher than those measurements in laboratories, however, the difference in K_d between the CF_2 moieties should be consistent and allow comparison between studies. As the field-based distribution of PFASs is consistent with previous literature values, the distribution coefficient measured for PFECHS can be used for future transport and fate modeling that are used to inform risk assessments for this cyclic compound.

PFAS Concentrations in Aquatic Macrophytes and Grass. PFCAs and PFSAs were the most detected compounds in aquatic macrophyte samples. Concentrations of $\sum_{11}$PFCAs and $\sum_{9}$PFSAs averaged 38 ng g^{-1} dw and 11 ng g^{-1} dw, respectively (SI Figure S6). The mean concentration of PFASs in order of decreasing concentration was PFDA (15 ± 4.0 ng g^{-1} dw), PFOS (7.5 ± 1.4 ng g^{-1} dw), and PFOA (6.8 ± 2.0 ng g^{-1}). The mean concentration of PFECHS in macrophyte samples was 0.4 ± 0.1 ng g^{-1} dw and as far as the authors are aware, is the first report of PFECHS in aquatic macrophytes. PFOS and PFOA were found in one species of aquatic macrophyte from an estuarine lagoon in Italy were reported at considerably lower average concentrations of 0.5 ng g^{-1} dw, where anthropogenic discharge to the river was the only identified source of contamination.[58] PFOS concentrations reported in aquatic macrophytes from Albert Park Lake are orders of magnitude lower than toxicity thresholds for the plant and secondary consumers where impacts on biomass, chlorophyll, soluble protein, and enzyme activity are measured in mg L^{-1}.[59]

Grass from the foreshore had four PFCAs, two PFSAs and 4:2 FTSA were detected in >50% of samples from three sites. In order of decreasing concentrations, PFBA, 4:2 FTSA, and PFHxA average concentrations in grass were 36 ± 7.5 ng g^{-1} dw, 21 ± 9.0 ng g^{-1} dw, and 16 ± 3.2 ng g^{-1} dw, respectively, and mean PFOS concentrations were lower at 1.9 ± 0.5 ng g^{-1} dw. Short-chain PFASs are more readily transpired in plants due to their higher solubility and lower sorption to soils, which can lead to higher concentrations.[60] N:2 FTSAs and their precursors, such as 6:2 FTSA, have been primarily used in the formulation of fluorotelomer-based AFFF and food packaging materials.[61] Given 4:2 FTSA is not commonly reported in environmental studies of PFASs,[62] it does not infer the presence of 6:2 FTSA due to the limited information on the correlation between the compounds. The trend for short-chain PFAS to accumulate in grasses is consistent with concentrations of PFAS detected in grass irrigated with AFFF-impacted groundwater, where PFBA and PFHxA were reported at 11 ± 8 ng g^{-1} ww and 3 ± 2 ng g^{-1} ww, respectively, but PFOS concentrations were considerably higher compared with this study at 32 ± 28 ng g^{-1} ww.[63]

PFAS Concentrations in Black Swans. Seven PFCAs, four PFSAs, and PFECHS were detected in >50% of swan serum samples (n = 22). There was no clear difference between $\sum_{46}$PFASs between male (n = 10) and female (n = 12) swans (Welsh's t = −0.27606, df = 18.392, p = 0.7856, Figure 3), although due to the uncertainty in the age of the birds, further research is needed to confirm the distribution of PFASs among the population with regards to age. The compounds most abundant in swan serum were PFOS (34 ± 3.1 ng mL^{-1}), PFDA (32 ± 4.1 ng mL^{-1}), and PFECHS (16 ± 1.6 ng mL^{-1}). The individual mean concentrations of other PFASs ranged between 0.3 and 5.4 ng mL^{-1} (SI Table S5). Mean PFOS concentrations

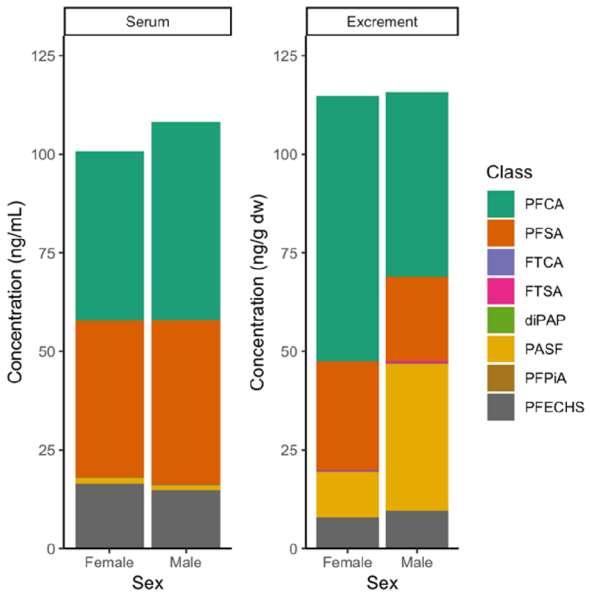

Figure 3. Mean concentrations of PFASs from seven classes of compounds and PFECHS from serum from male (n = 10) and female (n = 12) Black Swans (ng mL^{-1}) and excrement (ng g^{-1} dw) resident to Albert Park Lake.

in swans were similar to levels detected in plasma from White-tailed Eagle nestlings in the Arctic from 2008 to 2010 (25.4−38.7 ng g^{-1} ww),[64] and from 2011 to 2012 (40.9 ng g^{-1} ww),[65] despite this species occupying a much higher trophic level compared to swans. Concentrations of PFOS in swans were, as expected, less than levels typically detected in bird species near fluorochemical facilities or AFFF-impacted sites.[6,66,67] On the other hand, PFDA concentrations in serum from swans in this study were equivalent to birds from highly impacted areas from AFFF and manufacturing (SI Table S7).

Additionally, the PFECHS concentrations found in serum were significantly higher than those previously detected in species from the northern hemisphere, where maximum concentrations for guillemot eggs averaged 2.9 ng g^{-1} ww (Table 1). Although it is difficult to compare the concentrations from blood, liver and egg, typically elevated concentrations of PFASs in one tissue are correlated with elevated concentrations in another tissue−particularly liver and blood.[68] It is likely that there are also elevated concentrations of PFECHS in the liver and eggs of this population of swans given the evidence for the enrichment of some PFAAs during maternal transfer,[69] potentially causing negative impacts to the birds (see Bioaccumulation and Impact).

In swan serum, the relationship between PFAS concentrations is more complex, PFCAs were strongly correlated with compounds that had the nearest number of CF_2 moieties and PFCAs between C8 and C12 were correlated with PFOS (SI Figure S7). PFOS was also strongly correlated with concentrations of PFECHS and PFHpS. Differences between the correlations of PFASs in water and serum are likely due to the uptake and depuration rates of each of the compounds, indicated by the BAF reported below (Table 2). Long-chain PFCAs and PFOS bioaccumulate in swans more strongly than short-chain PFCAs, PFSAs, and PFECHS.

A further six PFCAs, PFOS, PFECHS, and FBSA were detected in >50% of excrement samples (SI Table S5). In order of decreasing mean concentration PFOS (26 ± 5.1 ng g^{-1} dw),

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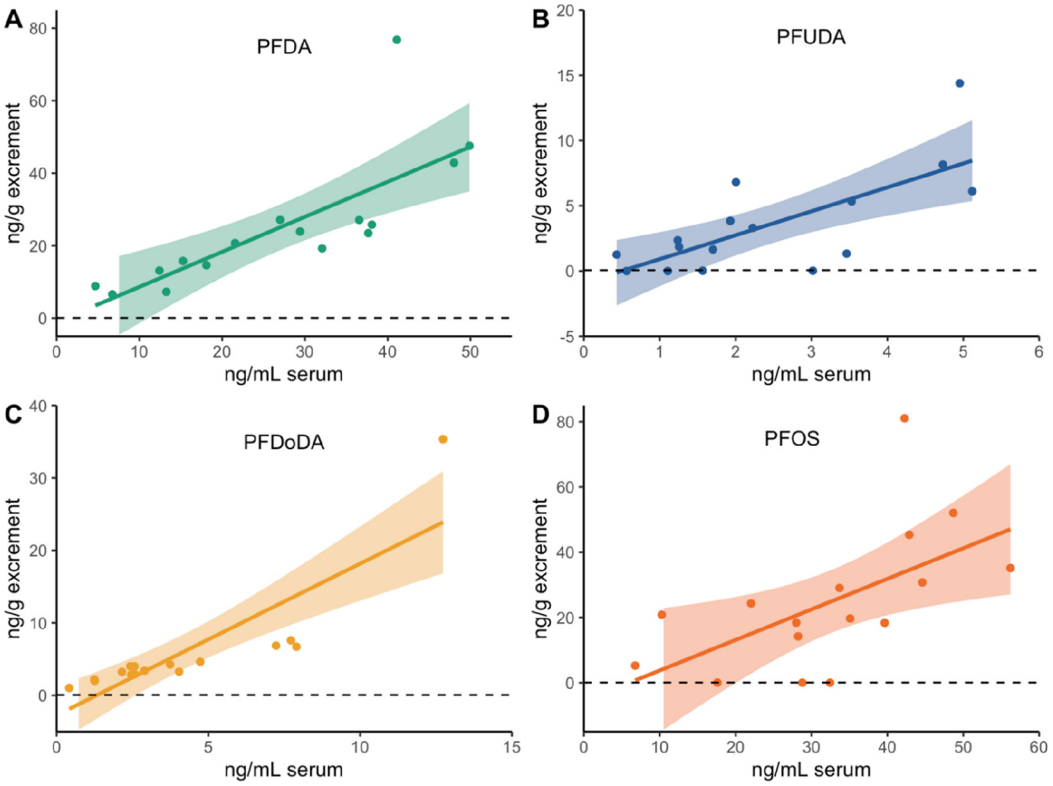
Table 3. Plot of Excrement (ng g^{-1} dw) and Serum (ng mL^{-1}) Concentrations in Swans for PFDA, PFUDA, PFDoDA, and PFOS[a]



