Supporting Information

(25 pages, 5 figures, 12 tables)

Occurrence and Tissue Distribution of Novel Perfluoroether Carboxylic and Sulfonic Acids and Legacy Per/Polyfluoroalkyl Substances in Black-Spotted Frog (*Pelophylax nigromaculatus*)

Qianqian Cui,[1,2,#] Yitao Pan,[1,#] Hongxia Zhang,[1] Nan Sheng,[1,2] Jianshe Wang,[1] Yong Guo,[3] and Jiayin Dai[1,*]

[1]Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P. R. China; [2]University of Chinese Academy of Sciences, Beijing 100049, P. R. China; [3]Key Laboratory of Organofluorine Chemistry Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, P. R. China

# These authors contributed to this work equally.

***Correspondence author:** Jiayin Dai, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P. R. China. Telephone: +86-10-64807185. E-mail: daijy@ioz.ac.cn

Competing financial interests: The authors declare no conflicts of interest.

Table of Contents

Standards and Reagents

The 17 target PFASs included perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnDA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTriDA), perfluorotetradecanoate (PFTeDA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), chlorinated polyfluorinated ether sulfonates (6:2 and 8:2 Cl-PFESA), and hexafluoropropylene oxide trimer acid (HFPO-TA). Except for HFPO-TA and Cl-PFESAs, all native and mass-labelled internal standards were purchased from Wellington Laboratories (Guelph, ON, Canada). Native standards of HFPO-TA, 6:2, and 8:2 Cl-PFESA (purity > 98%) were synthesized by Dr. Yong Guo at the Key Laboratory of Organofluorine Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

Tetra-*n*-butyl ammonium hydrogen sulfate (TBAS), ammonium hydroxide, ammonium acetate, potassium hydroxide, sodium carbonate, and sodium bicarbonate were obtained from Sigma (St. Louis, MO, USA). LC-MS grade water and methanol and LC grade methyl *tert*-butyl ether (MTBE) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Solid phase extraction (SPE) weak anion exchange cartridges (strata X-AW, 200 mg/6 mL) were purchased from Phenomenex (Torrance, CA, USA). Oasis weak anion exchange (WAX) cartridges (200 mg/6 mL) were purchased from Waters (MA, USA).

Sample Extraction.

Water sample: Water (200 mL) was spiked with 0.5 ng of internal mass-labelled standard and then loaded onto a solid phase extraction (SPE) cartridge (Phenomenex strata X-AW, 200 mg/6 mL) preconditioned with 8 mL of 0.5% ammonium hydroxide in methanol, 8 mL of methanol, and 4 mL of ultrapure water. The cartridges were washed with 4 mL of buffer solution (25 mM acetic acid/ammonium acetate, pH = 4) and centrifuged for 30 min at 4000 rpm to remove residual water. Target compounds were eluted into fractions separately by adding 4 mL of methanol (fraction 1) and then 4 mL of 0.5% ammonium hydroxide in methanol (fraction 2); fraction 2 was evaporated to dryness under nitrogen at 40 °C and reconstituted with 200 µL of methanol for instrumental analysis.

Biota sample: For the extraction of frog tissues (except muscle and carcass), generally 1 mL of homogenate (containing 0.2 g of tissue) was spiked with 0.5 ng of mass-labelled standard, 1 mL of tetra-*n*-butylammonium hydrogen sulfate solution (TBAS, 0.5 M), 2 mL of $NaHCO_3/Na_2CO_3$ buffer solution (pH = 10), and 4 mL of methyl *tert*-butyl ether (MTBE). After shaking and centrifugation, the supernatant was collected by a Pasteur pipette, with the remaining residue extracted twice more with 4 mL of MTBE. All three extracts were combined and evaporated to dryness under nitrogen at room temperature and reconstituted with 200 μL of methanol. Additional cleanup was performed for the tissue samples, with the extract further diluted to 10 mL with water, and then loaded onto the Oasis WAX cartridge (200 mg/6 mL, Waters, MA, USA) following the same procedure as that for the water samples.

For frog muscle and carcass, 2 mL of homogenate (0.4 g of muscle or 0.8 g of carcass) was spiked with 0.5 ng of mass-labelled standard, then sonicated for 1 h in 10 mL of 10 mM KOH methanol solution, and shaken at 200 rpm overnight. The supernatant was concentrated to dryness and diluted to 10 mL with water for further SPE cleanup (200 mg/6 mL Oasis WAX cartridge, Waters, MA, USA) following the same procedure as that for the water samples.

