Supplementary Information for **Metabolomic Profiles associated with exposure to poly- and perfluoroalkyl acids (PFAAs) in aquatic environments**

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1. PFAS Extraction and analysis

1.1 Chemicals and reagents

LiChrosolv® Methanol (99,8%) and Acetonitrile (99,9%) were purchased from Merch (Darmstadt, Germany). Solvents used were of analytical grade. Ammonium acetate (>97%, C2H7NO2) was purchased from ChemSupply (Gillman, SA, Australia), ammonium hydroxide (NH3(aq)) and sodium hydroxide (NaOH) and were purchased from Sigma Aldrich. Hydrochloric acid (HCl, 37%) was obtained from Riedel-de Haën (Seelze, Germany). MilliQ Water filter system pores were 0.22 µm, 18.2 mΩ cm^{-1}.
Standards were purchased from Wellington Laboratories, Guelph, Ontario, Canada. Mass labelled standard mix include 0.2ppm; $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{18}O_2$-PFHxS, $^{13}C_4$-PFOS, $^{13}C_2$PFUdA, $^{13}C_2$PFDoA $^{13}C_2$6:2FTS and $^{13}C_3$PFBS. The corresponding PFASs to the mass labelled standards are specified in table S1**.** Instrumental recovery standard included 0.2 ppm; $^{13}C_8$-PFOA and $^{13}C_8$-PFOS. The native standard mix includes PFAAs (perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorobutanesulfonate (PFBS), perfluorohexane sulfonate (PFHxS), and perfluorooctane sulfonate (PFOS)), and the fluorotelomer sulfonates 8:2 FTS, 6:2 FTS, and 4:2 FTS.

1.2 Extraction Method

One gram homogenized muscle-tissue was spiked with 10µL of a 0.2ppm mass-labelled PFAS standard mix. After 30 minutes 400µL 200 mM NaOH/MeOH solution was added and the samples were vortex-mixed and allowed to digest for 30 minutes. Acetonitrile (4mL) was added and the samples were vortex-mixed and ultra-sonicated for 15 minutes. The tissue was neutralised by adding 20µL 4M HCl/MeOH solution before the samples were centrifuged (2500 rpm for 15 minutes). The supernatant was transferred to 15 mL Falcon polypropylene tubes. The samples were extracted with a second aliquot of 4 mL ACN followed by ultra-sonication (15 min) and centrifugation (4750 rpm, 15 minutes). The supernatant was combined and the volume was reduced to 2mL under a stream of high purity nitrogen at 40 °C. Liquid-liquid extraction was performed by adding 1mL n-Hexane and vortex the samples for 30 seconds, and centrifuged (4750 rpm, 5 minutes) before the n-hexane was discarded and this was repeated twice. Samples were concentrated to a volume of 250µL and thereafter MeOH was added to make the sample up to a volume of 1mL. Further clean-up was performed by pushing the samples through (MeOH preconditioned) Bond Elut Carbon cartridges (100mg, Agilent Technologies), with the use of a syringe. Samples were collected into 1.5mL polypropylene vials. The cartridges were then washed by pushing 0.5mL MeOH through the cartridges into the corresponding vials. The samples were concentrated to a volume of 200µL under high purity nitrogen at 40 °C. A total final volume of 500µL was made up by adding 300µL 5mM ammonium acetate in water and 1% MeOH. The samples were spiked with 10µL 0.2ppm instrument standard before HPLCMS/MS analysis.

1.3 Instrumental Analysis

PFAS analysis was performed using a high performance liquid chromatography (HPLC, Nexera HPLC, Shimadzu Corp, Kyoto Japan) coupled to a tandem mass spectrometer (SCIEX Quad 6500+,Concord, Ontario, Canada) with negative electrospray ionisation operating in multiple reaction monitoring (MRM) mode. The injection volume was 5 µL. To

separate the target PFASs, a gradient elution of mobile phase 1% (Aqueous Phase A) and 90% (Organic Phase B) MeOH, respectively, with 5 mM ammonium acetate were used. The column was a NX C18 column (50 × 2 mm, 3 µm, 110 Å, Phenomenex, Lane Cove, Australia), and the temperature was held constant at 50°C. Integrations of peak areas were performed using MultiQuant (3.0.2). Retention times and comparison of MRM transitions between samples and the calibration was used for confirmation and identification of peaks. Calibration standards (linear isomers of each compound, 500 µL; 200 µL methanol and 300 µL 5 mM ammonium acetate in water) had a concentration range of 0.1–100 µg L-1 (0.1; 0.4; 1; 4; 10; 20; 40; 60; 80; 100).