compound	coefficient	intercept	*p*-value	R^2_{adj}	*F*-statistic	log-likelihood	censored observations
PFDA	0.9668	−1.0457	0.0004	0.5678	20.7	–	0
PFUDA	2.2158	−2.1425	0.0002	–	–	−34.25347	4
PFDoDA	2.0917	−2.741	0.0001	0.6876	34.02		0
PFOS	1.0212	−10.4599	0.0076	–	–	−60.33017	3
$\sum_{46}$PFAS	1.0752	13.4379	0.048	0.1976	4.694	–	0

[a]The dotted line represents the method reporting limit. Estimates for the coefficient and intercept of the linear model calculated by GLM or MLE with *p*-value and test-statistics where applicable. Note: there is no equivalent of R^2 for MLE analysis.

PFDA (25 ± 4.5 ng g^{-1} dw), PFBA (20 ± 3.8 ng g^{-1} dw), and PFECHS (8.4 ± 2.2 ng g^{-1} dw) were detected in excrement. The only other field-based study on PFASs in animal excrement reported concentrations of PFOS and PFDA, among others in domestic cats (2.67 ± 5.70 ng g^{-1} dw and 7.86 ± 7.87 ng g^{-1} dw, respectively) and dogs (3.34 ± 1.97 ng g^{-1} dw and 15.9 ± 50.1 ng g^{-1} dw, respectively) from the U.S., indicating potentially elevated contamination in their food.[70]

From the compounds most frequently detected in both serum and excrement, there was a strong linear relationship between the concentrations of PFDA, PFUDA, PFDoDA, PFOS, and $\sum_{46}$PFAS (Table 3). Conversely, there was no statistically clear relationship between concentrations of PFOA, PFNA, and PFECHS between serum and excrement (−0.4 < Spearman's *R* < 0.4, $p > 0.05$). Birds exposed to dietary PFASs accumulate the compounds in their tissues,[71] and it is accepted that diet is a primary pathway of exposure in the environment.[72] As many PFASs are highly persistent and resistant to degradation or metabolisation,[61] urine, excrement, and maternal transfer are the only known depuration pathways.[73,74] Typically, feathers have been used to estimate the body burden of persistent organic pollutants in birds, but this technique gives inconsistent results for PFASs.[75,76] There is potential for the estimation of PFAS exposure and body burden in animals via the analysis of excrement, representing a novel noninvasive sampling technique and allow for more routine and frequent monitoring campaigns in the future. The interpretation of the current results must be considered as there is a limited sample number (n = 22) with only one residential species, with a diet that is not well described and unknown ages of the birds. Further research is recommended to confirm the faecal model presented in this study in other avian species, both migratory and residential, marine and terrestrial.

Bioaccumulation and Impact. The field-based bioaccumulation factor (BAF) was determined in swans for nine PFASs, with detection frequencies >50% from both water and serum (Table 4). The BAF for PFCAs increased from PFOA (15.7 L kg^{-1} ww) to PFDoDA (3615 L kg^{-1} ww), indicating a positive trend in BAF for compounds with increasing carbon lengths. The increase in BAF for PFCAs with additional CF_2 moieties is consistent with previous studies. For example, Kelly et al.[34] described the trophic biomagnification of long-chain PFCAs in ducks from an Arctic food-web study, where the log-biomagnification factor increased from PFOA (3.28) to PFDA (8.29). The BAF for PFSAs in this study increased with additional CF_2 moieties from PFHxS (408 L kg^{-1} ww) to PFOS (1097 L kg^{-1} ww), although elevated concentrations of PFHpS in serum resulted in an unusually high BAF (2069 L kg^{-1} ww). The BAF for PFSAs in fish will increase with additional CF_2 moieties,[77] although the BAF for PFHpS in birds is not well

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Table 4. Summary of Field-Based Bioaccumulation Factors (BAF) for Macrophytes, Grass and Swans from Water and Soil, Respectively, from Albert Park Lake, Melbourne, Australia 2019[a]

compound	macrophyte (mL g^{-1} dw)	grass (mg kg^{-1} dw)	swan (L kg^{-1} ww)
PFOA	145 ± 2	NC	15.7 ± 0.3
PFNA	690 ± 2	NC	931 ± 2
PFDA	625 ± 2	NC	1333 ± 3
PFUDA	1125 ± 3	NC	1813 ± 3
PFDoDA	2769 ± 3	NC	3615 ± 3
PFHxS	NC	NC	408 ± 2
PFHpS	NC	NC	2069 ± 2
PFOS	242 ± 2	198 ± 2	1097 ± 3
PFECHS	14 ± 1	NC	593 ± 2

[a]NC = not calculated for compounds detected <50%.

understood. Normally, the estimated ratio of predator−prey concentrations for PFHpS is lower than PFOS, but in certain circumstances, PFHpS can be higher than PFOS.[35] It can be difficult to compare the results of this study to previous findings due to the differences in matrices studied (i.e., serum vs liver) and also that swans occupy a lower trophic order than the species typically studied. In piscivorous and carnivorous birds, the biomagnification factor reported for PFOS is typically greater than that of PFHxS and PFHpS,[33,35] indicating that a higher trophic level may be a primary factor in a higher BAF, rather than simply the chain length of the PFASs. Overall, the results from this study are congruent with reports for other species, where the BAF for swans is in the same order of magnitude as those found for other birds, as well as fish and invertebrates.[78]

The BAF for PFECHS was determined to be 593 L kg^{-1} ww and is lower than PFHpS (2069 L kg^{-1} ww) and PFOS (1097 L kg^{-1} ww), which the latter is its acyclic C8 equiv. To our knowledge, this is the first reported bioaccumulation factor for the cyclic PFAS in birds. The elevated PFECHS concentrations in swan serum indicate a significant exposure to contaminated water which may be of importance in other urban lakes throughout the world. Currently, information on PFECHS is limited by few, if any, peer-reviewed studies on its occurrence and toxicity and the risk to aquatic organisms are difficult to assess. Laboratory-based ecotoxicological exposure experiments found that chronic PFECHS exposure at 6×10^5 ng L^{-1} has resulted in the up- and down-regulation of gene transcription in *Daphnia magna* but no acute end points (i.e.: lethality, reproduction) were observed at these relatively high concentrations.[22] However, PFECHS concentrations in water negatively impact the growth of aquatic algae species at environmentally relevant concentrations as low as 100 ng L^{-1}.[79] As a result, PFECHS has been identified as an emerging PFAS that requires further study into the toxicity specifically to apex predators in the environment.[80] More investigation is required to assess the impact of PFECHS on this urban ecosystem and the swans in this study.

Alongside BAFs calculated in swans, the BAF for seven PFASs were determined from water to aquatic macrophytes sampled in Albert Park Lake in 2019 (Table 4). The BAF for macrophytes increased with increasing chain length for PFCAs from PFOA (145 mL g^{-1}) to PFDoDA (2769 mL g^{-1}). The BAF for PFOS and PFECHS were 242 and 14 mL g^{-1}, respectively, indicating each of these compounds are not strongly bioaccumulated to aquatic macrophytes. Similarly to the BAF in macrophytes, the BAF for PFOS from soil to grass was determined to be 198 mg kg^{-1} dw soil−the only compound to be detected in >50% of samples of both matrices. The BAF of PFASs in nonagricultural plants is not frequently reported in the literature, however, the use of plants has been explored as a bioremediation strategy.[60] In contrast to the results from this study, edible crops exposed to biosolid-impacted soil, the BAF of PFASs decreased with increasing carbon chain length for both PFCAs and PFSAs.[81] More investigation is needed to elucidate the observed differences in the behavior of PFASs in aquatic macrophytes.