Skeletochronological Age Determination

We followed the procedures of previously published research.[1] Clipped toes from 56 individuals were cleaned in running water for 30 min, and then digested with 1% trypsin. Toes were decalcified by submersing them in 5% nitric acid for 2 d and then neutralized with 5% sodium hydrogen carbonate solution. The toes were cut into 15- to 20-mm-thick sections using a freezing microtome, stained using hematoxylin, and then observed under a microscope. The number of lines of arrested growth was counted to estimate frog age.

References:

[1] Seokwan Cheong, JiHye Yoo, ShiRyong Park, HaCheol Sung; Age estimation by skeletochronology and advertisement call variation in the black-spotted pond frog (*Rana nigromaculata*). Anim Cells Syst 2013, 17(2):141-146.

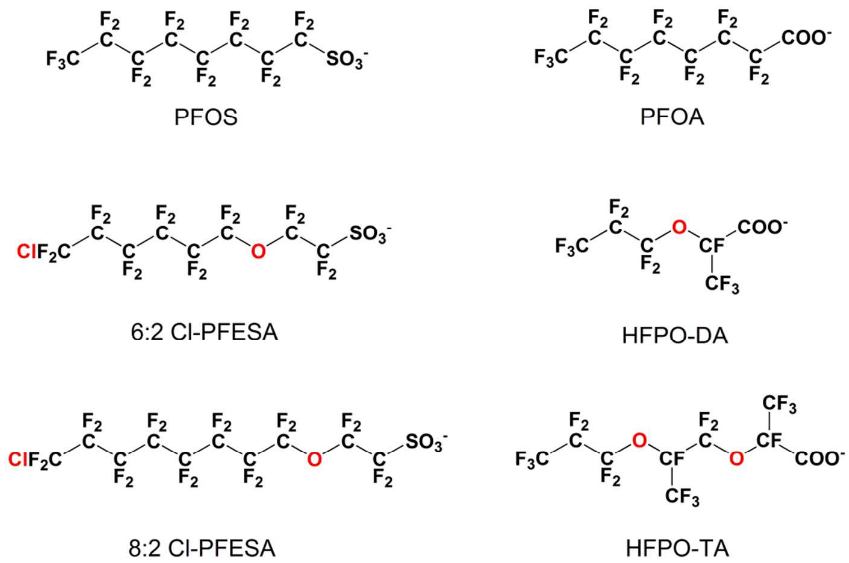

Figure S1. Molecular structures of novel PFAS alternatives Cl-PFESAs and PFECAs compared to their predecessors PFOS and PFOA.

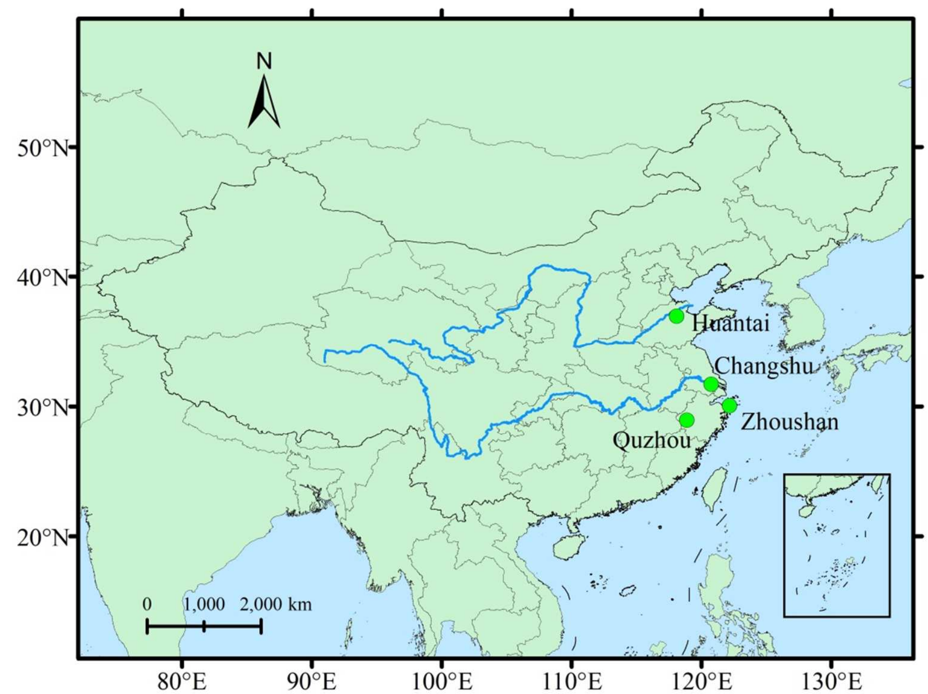

Figure S2. Sampling locations in east China.