1.4 Internal standards and MRM transitions

Table S1 Internal standards and MRM transitions

Class	Analyte	Abbrevation	Internal Standard	MRM transition
PFCA	Perfluorobutanoic acid	PFBA	$^{13}C_4$-PFBA	**213 / 169**
	Perfluoropentanoic acid	PFPeA	$^{13}C_4$-PFBA	**263 / 219**
	Perfluorohexanoic acid	PFHxA	$^{13}C_2$-PFHxA	**313 / 269** 313 / 119
	Perfluoroheptanoic acid	PFHpA	$^{13}C_4$-PFHxA	**363 / 319** 363 / 169
	Perfluorooctanoic acid	PFOA	^{13}C4-PFOA	**413 / 369** 413 / 169
	Perfluorononanoic acid	PFNA	$^{13}C_5$-PFNA	**463 / 419** 463 / 169
	Perfluorodecanoic acid	PFDA	$^{13}C_2$-PFDA	**513 / 469** 513 / 269
	Perfluoroundecanoic acid	PFUnDA	$^{13}C_2$PFUdA	**563 / 519** 563 / 269
	Perfluorododecanoic acid	PFDoDA	$^{13}C_2$PFDoA	**613 / 569** 613 / 169
	Perfluorotridecanoic acid	PFTriDA	$^{13}C_2$PFDoA	**663-619** 663-169
	Perfluorotetradecanoic acid	PFTreDA	$^{13}C_2$PFDoA	**713-669** 713-169
PFSA	Perfluorobutane sulphonic acid	PFBS	$^{13}C_3$ PFBS	**299 / 80** 299 / 99
	Perfluorohexane sulphonic acid	PFHxS	$^{18}O_2$-PFHxS	**399 / 80** 399 / 99
	Perfluorooctane sulphonic acid	PFOS	$^{13}C_4$-PFOS	**499 / 80** 499 / 99 499 / 169 549 / 80
	Perfluordecanesulphonate	PFDS	$^{13}C_4$-PFOS	**599 / 80** 599 / 99
FTS	8:2 Fluorotelomer sulfonate	8:2 FTS	$^{13}C_2$-6:2 FTS	**527 / 507** 527 / 81
	6:2 Fluorotelomer sulfonate	6:2 FTS	$^{13}C_2$6:2 FTS	**427 / 407** 427 / 81
	4:2 Fluorotelomer sulfonate	4:2 FTS	$^{13}C_2$ 6:2 FTS	**327 / 307** 327 / 81

1.5 Quality Assurance Details and Results

Samples were extracted in batches of 10 samples. In each batch, a blank (acetonitrile) was included to be able to detect potential contamination. No PFAS analytes were detected above limit of reporting in any blanks. Quality control also included a duplicate sample and native spiked duplicate, which both followed the same extraction procedure as the samples in each batch. The recovery of the native spike was determined by subtracting the analyte concentrations detected in the spiked samples by the analyte concentration found in the un-spiked duplicate sample and comparing this concentration to the known spiked concentration (quantified in a non-extracted side spike). In each batch a QAQC matrix sample with a known PFAS concertation also followed the same extraction procedure as the samples and was used to identify any potential between batch-variation. Details of QAQC matrix sample variations, duplicate variation, and average native recovery are presented in table S2, S3 and S4 respectively.

During instrumental analysis, calibration standards were injected twice in each run. Quality control standards were added between every 10 samples to monitor instrumental variations. Linear (weighted by 1/x) or hill regression fits were applied on the calibration curves for each linear isomer, and were used to quantify the analytes. Regression coefficients (r^2) were only accepted >0.993. Limits of detections (LODs) were set to three times the standard deviation of the concentration of the lowest standard after 10 injections of the standard with a signal to noise superior to 3. Limits of Reporting (LORs) were set 10 times this standard deviation.