For the most frequently detected PFASs in swan serum, only PFHxS had a moderate negative correlation between serum concentration and scaled mass index (Spearman's $R = -0.53$, $p = 0.012$). Complete information on swan age, sex, body morphometrics, and body condition is reported in SI Table S4. While PFHxS is a suspected thyroid endocrine disrupting compound in birds,[82,83] it remains to be determined if the exposure of PFHxS is inducing a negative impact on the birds condition, as it is possible that there are impacts of unknown contaminants that the birds are exposed to from the lake. High concentrations of PFOS and PFHxS negatively impacted weight gain in female Northern Bobwhite Quails (water: 375 ng[PFOS +PFHxS] L^{-1}),[84] which may be due to the decreased thyroid hormone activity in birds[82,83,85] and increase in the basal metabolic rate.[86] Due to the relatively small sample size and small variation in concentrations of PFASs between swans, further studies on the thyroid hormone levels in the population of swans and on the presence of other toxic compounds on the lake is recommended to confirm or deny the impact and risk of PFHxS and other contaminants in this system.

Implications and Future Work. Concentrations of PFASs in Albert Park Lake exceed the concentrations of nearby surface waters in Melbourne[50] and the Australian guideline values for the conservation of 99% of species.[51] This result was unexpected given the lack of any sources typically associated with PFAS contamination in the residential and light commercial catchment area (SI Figure S1). The concentrations of PFECHS, a cyclic PFAS, exceeded concentrations detected in other negatively impacted surface waters and sediments from around the world, although there are few other measurements to compare with the results reported in this study. Sources of PFASs to the surface water are likely from two distinct sources; however, the source of PFECHS is unknown but may be linked to the use of aviation-grade hydraulic fluid in high-performance vehicles on the Albert Park Formula One Circuit. Further investigation is recommended to confirm the potential contamination as a result of motorsports at this site and others internationally. Environmental distribution and bioaccumulation were described for the cyclic PFECHS, where the distribution coefficient (log K_d) was determined for concentrations of PFECHS in water and sediment (1.83 L kg^{-1} dw), which were congruent with previous studies.[87] PFASs were present in the serum of a resident avian species and the field-based bioaccumulation factor (BAF) was determined for several substances, including PFECHS, in an avian species for the first time (593 L kg^{-1} dw). Occurances of PFASs in swan serum and excrement were strongly related in this resident population, There were no observable impacts from PFASs on the body condition of the Black Swan, except for PFHxS, which was showed a moderate negative correlation. This study has demonstrated that urban lakes may be important field locations for developing modeling data necessary for risk assessment for

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novel PFAS. As more than 60% of humanity will be living in cities by 2030 and parks such as these are important areas of leisure and recreation, pressure is increasing on the local environment and public health. Therefore, continued research on the occurrence and impact of emerging contaminants, such as PFASs, is highly recommended in urban areas and remote protected areas equally.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01965.

Site information; details on methods; confirmation of PFECHS; supplementary figures and summary tables (PDF)

Raw concentrations of compounds detected for each sample in each matrix (XLSX)

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Author Contributions

D.S.: Writing − original draft, methodology, investigation, formal analysis, visualization. D.M.: Methodology, investigation, writing − review & editing. R.A.M.: Project administration, writing − review & editing, investigation, supervision. M.P.G.: Writing − review & editing, investigation, supervision. B.O.C. Project administration, Funding acquisition, conceptualization, investigation, supervision, writing − review & editing.

Notes

The authors declare no competing financial interest.

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High-resolution temporal wastewater treatment plant investigation to understand influent mass flux of per- and polyfluoroalkyl substances (PFAS)

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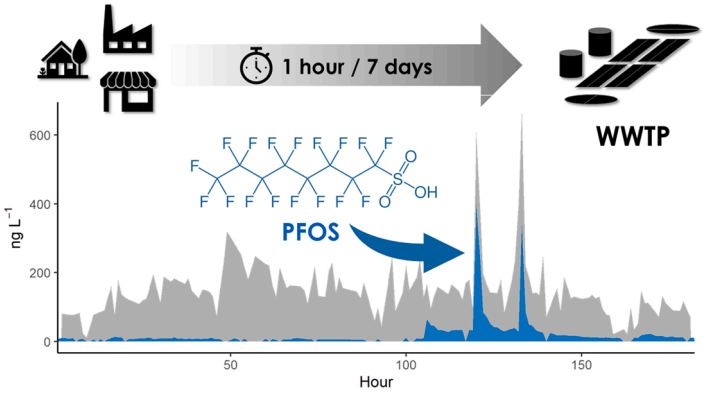
HIGHLIGHTS

- Concentrations of 50 PFAS were measured for 168 consecutive hours in WWTP influent.
- Daily average $\sum_{50}$PFAS concentrations differed significantly over 7-days.
- A minimum of nine randomly collected grab samples per day is recommended.
- Pulse events increase PFAS concentrations up to 10-times compared with daily averages.
- Instantaneous grab samples should not be used to estimate annual mass flux.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to identify sources of per- and polyfluoroalkyl substances (PFAS) to wastewater treatment plants (WWTPs) and reveals previously undescribed variability in daily PFAS concentrations by measuring their occurrence in WWTP influent each hour over the course of a week. $\sum_{50}$PFAS concentrations ranged between 89 ± 38 on Monday and 173 ± 110 ng L^{-1} on Friday, where perfluoroalkyl carboxylic acids (PFCAs), disubstituted phosphate esters (diPAPs), and perfluoroalkyl sulfonic acids (PFSAs) contributed the largest proportion to overall weekly concentrations 37%, 30%, and 17% respectively. Simultaneous pulse events of perfluorooctanesulfonic acid (PFOS; 400 ng L^{-1}) and perfluoroheptanesulfonic acid (PFHpS; 18 ng L^{-1}) indicate significant industrial or commercial waste discharge that persists for up to 3 h. The minimum number of hourly grab samples required to detect variation of PFOS and PFHpS concentrations are 7 and 9 samples respectively, indicating a high degree of variability in PFAS concentrations between days. Overall, the risk of sampling bias from grab samples is high given the variability in PFAS concentrations and more frequent sampling campaigns must be balanced against the cost of analysis carefully to avoid the mischaracterisation of mass flux to receiving surface waters.

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1. Introduction

Identifying the sources of per- and polyfluoroalkyl substances (PFAS) emitted to wastewater treatment plants (WWTPs) would allow chemical manufacturers and industrial users of these substances to prevent their inevitable release to the environment. Contaminated waste can then be diverted to landfill or emerging PFAS remediation strategies, such as incineration, sonication, or advanced oxidation and reduction [1]. PFAS are a family of emerging contaminants that contain at least one saturated and aliphatic CF_3 or CF_2 moiety [2], which includes over 6 million compounds in the PubChem database [3], and approximately 4700 compounds of interest identified earlier by the OECD [4]. Perfluorooctanoic acid (PFOA, $C_8HF_{15}O_2$) and perfluorooctanesulfonic acid (PFOS, $C_8HF_{17}SO_3$) were discovered to be widespread in the environment, wildlife and humans in the early 2000s due to their historic use in commercial and industrial products [5]. The negative impacts associated with exposure to these compounds resulted in their use being restricted by the Stockholm Convention in many countries [6]. Generally, the chemical industry has shifted to the production of PFAS that are more quickly eliminated by organisms, such as short-chain perfluorocarboxylic acids (PFCAs, $C_{n<7}$) and perfluorosulfonic acids (PFSAs, $C_{n<6}$) or compounds that degrade to short-chain PFCAs and PFSAs, in an attempt to mitigate the risk associated with their exposure [7].

Waste contaminated with PFAS from commercial and industrial applications (hereafter "*trade waste*") results in discharge to the environment through direct emissions from the source and secondary emissions through waste streams such as WWTPs [8]. Regardless of aerobic and anaerobic treatments performed at WWTPs, PFCAs and PFSAs are poorly metabolised or degraded due to the strong CF bond [9]. Moreover, concentrations of PFCAs and PFSAs can increase throughout the primary and secondary treatment of wastewater due to the transformation of precursor compounds into their stable end products [10,11]. Discharge of WWTP effluent contaminated with PFAS to receiving waters can increase the risk of negative human [12,13] and ecological health impacts [14].

Estimates of total PFAS mass flux (kg $year^{-1}$) from WWTPs are commonly employed to help complete mass balance inventories and risk to downstream environments [11,15,16]. However, the variability in concentrations of PFAS in wastewater over time is not well understood and often overlooked due to its complexity. Discharge of trade waste to municipal sewer systems can account for the majority of the fraction of PFAS to WWTPs [17] and can account for significant variability of PFAS concentrations in WWTPs over periods of weeks or months [18]. Other credible sources of temporal variation in concentrations of emerging contaminants include residential inputs from cosmetics [19], food packaging [20], numerous household products [21], and infiltration from contaminated groundwater during periods of high precipitation [22]. In each case, the impacts of each of these sources on PFAS concentrations in WWTP influent will change depending on the conditions of the local sewer network, further limiting comparative temporal analysis between regions.