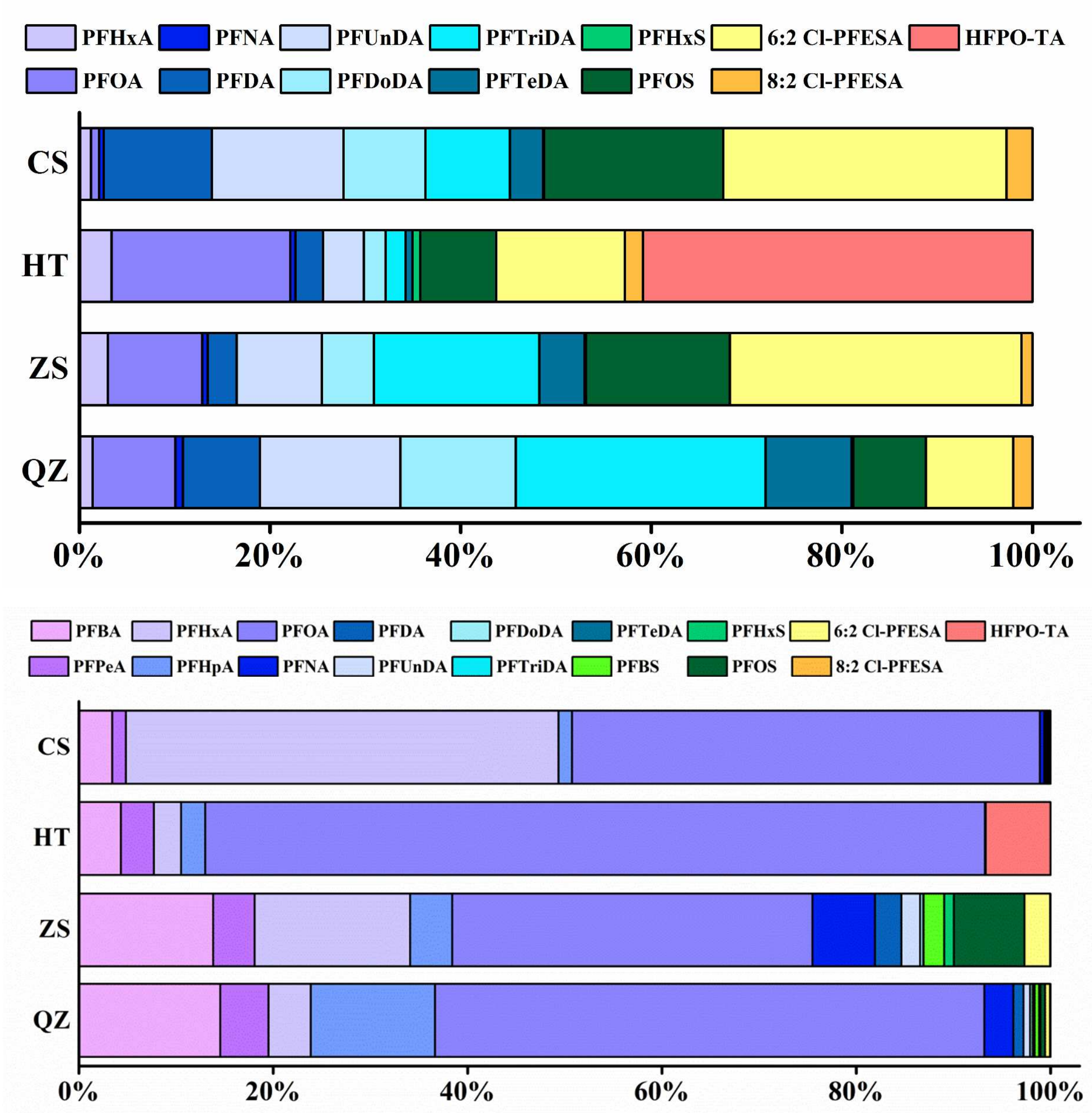

Figure S3. Compositions of PFASs in frog livers (up) and corresponding water samples (down) from Changshu (CS), Huantai (HT), Zhoushan (ZS), and Quzhou (QZ).