Table S2 Average relative Standard Deviation (%) of QAQC samples from the different batches (n=4) Only PFASs detected >LOR in at least one sample are presented. (Average calculations only include PFASs where PFASs have been detected >LOR in all QAQC samples)

Analyte	**RSD (%)**
PFDA	18.7
PFOS(linear)	11.7
PFOS(branched)	17.3

Table S3 Average ratios of PFASs in duplicate samples (n=4). Only PFASs detected >LOR in at least one sample are presented. (Average calculations only include duplicates where PFASs have been detected >LOR in both duplicate samples)

Analyte	Average Ratio
PFHpA	1.19
PFOA	1.10
PFNA	1.12
PFDA	1.18
PFHxS	1.03
PFOS (Linear)	1.14
PFOS(Branched)	1.54

Table S4. The average percent recovery of all native spiked matrix samples (n=4)**,** and percent recoveries of ^{13}C and ^{18}O labelled standards for each PFAS analyte (n=45). Standard deviation is presented in brackets.

Analyte	Average Native Recovery	Average Labelled Standard Recovery
PFBA	100(6)	63(10)
PFPeA	107(4)	N/A
PFHxA	101(3)	79(11)
PFHpA	102(1)	N/A
PFOA	95(13)	86(12)
PFNA	104(5)	82(10)
PFDA	102(6)	87(10)
PFUnDA	104(4)	79(12)
PFDoDA	106(6)	55(17)
PFTriDA	68(10)	N/A
PFTreDA	70(32)	N/A
PFBS	109(9)	94(16)
PFHxS	99(6)	94(14)
Linear PFOS	111(5)	90(11)
PFDS	79(11)	N/A
8:2 FTS	88(18)	N/A
6:2 FTS	101(4)	61(10)
4:2 FTS	122(21)	N/A

Table S5 Limits-of-reporting (LORs) ug kg^{-1} for each PFAS analyte

Analyte	LOR (ug kg^{-1})
PFBA	0.07
PFPeA	0.07
PFHpA	0.02
PFOA	0.06
PFNA	0.05
PFDA	0.03
PFUnDA	0.04
PFDoDA	0.12
PFHxS	0.06
Linear PFOS	0.05

8:2 FTS	0.09
6:2 FTS	0.09
4:2 FTS	0.38

1.6 Metabolome analysis QAQC

Table S6 Recoveries of spiked of linolenic and linoleic acid.

µg spiked	µg recovered	%recovery
Linolenic acid		
80	83.12976708	103.91221
80	81.34815999	101.6852
80	81.30343058	101.62929
Linoleic acid		
80	77.32142833	96.651785
80	73.78434728	92.230434
80	76.11085773	95.138572

Table S7 Limits-of-reporting (LORs) ng uL^{-1} for each fatty acid analyte

Fatty acid	LOD
C10:0	1 ng μL^{-1}
C12:0	0.3 ng μL^{-1}
C13:0	0.5 ng μL^{-1}
Cis-9-C14:1	0.3125 ng μL^{-1}
C14:0	0.65625 ng μL^{-1}
C15:0	0.15625 ng μL^{-1}
Cis-9-C16:1	0.625 ng μL^{-1}
C16:0	0.75 ng μL^{-1}
C17:0	0.34375 ng μL^{-1}
Cis-9,12-C18:0	0.3125 ng μL^{-1}
Cis-9,12,15-C18:3	0.45 ng μL^{-1}
C18:0	0.3125 ng μL^{-1}
C19:0	0.171875 ng μL^{-1}
Cis-11-C20:1	0.3125 ng μL^{-1}
C20:0	0.84375 ng μL^{-1}
C21:0	0.328125 ng μL^{-1}
C22:0	0.3125 ng μL^{-1}l
Cis-9-C18:1	0.625 ng μL^{-1}
iC15:0	0.453125 ng μL^{-1}
aiC15:0	0.378125 ng μL^{-1}
C20:1(n-8)	2.7 ng μL^{-1}