Chemical inventories of PFAS can reveal trends in overall usage and disposal of these chemicals over time [7], where international restrictions and concerns related to long-chain PFCAs and PFSAs appear to reflect decreasing concentrations of these compounds in WWTPs [10]. Between 2010 and 2020, decreasing concentrations in WWTP influent was reported for numerous compounds, including PFOS (−23.7%) and PFOA (−10.2%), with evidence for a shift from long-chain to short-chain replacements in that time [23]. There were no long-term temporal trends for the concentrations of PFOS and PFOA from eight WWTPs in Switzerland between 1993 and 2008 [24], as the sampling was conducted before PFOS had its use restricted by the Stockholm Convention in 2009 [6]. In a study of two WWTPs over four years between 2014 and 2017, there was no observable change in overall mass flux per capita (μg day^{-1} $person^{-1}$) due to insufficient resolving power from the number of samples taken [25]. Longer or more frequent sampling campaigns are required to resolve temporal trends in usage and concentrations of PFAS in WWTPs. To date, there are no short-term temporal studies for the occurrence of PFAS in WWTPs over a day or a week, limiting the accuracy for 1) the identification of sources throughout the sewage network, and 2) the estimation of overall average concentrations received or discharged from WWTPs. To help identify sources and temporal trends of legacy and emerging PFAS to WWTPs, sampling techniques must be applied correctly in influent samples to ensure confident estimations of their concentrations.

This study aims to investigate the short-term temporal variability of 50 PFAS concentrations in WWTP influent each hour for a week (n = 168), the highest resolution temporal study to date. A single plant was selected as a case study due to the lack of consistent inputs to respective WWTPs and to provide an example of the variability of PFAS concentrations to WWTPs. Changes in the concentration and detection frequency of each compound over a week were monitored to exemplify various sources of PFAS, including suspected pulsed trade waste discharge. The number of random grab samples required each day to detect changes in daily mean concentrations was determined to allow for more cost effective sampling campaigns to measure the mass flux of PFAS in aqueous systems. Variations in mean PFAS concentrations over daily time intervals were expected to significantly impact the estimation of mean input to WWTPs. This study will help to ensure the accuracy of future assessment and monitoring of a wide range of PFAS in WWTPs, where the associated risks are better reflected by the true rates of discharge to the environment.

2. Methodology

2.1. Chemicals and reagents

High-purity methanol (MeOH, 67–56–1), sodium azide (>99.95%, 26628–22–8), ammonium acetate (>99.99, 631–61–8), anhydrous sodium acetate (>99.99%, 127–09–3), and acetic acid (>99.99%, 64–19–7) were purchased from Merck Millipore (Victoria, Australia). Type I ultrapure water was obtained from reverse osmosis water coupled with a MilliQ Reference A+ system (18.2 Ω TOC <5 ppm, Merck, Victoria, Australia). Weak anion exchange solid-phase extraction cartridges (WAX, 3 cc, 60 mg) were obtained from Waters Corporation (New South Wales, Australia). The pH 4 buffer solution was prepared with 15 mM sodium acetate and 0.1 mM acetic acid in ultrapure water.

Primary PFAS standards (n = 50) and mass-labelled surrogates (n = 18) were obtained individually from Wellington Laboratories (Ontario, Canada). A complete list of PFAS, their names and CAS numbers are available in Table S1. Briefly, PFAS included in this study include eleven perfluoroalkyl carboxylic acids (PFCAs), nine perfluroalkanesulfonic acids (PFSAs), five fluorotelomersulfonic acids (FTSAs), ten perfluoroalkanesulfonyl fluorides (PASFs), three perfluoroalkyl phosphinic acids (PFPiAs), four fluorotelomer phosphate diesters (diPAPs), and eight perfluoroalkyl ether acids (PFEAAs).

2.2. Study area and autosampling collection

Raw influent from an undisclosed WWTP in Southeast Australia was macerated and pumped to an above-ground polyvinyl chloride (PVC) manifold. Wastewater from the manifold was sampled every hour for 182 h (n = 182) with a silicone tube by peristaltic pump using an automated collection device into cleaned and rinsed 1 L high-density polyethylene (HDPE) bottles (Hach Sigma 900 Auto Sampler). The population normalised total influent flow rate (L day^{-1} pp^{-1}) was measured each hour for the entire sampling period and is reported in Fig. S1. Sampling began at 19:00 on 10 November 2019 and concluded at 04:00 on 18 November 2019 AEDT (UTC +11). Each day, a homogenised aliquot from each hourly sample was transferred to a 50 mL polypropylene centrifuge tube and sent to the laboratory for analysis.

The samples were then preserved (~0.5 g sodium azide) and stored at 4 °C until extraction and analysis.

The majority of stormwater is not collected in the WWTP catchment but rather diverted to local surface water catchments for direct discharge to the environment. Nonetheless, sampling was scheduled for a period with low precipitation to minimise the effect of inflow and groundwater intrusion throughout the sewer network over the sampling period. During the sample collection period, an average of 0.3 ± 0.8 mm day^{-1} (0–4.8 mL) of rainfall was recorded from six weather stations in the catchment area of the WWTP [26].

2.3. Per- and polyfluoroalkyl substance extraction

The extraction method was based on a method described by Szabo et al. [27]. Briefly, an aliquot of the mass-labelled internal standard mix (1 ng[^{13}C-PFAS]) was added to each water sample (50 mL). The samples were then centrifuged to separate suspended solids from the aqueous phase (3200 *g*, 10 min). WAX cartridges were pre-conditioned by elution of 3 mL 0.1% ammonium hydroxide in methanol, methanol and water respectively. The supernatant from the centrifuged water samples was loaded at a rate of approximately 1 mL min^{-1} under vacuum (17 kPa), after which the cartridges were treated with 4 mL pH 4 buffer and allowed to dry. Methanol (2 mL) was added to the sample container, including suspended solids, and then eluted through the cartridge with a further 4 mL 0.1% ammonium hydroxide in methanol. The organic eluent was captured in a 15 mL polypropylene centrifuge tube where the solvent was evaporated to dryness and reconstituted to 0.200 mL with methanol until transferred to a 0.250 mL polypropylene vial for analysis.

2.4. LC-MS/MS analysis

Instrument analysis was performed with an Agilent 1290 Infinity II liquid chromatography system coupled with a 6495 C tandem mass spectrometer [28]. Chromatography was achieved over a 15-minute acquisition using an Agilent C18 Zorbax Eclipse column (1.8 μm x 50 mm) and a short guard column with an identical stationary phase. The mobile phase consisted of 2 mM ammonium acetate aqueous phase and 100% MeOH organic phase with the following gradient of MeOH: t_0 = 10%, $t_{0.5}$ = 10%, $t_{2.5}$ = 55%, t_9 = 90%, $t_{9.1}$ = 100%, $t_{11.5}$ = 100%, $t_{11.6}$ = 10%. Transitions and collision energies were optimised for each compound with analytical standards and a summary can be found in Table S1. The source conditions were as follows: drying gas temperature and flow = 250 °C at 11 L/min, sheath gas temperature and flow = 375 °C at 11 L/min, nebuliser pressure = 25 psi, capillary and nozzle voltage = 2500 and 1500 V, and iFunnel high and low-pressure RF = 90 and 60 V. A ten-point internal standard corrected calibration curve was used to quantify the concentrations of each analyte ranging between 0.05 and 50 ng mL^{-1} ($R^2 > 0.99$), whilst mass-labelled concentrations remained constant in calibration levels at 5 ng mL^{-1} in MeOH.

2.5. Quality assurance and quality control (QAQC)

Field blanks were collected by transferring a 50 mL aliquot of ultrapure water into the centrifuge tubes used for analysis at the same time as the samples were transferred at the WWTP. Quality of extraction was verified by the addition of a laboratory control sample (LCS) and method blank (MB) each with the corresponding batch of a maximum of 10 samples randomly selected from the total pool. The LCS and MB were prepared using 50 mL of ultrapure water, where the LCS was spiked with a 5 ng mL^{-1} native PFAS mix (1 ng[PFAS]) and both the LCS and MB were spiked with a 5 ng mL^{-1} mass-labelled internal standard mix (1 ng [^{13}C-PFAS]). Concentrations of PFAS from the LCS were compared with the spiked concentration to calculate the recovery of each compound. The concentration of PFAS in the field blanks and method blanks were below the method detection limit for each compound. A detailed report on QAQC results can be found in Fig. S2. The average internal standard response from the samples and QAQC were compared to the average internal standard response from the calibration curve. Briefly, the mean internal standard corrected recoveries for LCS fell between 75% and 137%, except for 8:2 diPAP (169 ± 47%) which were omitted from the results.

The method reporting limit (MRL) was defined by the lowest calibration level for compounds with S/N > 10:1. Samples with S/N < 3 are defined as non-detects (n.d.) and samples 3 < S/N < 10 were defined as <MRL. Concentrations of PFAS in samples had to meet the following conditions for quantification: (1) S/N > 10:1, (2) concentration greater than the lowest calibration level, (3) retention time within 5% of highest calibration result, (4) qualifying ion ratio (where available) within 20% of highest calibration result. Results that did not meet one or more of these conditions were treated as <MRL. All PFAS concentrations in wastewater are internal-standard corrected and are reported in ng L^{-1}.