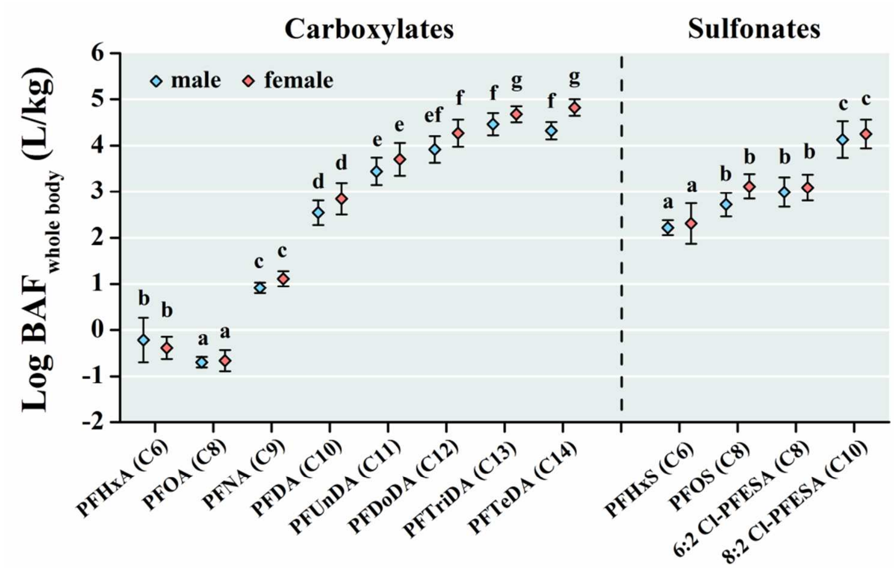

Figure S4. Log $BAF_{whole\ body}$ of PFAS carboxylates (left) and sulfonates (right) with increasing molecular chain length in black-spotted frogs from Changshu (n = 10, five males and five females). Different letters indicate statistically significant differences in BAFs at a statistical threshold of $p < 0.05$.

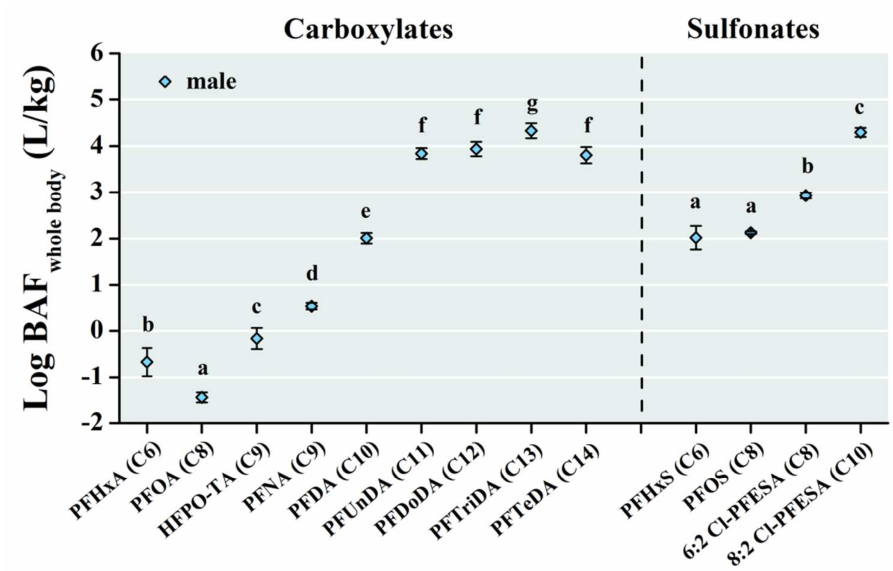

Figure S5. Log $BAF_{whole\ body}$ of PFAS carboxylates (left) and sulfonates (right) with increasing molecular chain length in black-spotted frogs from Huantai (n = 4). Different letters indicate statistically significant differences in BAFs at a statistical threshold of $p < 0.05$.

Table S1. Information on sample collection, sex, and body size for the studied frogs (n = 56).