2.6. Data analyses

Data were acquired and quantitated using Agilent MassHunter Workstation 10.0 and Quantitative Analysis 10.1 respectively. Descriptive statistics, statistical analyses and data visualisation were performed with R [29] and RStudio (1.2.5019, Boston, Massachusetts, USA) with tidyverse v1.3.1 [30], ggplot2 v3.3.5 [31] and rstatix v0.7.0 [32] packages. From the pool of 182 samples collected between 10 November and 18 November 2019, only samples between 11 November and 17 November were used in the analysis (n = 168), representing days with comprehensive sampling. For statistical analysis, concentrations for PFAS <MRL were substituted with MRL/2. The arithmetic mean, standard deviation, Spearman's correlation, and principal component analysis were calculated for compounds with > 50% detection frequency in at least one 24-hour period. The normality of concentrations each day was tested by Shapiro-Wilks for compounds with > 50% detection frequency in at least one day. Differences in concentrations of $\sum_{50}$PFAS between days were compared by analysis of variance (ANOVA) with Tukey's post hoc analysis. The effect size for ANOVA was calculated by generalised eta squared (η^2_G). The compact letter display indicates a significant difference in $\sum_{50}$PFAS between days. G*Power v3.1.9.6 [33] was used to calculate the minimum number of hourly grab samples required each day to detect a significant change in concentration for each compound detected in over 50% of samples with a statistical power of 0.80 and using their respective eta squared effect sizes. The level of confidence for all tests is reported as $\alpha = 0.05$. Outliers were determined for values > 99% confidence interval for total concentrations over the seven days.

3. Results and discussion

3.1. Overall PFAS frequencies of detection and concentrations

Fifty PFAS were measured in influent wastewater from an Australian WWTP each hour for one week (n = 168) during a dry weather period. PFAS were detected in 100% of the samples and the most abundant classes of PFAS over the seven-day sampling period were PFCAs (53 ± 52 ng L^{-1}), diPAPs (44 ± 25 ng L^{-1}), PFSAs (25 ± 50 ng L^{-1}), FTSAs (14 ± 16 ng L^{-1}), and PASFs (8.4 ± 17 ng L^{-1}). PFEAAs and PFPiAs were not frequently detected above the MRL with average concentrations of 0.66 ± 0.74 ng L^{-1} and 0.23 ± 0.13 ng L^{-1} respectively, so will not be discussed further (Table S3).

The average concentration of PFCAs tended to decrease with increasing chain length, from perfluorobutananoic acid (PFBA) (20 ± 35 ng L^{-1}; *f*= 68%) to perfluoropentanoic acid (PFPeA) (15 ± 38 ng L^{-1}; *f* = 44%), perfluorohexanoic acid (PFHxA) (6.3 ± 11 ng L^{-1}; 42%), perfluoroheptanoic acid (PFHpA) (1.1 ± 1.2 ng L^{-1}; *f*= 56%), except for PFOA (8.2 ± 7.7 ng L^{-1}; *f* = 97%). The C_9 to C_{14} PFCAs all presented low concentrations < 1 ng L^{-1} (Table S3). PFOS (*f* = 81%) and PFHxS (*f*= 40%) were the most abundant PFSAs, with average concentrations of 19

± 44 ng L^{-1} and 3.3 ± 6.0 ng L^{-1} respectively. Concentrations of PFCAs and PFSAs from this WWTP are consistent with previous reports from influent from WWTPs in Australia from 2014 [23] and 2017 [11], where compounds of shorter chain length are more frequently detected at higher concentrations. This effect has been well described in previous literature and is due to the increased water-sediment partition coefficient (K_d) with increasing CF_2 moieties, thereby decreasing their concentrations in the aqueous phase [34,35]. PFOS concentrations remained consistent in WWTP influent from these studies across numerous Australian WWTPs in 2014 (17 ± 35 ng L^{-1}) [23] and 2017 (17 ± 28 ng L^{-1}) [11], which may indicate that sources of this compound have not changed since import and usage controls were placed on PFOS and its salts by the Australian Government in 2014 [36]. Although, regional WWTPs in Australia have recorded an 18% decrease in background PFOS concentrations from 2010 to 2020, elevated levels of PFAS on specific days were attributed to discharges of trade waste [18].

Both 6:2 diPAP and 6:2, 8:2 diPAP were detected in wastewater influent at average concentrations of 8.2 ± 6.7 ng L^{-1} (f = 83%) and 35 ± 21 ng L^{-1} (f = 92%) respectively, with concentrations of diSAmPAP were lower at 0.04 ± 0.04 ng L^{-1}. diPAPs are generally used as grease-proofing agents in food-contact materials and levelling agents in personal care and cosmetic products [37], and they have demonstrated the ability to degrade into PFCAs in WWTPs [38]. The presence diPAPs in wastewater has been described in WWTPs from Hong Kong (range: <0.25 – 11.65 ng L^{-1}) [39] and Sweden (58 ng L^{-1}) [17], where the compounds could be found in higher concentrations compared to many PFAAs, indicating local uses such as food-contact material and may be contributing to the contamination of surface waters in the area [40]. Given the relatively high water-sediment distribution (log K_{OC} = 4.51 – 4.62) of diPAPs [41], high concentrations of these compounds in water have resulted in high concentrations in biosolids, including 8:2 diPAP [42].

6:2 fluorotelomer sulfonamide betaine (6:2 FTAB or 6:2 FTSA-PrB) was detected in 77% of WWTP influent samples in November 2019 with an average concentration of 13 ± 16 ng L^{-1} (range: <0.26–100 ng L^{-1}). The only reported use of 6:2 FTAB in Australia is as an active ingredient in fluorotelomer-based AFFF [43] that were developed in response to the regulation of PFOS. Fluorotelomer-based AFFF is currently only used by the Australian Defence Force [44] since civilian airports and municipal fire services in the area ceased the use of PFAS-containing AFFF in 2010 [45] and 2007 [46] respectively. Although since the import and uses of 6:2 FTAB are not currently regulated or controlled by the Australian Government, it is difficult to identify other potential sources. 6:2 FTAB was also recently reported in a regional Australian WWTP in relatively high proportions compared with other PFAS, with comparable mean concentrations of 18 ± 73 ng L^{-1}, which was attributed to increased industrial pulse inputs to the system [18]. Since there is evidence for the biotic and abiotic degradation of 6:2 FTAB to short-chain PFCAs over 30 – 100 days [47,48], it is reasonable to conclude that there was ongoing discharge of this compound to these WWTP networks in Australia at the time of sampling. Concentrations of 6:2 FTAB in Australian WWTPs were lower than surface waters impacted by WWTP discharge in China (range: <11–1300 ng L^{-1}) [49], and orders-of-magnitude lower than those found in a French WWTP that received industrial effluent from a fluorochemical manufacturer (range: 4 – 45.5 mg L^{-1}).

3.2. Variability in daily concentrations and detection frequency

The mean total daily $\sum_{50}$PFAS concentrations in WWTP influent ranged from 89 ± 38 ng L^{-1} on Monday to 173 ± 110 ng L^{-1} (n = 168) on Friday. The $\sum_{50}$PFAS mean concentrations variations between days were significantly different despite the high variability of concentrations over the 24 h (F_6 = 5.164, $p < 0.0001$). $\sum_{50}$PFAS concentrations on Monday and Sunday were significantly less than concentrations on Tuesday, Wednesday, Friday, and Saturday (Fig. 1). $\sum_{11}$PFCA and $\sum_{9}$PFSA concentrations had the greatest range between days, between 22 ± 11–93 ± 49 ng L^{-1} and 5.3 ± 4.7–77 ± 92 ng L^{-1} respectively. The concentrations of $\sum_{3}$diPAPs were the next most abundant class (range: 31 ± 20–60 ± 38 ng L^{-1}), followed by $\sum_{5}$FTSAs (range: 3.6 ± 3.0–28 ± 18 ng L^{-1}), and $\sum_{11}$PASFs (range: 0.83 ± 0.51–14 ± 16 ng L^{-1}), whilst PFEAAs and PFPiAs were the least abundant classes with consecutive daily average concentrations < 1 ng L^{-1}.

The variability in average PFAS concentrations observed between Monday and Sunday may be indicative of various sources of contamination to the sewer network, particularly increased discharge of trade waste from commercial and industrial emitters that operate during business hours. Here, the observed 12 – 24 h delay in increased concentrations may be caused by the relative size of the sewer network in the study area – only reaching the WWTP hours after the primary emission.

A total of fifteen PFAS were detected in at least > 50% of samples on each respective day (n = 168), from four classes, namely 7 PFCAs, 3 PFSAs, 3 PASFs and 2 diPAPs (Fig. 2). Spearman's correlation analysis revealed two groups of compounds, where concentrations of PFAS within groups were related. There was a significant positive correlation between concentrations of PFHxA, PFHpA, PFOA, PFNA, PFDA, PFHxS, FBSA and FOSA (0.20 < R < 0.82, Table S2). These compounds were also clustered on the first principal component with concentrations measured early in the week from Monday to Thursday (Fig. S3). PFHpS, PFOS, and 6:2 FTAB were also positively correlated in concentration (0.30 < R < 0.50, Table S2) and clustered on the second principal component with concentrations measured on Friday and Saturday (Fig. S3). The high correlation between concentrations of PFAS within these two groups provides further evidence for independent sources of contamination to the sewer network, although it remains unclear if these trends in variability will continue from week to week over the year. Compounds from the former group (PFCAs and PASFs) are commonly used in a variety of industries, including but not limited to: metal manufacture and plating, oil and gas operations, photography, textiles, and automotive [21]. Further investigation is recommended to identify potential sources of these PFAS to the WWTP influent, specifically concerning high spatial and temporal resolution.

The frequency of detection for each compound measured in > 50% of samples differed between days, where compounds such as PFOA, PFOS and 6:2 FTAB were detected in > 50% of samples each day during the sampling period, and PFHpS were only detected > 50% of samples for a single day. A pattern of increased detection frequency early in the week and decreased frequency later in the week was observed for PFHpA, perfluorodecanoic acid (PFDA), PFHxA, perfluorononanoic acid (PFNA), perfluorooctane-1-sulfonamide (FOSA) and perfluorobutane-1-sulfonamide (FBSA), which is also supported by the strong positive correlation in their concentrations (R > 0.46, p < 0.001, Table S2).

Parametric and non-parametric data analysis, including the calculation of mean and standard deviation, typically require detection frequencies > 50% [50]. As a result, the number of compounds that could have statistical tests run varies greatly as a function of the day that samples are collected. As the abundance (Fig. 1) and detection frequency (Fig. 2) of PFAS in WWTP influent change daily over a week, grab samples collected from a single day may not be representative of average PFAS concentrations over time.

3.3. Pulse events

Concentrations of PFOS and PFHpS that exceeded the 99% confidence interval on two occasions are strong indicators of pulse inputs to the WWTP. The mean weekly concentration of PFOS was 19 ± 44 ng L^{-1} and the mean concentration between Monday to Thursday was 5.6 ± 3.9 ng L^{-1} (range: <0.04–13 ng L^{-1}). Mean PFOS concentrations then increased from Friday to Sunday to 35 ± 63 ng L^{-1} (range: <0.04–400 ng L^{-1}), with two clear pulse events occurring on Friday at

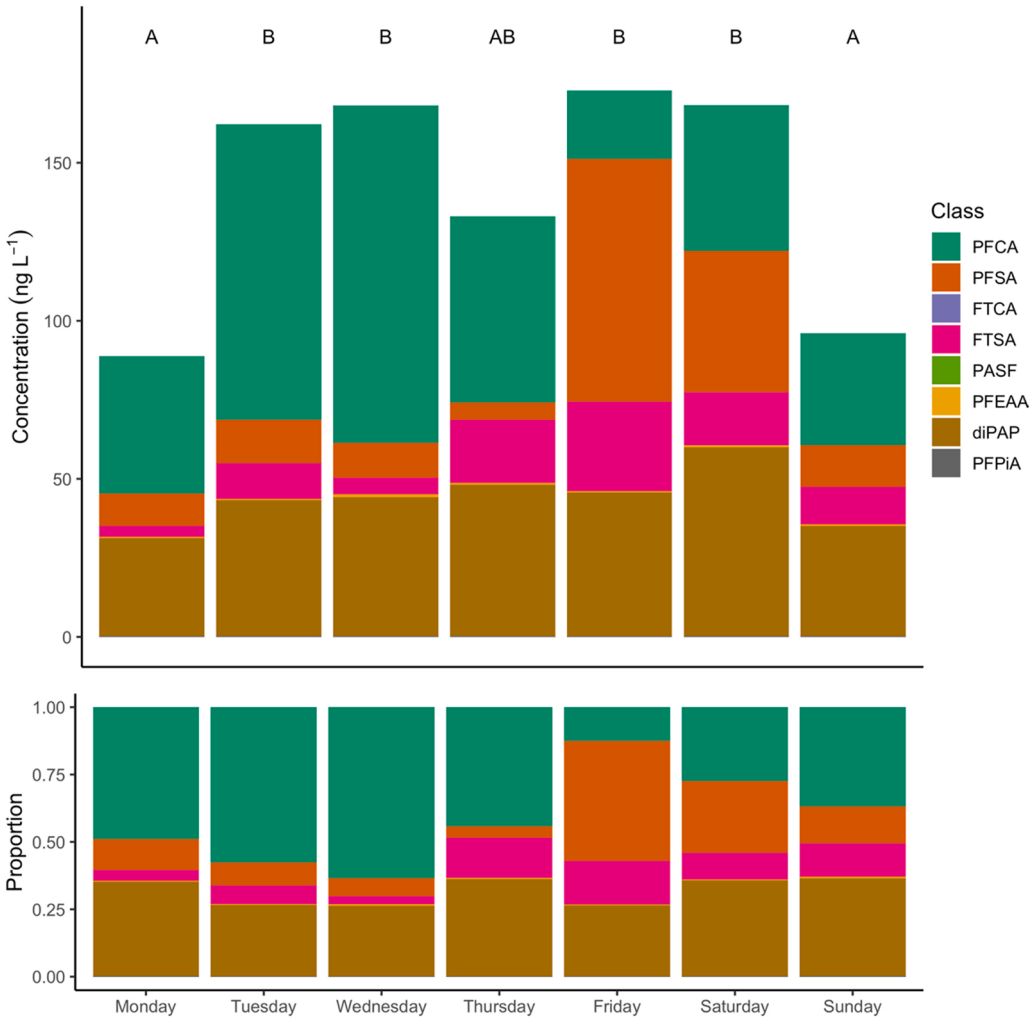


Fig. 1. Average concentrations and proportion of 50 PFAS from seven classes sampled hourly each day (n = 24) from a WWTP in southeast Australia between 11 and 17 November 2019. Compact letter display of Tukey's posthoc analysis for ANOVA for $\sum_{50}$PFAS concentrations denoting differences between days ($\alpha = 0.05$).

16:00 (Event 1: 400 ng L^{-1}) and Saturday at 05:00 (Event 2: 340 ng L^{-1}) that exceeded the 99% confidence interval (Fig. 4A). In Event 1, elevated concentrations of PFOS persisted for three consecutive hours before returning to baseline levels and Event 2 persisted for approximately 2 h before returning to baseline levels. Elevated concentrations of PFHpS were also detected during each event reaching maximum concentrations of 14 ng L^{-1} and 18 ng L^{-1} respectively, which was greater than the weekly average concentration of 0.69 ± 2.1 ng L^{-1}. Concentrations of PFOS and PFHpS in WWTP influent over the week were significantly correlated (R = 0.50, p < 0.0001, Table S2) and were grouped across the second principal component for Friday and Saturday (Fig. S3), indicating a common source of these compounds, particularly during the pulse events (Fig. 4). PFOS and PFHpS are not typically linked with fluorotelomer-based PFAS production [51], so their presence may be related to the use of products produced by electrochemical fluorination (ECF) [5].

Concentrations of PFHxS, PFOA and FOSA were also recorded at higher concentrations during specific periods throughout the week (Fig. S4), however, a pulse event is not as clear with these compounds as the elevated concentrations persisted for more than 24 h in two events between Monday and Thursday. Concentrations of these compounds over the entire week were significantly correlated (R > 0.52, p < 0.0001, Table S2) which may also indicate common sources, unrelated to the discharge of PFOS and PFHpS to the WWTP.

This is the first study to report concentrations of PFAS on an hourly frequency so it is difficult to make comparisons with the literature. Our data are consistent with intraday variability for concentrations of pathogens [52], inorganic byproducts [53], pharmaceuticals and personal care products [54], in WWTPs where concentrations typically increase with peak residential usage times. In contrast to these studies investigating other WWTP inputs, we found PFAS concentrations increased overall between Monday and Saturday and further investigation is needed to determine the impact of residential and industrial inputs to this WWTP. It should be noted that these trends may be specific to this site and may not be representative of PFAS inputs to all WWTPs. Overall, the differences in concentrations over time that are provided in this study may indicate inputs from residential or trade waste. In this case, however, the water retention time in the network varies by several hours throughout the catchment for this WWTP making it difficult to elucidate precise discharge times or locations. Other general factors that should be considered to impact the variation in concentrations of contaminants in a given WWTP catchment include wet weather, storage capacity, discharge volume, and the capacity of the infrastructure to receive discharge.