Sampling site	Longitude (°E)	Latitude (°N)	Habitat type	Sampling date	Gender	No	Age (year) Mean ± SD	Snout-vent length (cm) Mean ± SD	Weight (g) Mean ± SD	Condition factor (g/cm^3) Mean ± SD	Liver somatic index (%) Mean ± SD
Changshu	120.8543	31.6584	Paddy field	June 18, 2016	female	18	2.2 ± 0.8	6.3 ± 0.9	18.74 ± 9.35	6.96 ± 0.70	2.89 ± 0.45
					male	11	1.7 ± 0.8	5.3 ± 1.0	10.50 ± 6.95	6.57 ± 0.75	1.87 ± 0.70
Huantai	118.1220	37.0823	Corn field	Sept 23, 2016	male	4	1.5 ± 0.6	5.0 ± 0.5	9.90 ± 1.84	7.97 ± 1.07	5.22 ± 0.32
Quzhou	118.8810	28.9193	Paddy field	May 06, 2016	female	7	2.0 ± 0.8	5.2 ± 1.5	19.15 ± 20.8	9.77 ± 1.08	2.74 ± 1.11
					male	5	2.0 ± 1.0	5.1 ± 1.6	12.88 ± 9.50	8.47 ± 2.09	2.08 ± 0.61
Zhoushan	122.2755	29.8379	Paddy field	Aug 29, 2016	female	5	2.4 ± 0.9	8.2 ± 2.1	46.07 ± 31.54	6.95 ± 0.88	2.73 ± 0.93
					male	6	2.0 ± 0.9	6.0 ± 0.8	15.51 ± 6.80	6.97 ± 0.85	1.83 ± 0.23

Female frogs from Changshu and Quzhou were in the reproductive period and carrying eggs. Frogs from Zhoushan were not gravid.

Fulton's condition factor (K) was calculated based on the equation: $K = 100\ W/L^3$, W, body weight; L, body length.

Liver somatic index (LSI) was calculated based on the equation: LSI = Liver weight/Body weight × 100.

Table S2. Information on the body and organ weights for selected frog individuals from Changshu (*n* = 10) and Huantai (*n* = 4).

Sample site	Sex	Body weight (g)	Skin (g)	Liver (g)	Kidney (g)	Gonad (g)	Lung (g)	Heart (g)	Stomach (g)	Intestine (g)	Muscle (g)	Carcass (g)
Changshu	Male (*n* = 5)	27.98	2.56	0.38	0.0603	0.0273	0.0483	0.0842	0.5434	0.3914	15.31	8.58
		17.69	1.52	0.26	0.0439	0.0167	0.0833	0.0578	0.4181	0.4369	10.01	4.84
		5.68	0.52	0.02	0.0190	0.0092	0.0206	0.0081	0.0915	0.0911	3.25	1.65
		13.54	1.35	0.34	0.0744	0.0033	0.0597	0.0211	0.2971	0.4425	7.00	3.95
		8.15	0.85	0.18	0.0200	0.0132	0.0307	0.0133	0.1448	0.1451	5.12	1.63
	Female (*n* = 5)	48.87	4.52	1.18	0.0821	6.3416	0.2044	0.0846	0.9137	0.9438	22.07	12.53
		17.94	1.66	0.41	0.0429	1.6080	0.0728	0.0332	0.4206	0.4166	7.53	5.75
		17.34	1.41	0.52	0.0454	2.4511	0.0732	0.0370	0.4072	0.6145	6.77	5.01
		26.23	2.26	0.80	0.0886	5.7404	0.0726	0.0399	0.5522	0.6886	11.18	4.81
		22.83	2.61	0.62	0.0673	1.4167	0.0844	0.0499	0.4523	0.5007	9.46	7.57
Huantai	Male (*n* = 4)	11.91	1.12	0.64	0.0549	0.0069	0.0437	0.0269	0.2445	0.2834	5.96	3.53
		10.99	1.00	0.53	0.0483	0.0089	0.0502	0.0276	0.2557	0.2521	5.28	3.54
		8.42	0.83	0.43	0.0284	0.0053	0.0251	0.016	0.1729	0.1948	4.15	2.57
		8.26	0.81	0.46	0.0296	0.0036	0.0337	0.0123	0.1696	0.1711	4.04	2.53

Table S3. LC-MS/MS instrument parameters for the quantification of target analytes.