3.4. Recommended minimum number of grab samples

In compounds detected in > 50% of samples for at least one day, daily average concentrations of PFBA ($F_{6160} = 1.67$, $p = 0.131$) and 6:2 diPAP ($F_{6160} = 2.09$, $p = 0.057$) in the WWTP influent did not change

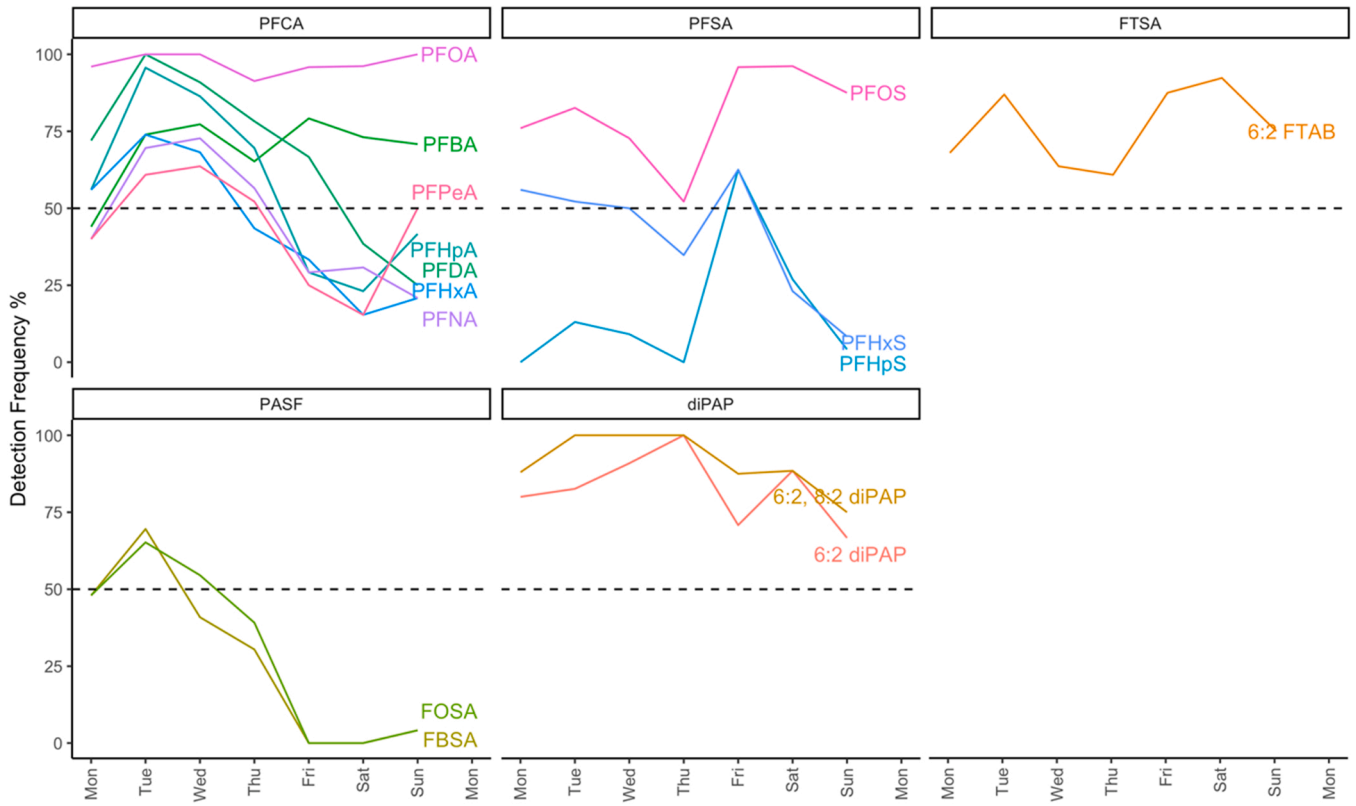


Fig. 2. Detection frequency for one week of each compound that was detected in > 50% of samples for at least one day collected between 11 Nov to 17 Nov 2019.

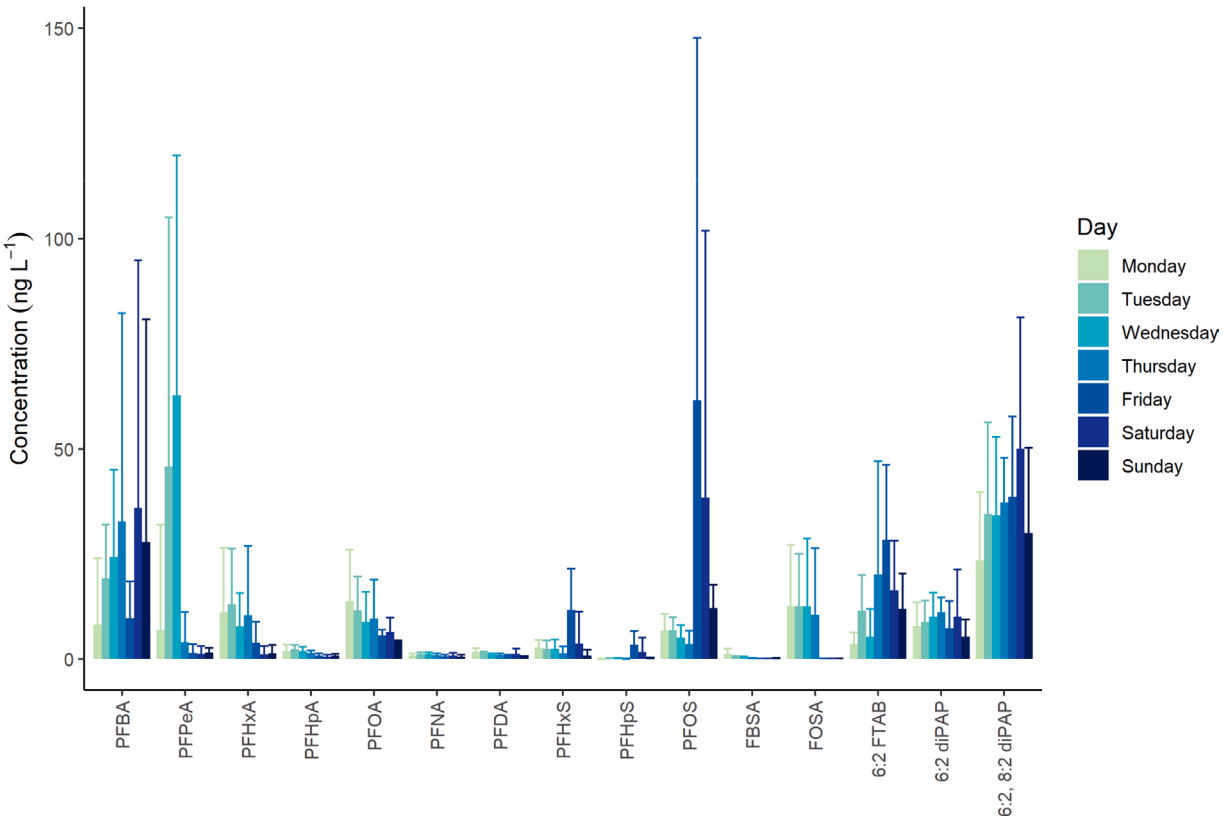
significantly throughout the week. Conversely, two compounds had differences in concentrations with moderate effect size PFPeA ($F_{6160} = 13.7$, $p < 0.0001$, $\eta^2_G = 0.339$) and PFHxS ($F_{6160} = 12.3$, $p < 0.0001$, $\eta^2_G = 0.316$). The mean daily concentrations of PFPeA ranged between 0.65 ± 1.6 ng L^{-1} on Saturday to 61 ± 59 ng L^{-1} on Wednesday, while PFHxS ranged between 0.56 ± 1.6 ng L^{-1} on Sunday to 12 ± 10 ng L^{-1} on Friday. There were also significant differences in daily average concentrations for a further eleven compounds outlined in Fig. 3, albeit with a smaller effect size. Previous studies of the average PFAS concentrations from 24-hour pooled samples (per 8000 m^3) indicated that there was no significant change from days throughout the week [25]. However, this may have been a result of the differences between the collection of pooled flow-proportional samples, where the effective sample size, $n = 1$, rather than the average of individual hourly sample analysis in this study where $n = 24$, which may have reduced the resolving power of the daily variability compared with this study. The increased temporal resolution in this study has revealed changes in PFAS concentrations over time that previous studies may have been lacking in statistical power.

The minimum number of hourly grab samples required per day to detect a significant change in the mean concentration range for each compound due to the difference in total variation (Fig. 3). Given the medium effect size determined for PFPeA ($\eta^2_G = 0.339$), only five samples are required each day to detect a significant difference in daily concentrations with a statistical power (1-β) of 80%. For compounds with small effect sizes like PFNA ($\eta^2_G = 0.099$), a total of 19 samples are required to detect changes in daily average concentrations. To determine changes in daily concentrations for compounds of high interest, such as PFOS and PFOA, a minimum of nine and eleven hourly grab samples per day respectively are required to detect changes in daily averages. Ideally, grab samples should be randomly collected throughout the entire day, rather than during business hours (09:00 – 17:00), to minimise sampling bias. The concentrations of PFAS in grab samples that are taken only during business hours may be over- or under-representative of the true daily mean concentration for any given compound. For any given flow rate, the mass flux estimate could be up to a 2-fold change in concentration between business hours and after-hours sampling (Fig. S5).