Instrument	Acquity UPLC coupled to a Xevo TQ-*S* triple quadrupole mass spectrometer (Waters, Milford, MA, USA) or API 5500 triple-quadrupole mass spectrometer (AB SCIEX Inc., Framingham, MA, USA)
Analytical column	Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 µm, Waters, MA, USA)
Trap column	C18 column (50 mm × 2.1 mm, 3.0 µm, Waters, MA, USA)
Column temperature	40 °C
Injection volume	2 µL
Mobile phase	2 mM ammonium acetate in water (A) and methanol (B)

Gradient

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0.0	0.30	90	10
1.0	0.30	80	20
4.0	0.30	10	90
6.0	0.30	10	90
6.1	0.30	90	10
9.0	0.30	90	10

Multiple reaction monitoring (MRM) transitions

Compound	Ion transitions	CV/DP (V)	CE (V)	Internal standard
PFBA	213→169*	30	11	$^{13}C_4$-PFBA
	213→115	22	18	
PFPeA	263→219*	2	8	$^{13}C_5$-PFPeA
	263→141	8	8	
PFHxA	313→269*	14	10	$^{13}C_2$-PFHxA
	313→119	14	15	
PFHpA	363→319*	30	10	$^{13}C_4$-PFHpA
	363→169	30	19	
PFOA	413→369*	30	10	$^{13}C_4$-PFOA
	413→169	30	18	
PFNA	463→419*	28	10	$^{13}C_5$-PFNA
	463→169	32	20	
PFDA	513→469*	12	10	$^{13}C_2$-PFDA
	513→219	12	18	
PFUnDA	563→519*	30	10	$^{13}C_2$-PFUnDA
	563→319	30	17	

	PFDoDA	613→569*	22	10	$^{13}C_2$-PFDoDA
		613→169	10	22	
	PFTriDA	663→619*	10	10	$^{13}C_2$- PFTeDA
		663→169	10	26	
	PFTeDA	713→669*	8	15	$^{13}C_2$- PFTeDA
		713→169	10	22	
	PFBS	299→80*	40	30	$^{18}O_2$-PFHxS
		299→99	40	28	
	PFHxS	399→80*	45	33	$^{18}O_2$-PFHxS
		399→99	30	31	
	PFOS	499→99*	30	39	$^{13}C_4$-PFOS
		499→80	30	37	
	6:2 Cl-PFESA	531→351*	12	24	$^{13}C_4$-PFOS
		531→83	12	22	
	8:2 Cl-PFESA	631→451*	20	28	$^{13}C_4$-PFOS
		631→83	20	35	
	HFPO-TA	495→185*	20	12	$^{13}C_5$-PFNA
		495→119	20	22	
	Mass-labeled internal standards				
	$^{13}C_4$-PFBA	217→172	30	11	
	$^{13}C_5$-PFPeA	268→223	2	8	
	$^{13}C_2$-PFHxA	315→270	14	10	
	$^{13}C_4$-PFHpA	367→169	30	19	
	$^{13}C_4$-PFOA	417→372	30	10	
	$^{13}C_5$-PFNA	468→423	30	10	
	$^{13}C_2$-PFDA	515→470	12	10	
	$^{13}C_2$-PFUnDA	565→520	30	10	
	$^{13}C_2$-PFDoDA	615→570	22	10	
	$^{13}C_2$- PFTeDA	715→670	10	14	
	$^{18}O_2$-PFHxS	403→103	45	30	
	$^{13}C_4$-PFOS	503→80	20	34	
	(*) represents quantitative ion transition of corresponding analyte CV: cone voltage; DP: declustering potential; CE: collision energy				
Other mass parameters	**<u>Xevo TQ-S, Waters</u>** Capillary voltage, -0.5 kV; Source temperature, 150 °C; Desolvation temperature, 450 °C; Desolvation gas flow, 850 L/h; Cone gas flow, 150 L/h;				

	API 5500, AB Sciex Ion Spray Voltage: -4.5 kV; Curtain Gas: 20 psi; Collision Gas: Medium; Temperature: 500 °C; Ion Source Gas 1: 50 psi; Ion Source Gas 2: 45 psi

Table S4. Limits of quantification (LOQs) of the target analytes in different tissues (unit: ng/g ww).

	Kidney	Ovary	Liver	Testis	Skin	Lung	Heart	Stomach	Intestine	Muscle	Carcass
PFBA	0.225	0.203	0.129	0.627	0.191	0.371	0.583	0.149	0.173	0.260	0.283
PFPeA	0.091	0.036	0.041	0.250	0.027	0.053	0.200	0.034	0.021	0.017	0.020
PFHxA	0.104	0.100	0.083	0.114	0.023	0.168	0.170	0.051	0.066	0.015	0.067
PFHpA	0.123	0.019	0.021	0.121	0.187	0.070	0.148	0.032	0.018	0.037	0.034
PFOA	0.100	0.025	0.031	0.200	0.062	0.100	0.200	0.053	0.038	0.014	0.010
PFNA	0.100	0.083	0.035	0.210	0.036	0.100	0.200	0.027	0.023	0.012	0.011
PFDA	0.100	0.020	0.029	0.420	0.020	0.100	0.200	0.020	0.020	0.010	0.010
PFUnDA	0.126	0.041	0.043	0.200	0.022	0.100	0.200	0.020	0.020	0.010	0.010
PFDoDA	0.098	0.043	0.029	0.100	0.014	0.063	0.100	0.011	0.014	0.005	0.005
PFTriDA	0.072	0.039	0.024	0.190	0.035	0.063	0.100	0.015	0.014	0.011	0.010
PFTeDA	0.078	0.036	0.024	0.200	0.021	0.050	0.116	0.020	0.020	0.014	0.012
PFBS	0.069	0.041	0.036	0.310	0.051	0.082	0.239	0.029	0.035	0.021	0.011
PFHxS	0.062	0.088	0.011	0.100	0.012	0.050	0.100	0.018	0.011	0.127	0.017
PFOS	0.136	0.036	0.010	0.230	0.045	0.061	0.145	0.035	0.067	0.016	0.005
6:2 Cl-PFESA	0.050	0.010	0.010	0.100	0.010	0.050	0.100	0.010	0.010	0.005	0.005
8:2 Cl-PFESA	0.083	0.010	0.010	0.100	0.010	0.050	0.100	0.010	0.010	0.008	0.005
HFPO-TA	0.250	0.050	0.050	0.500	0.050	0.250	0.500	0.050	0.050	0.025	0.025

Three criteria were used to evaluate LOQs: (1) Concentration resulting in a signal-to-noise ratio at or greater than 10 in matrices; (2) Lowest concentration of standard in the calibration curve with measured concentrations within ± 20% of its theoretical value; and, (3) Concentration or dilution factor. The LOQs based on criteria 1 and 2 were compared, with the higher value (lower sensitivity) chosen and further adjusted by criterion 3. The LOQs based on criterion 1 are presented in blue, and the LOQs based on criterion 2 are presented in yellow.

Table S5. Matrix spike recovery (mean ± SD, %) of target PFASs in different tissues (*n* = 3).