4. Conclusion

Concentrations of PFAS in wastewater, that were collected each hour for a week and measured in this study (n = 168), are consistent with WWTPs described in the literature, including fluorotelomer-based substances. Mean daily concentrations of PFAS varied significantly throughout the week, where samples collected on Monday and Sunday had lower levels compared with Tuesday, Wednesday, Friday, and Saturday. Strong correlations between concentrations of two groups of PFAS provide evidence for various sources of contamination discharged to the WWTP throughout the week. Furthermore, simultaneous pulse events of PFOS and PFHpS on Friday and Saturday resulted in concentrations over ten times greater than average for several hours.

We present information that can be used in a cost-benefit analysis for the water and waste industries to effectively estimate the concentrations of PFAS in WWTP influent. Variability in the concentrations of PFAS throughout the day resulted in a range of the minimum number of grab samples required to detect differences in daily average concentrations from 5 to 19 per day. To detect changes in the average daily concentration of PFOS, a minimum of nine randomly collected samples per day over the entire 24-hour period is recommended. This case study of a single plant has shown that concentrations of PFAS vary from day to day across the week, and also from hour to hour within each day. This is the first evidence of short-term temporal variability, however, given the average concentrations are supported by previous literature, it is likely that this effect is widespread across many WWTPs in Australia and internationally. Transformation of PFAS within the WWTP can drastically increase or decrease concentrations before discharge [11], so we recommended that further high-resolution temporal investigations are



Compound	DF_n	DF_d	F-statistic	p-value	η^2_G	n_{min}
PFBA	6	160	1.67	0.1310	0.059	-
PFPeA	6	160	13.7	**0.0000**	0.339	5
PFHxA	6	160	5	**0.0001**	0.158	12
PFHpA	6	160	9.2	**0.0000**	0.257	7
PFOA	6	160	5.23	**0.0001**	0.164	11
PFNA	6	160	2.94	**0.0100**	0.099	19
PFDA	6	160	6.64	**0.0000**	0.199	9
PFHxS	6	160	12.3	**0.0000**	0.316	6
PFHpS	6	160	9.11	**0.0000**	0.255	7
PFOS	6	160	6.91	**0.0000**	0.206	9
FBSA	6	160	7.33	**0.0000**	0.216	7
FOSA	6	160	7.59	**0.0000**	0.221	8
6:2 FTAB	6	160	8.86	**0.0000**	0.249	7
6:2 diPAP	6	160	2.09	0.0570	0.073	-
6:2, 8:2 diPAP	6	160	3.9	**0.0010**	0.128	15

Fig. 3. Average concentrations and standard deviation of the compounds detected in > 50% of samples in at least one day. Results of analysis of variance (ANOVA) and eta squared effect size (η^2_G) for concentrations within days for each compound. n_{min} = minimum number of hourly grab samples required each day to detect a significant change in daily concentrations.

performed in effluent to characterise the risk to receiving environments.

The probability of sampling during a pulse event may be low, although without context, these results would lead to erroneous estimates of the true concentration of PFAS in the WWTP. To more accurately evaluate the mass flux of PFAS in the influent and effluent of WWTPs, the use of passive samplers may be a suitable technology that would allow the cost-effective estimation of emerging contaminant mass flow by deploying them for up to 12 days in WWTPs and other surface waters [55]. It should be considered that instantaneous grab sampling is not applicable for mass flux estimates in WWTP influent, regardless of the number of days or months sampled. It is recommended that frequent and randomised 24-hour pooled samples (or longer) are used in conjunction with detailed hydrological measurements for future monitoring studies when estimating daily, weekly or annual PFAS mass flux

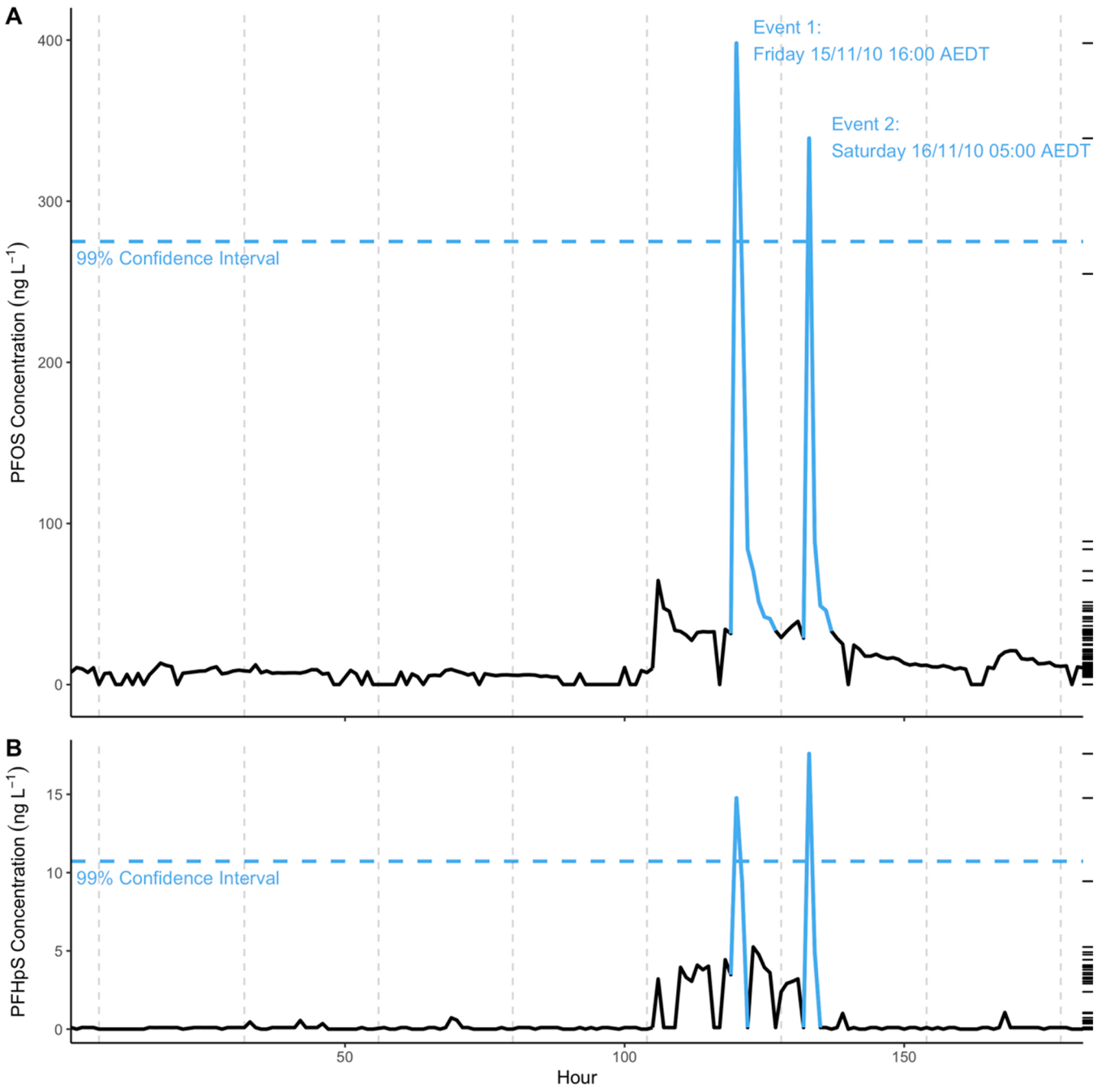


Fig. 4. Concentration of A) PFOS and B) PFHpS (ng L^{-1}) between 11 and 17 November 2019 and distribution of samples on the secondary y-axis (n = 182). The dotted blue line represents the 99% confidence interval for the concentration of PFOS (275 ng L^{-1}) and PFHpS (11 ng L^{-1}) over the 7 days. The dotted grey line indicates days from Monday to Sunday.

to receiving waterways.

Environmental Implications

Wastewater treatment plants (WWTPs) are an important factor in the fate of per- and polyfluoroalkyl substances (PFAS) in the environment. It is well known that PFAS are not eliminated by traditional WWTP processes and, in many cases, are discharged to the environment at potentially hazardous levels. The high temporal resolution monitoring described in this study is a technique that could be used to identify sources of PFAS to these plants. Identifying sources of PFAS to WWTPs more accurately is a key strategy to help reduce the emission and risk of exposure to receiving environments.

CRediT authorship contribution statement

Drew Szabo: Writing – original draft, Methodology, Investigation, Formal analysis, Visualization. **Jaye Marchiandi**: Investigation, Writing – review & editing. **Julia Johnston**: Investigation, Writing – review & editing. **Subharthe Samandra**: Investigation, Writing – review & editing. **Raoul A. Mulder**: Writing – review & editing, Supervision. **Mark P. Green**: Writing – review & editing, Project administration, Supervision. **Bradley O. Clarke**: Project administration, Funding acquisition, Conceptualization, Supervision, 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.

Data availability

Data have been provided in Appendix A using FAIR Guiding Principles. All other data may be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.130854.

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