	Kidney	Ovary	Liver	Testis	Skin	Lung	Heart	Stomach	Intestine	Muscle	Carcass
PFBA	88.1 ± 2.6	91.6 ± 7.3	87.2 ± 4.1	92.6 ± 4.0	91.7 ± 2.4	92.8 ± 2.3	95.3 ± 6.2	88.3 ±5.4	93.0 ± 4.3	90.4 ± 4.3	93.2 ± 5.2
PFPeA	93.9 ± 1.9	94.7 ± 4.5	92.2 ± 3.9	94.6 ± 2.7	96.3 ± 0.8	95.1 ± 1.8	84.5 ± 2.9	93.4 ± 2.3	80.5 ± 6.4	97.4 ± 1.6	95.6 ± 2.4
PFHxA	92.1 ± 1.6	89.1 ± 4.1	93.9 ± 2.7	94.6 ± 1.5	95.9 ± 2.0	94.7 ± 2.6	94.6 ± 1.1	91.2 ± 1.2	94.6 ± 1.7	94.8 ± 10.2	92.3 ± 11.5
PFHpA	90.8 ± 4.0	88.2 ± 5.1	94.6 ± 3.7	95.8 ± 4.3	93.6 ± 6.5	93.7 ± 3.0	94.1 ± 7.4	92.9 ± 4.6	93.5 ± 6.1	98.2 ± 6.7	99.5 ± 7.5
PFOA	95.0 ± 3.2	92.8 ± 4.5	98.6 ± 9.5	96.5 ± 3.3	93.7 ± 4.1	94.8 ± 5.1	95.8 ± 3.5	96.3 ± 2.8	98.8 ± 4.8	93.6 ± 10.5	94.5 ± 12.3
PFNA	96.6 ± 4.4	96.7 ± 8.1	95.3 ± 3.2	97.9 ± 3.0	101.3 ± 4.7	96.2 ± 2.7	98.8 ± 7.4	95.7 ± 2.9	98.2 ± 4.8	93.9 ± 12.3	96.3 ± 11.7
PFDA	96.7 ± 4.5	93.3 ± 7.4	93.8 ± 5.7	95.1 ± 1.9	99.4 ± 3.4	96.1 ± 0.7	93.3 ± 2.7	94.0 ± 6.4	95.8 ± 1.9	96.1 ± 9.8	94.6 ± 5.8
PFUnDA	94.0 ± 2.2	87.7 ± 9.1	94.2 ± 4.1	92.4 ± 3.9	99.2 ± 2.3	98.8 ± 2.1	95.7 ± 5.0	95.2 ± 3.0	95.1 ± 4.7	96.1 ± 10.8	97.9 ± 9.6
PFDoDA	94.4 ± 4.3	83.2 ± 5.6	94.7 ± 2.5	94.3 ± 2.6	93.4 ± 1.9	96.2 ± 2.2	94.0 ± 5.9	94.2 ± 8.3	96.8 ± 4.1	97.2 ± 7.3	98.6 ± 7.9
PFTriDA	103.3 ± 3.3	90.9 ± 8.4	123.6 ± 2.6	104.5 ± 7.4	144.4 ± 3.5	108.9 ± 2.1	100.3 ± 6.6	104.2 ± 10.2	107.9 ± 3.0	115.2 ± 16.2	112.8 ± 14.8
PFTeDA	97.7 ± 4.1	90.1 ± 6.4	95.0 ± 4.9	97.5 ± 3.0	98.1 ± 3.7	98.4 ± 0.5	94.9 ± 4.9	96.1 ± 10.4	93.7 ± 2.9	96.2 ± 9.5	96.2 ± 9.5
PFBS	90.2 ± 1.2	82.7 ± 7.1	80.5 ± 20.8	103.2 ± 2.5	100.8 ± 2.5	95.2 ± 1.8	81.3 ± 5.4	101.4 ± 1.4	101.3 ± 3.4	93.6 ± 10.3	94.9 ± 10.7
PFHxS	95.3 ± 0.2	97.5 ± 5.4	92.7 ± 3.2	94.8 ± 2.7	96.0 ± 2.0	98.4 ± 0.2	93.3 ± 5.0	92.8 ± 2.4	93.1 ± 3.1	93.5 ± 9.5	91.6 ± 10.6
PFOS	95.5 ± 7.8	81.7 ± 12.4	90.1 ± 3.2	93.1 ± 1.7	91.8 ± 2.0	93.8 ± 3.8	92.2 ± 1.5	93.2 ± 5.8	92.4 ± 1.3	98.8 ± 11.0	94.4 ± 10.2
6:2 Cl-PFESA	102.5 ± 6.3	92.5 ± 7.5	100.2 ± 3.5	99.4 ± 2.9	99.7 ± 1.5	103.7 ± 1.6	105.3 ± 9.7	97.7 ± 6.8	99.6 ± 1.3	89.4 ± 10.8	85.7 ± 9.6
8:2 Cl-PFESA	94.6 ± 6.6	89.2 ± 6.1	96.5 ± 2.5	94.0 ± 3.2	88.1 ± 4.5	96.4 ± 1.9	95.6 ± 11.3	91.6 ± 6.4	94.9 ± 2.3	76.7 ± 8.7	79.4 ± 9.3
HFPO-TA	87.1 ±4.6	93.2 ± 2.8	90.0 ± 4.9	83.3 ±5.7	85.9 ± 2.8	89.1 ± 3.8	90.2 ± 6.3	79.6 ± 5.8	87.2± 6.1	84.5 ± 3.2	86.6 ± 4.2

Table S6. Detection rates and measured PFAS levels (ng/g ww) in frog liver samples (*n* = 56).

	Detection rate	Arithmetic mean	SD	Geometric mean	Min	Max
PFHxA	74.5%	0.39	0.44	0.23	0.06	1.98
PFOA	100%	1.01	2.08	0.44	0.03	13.70
PFNA	81.8%	0.12	0.11	0.08	0.01	0.59
PFDA	100%	2.83	3.22	1.27	0.07	14.81
PFUnDA	100%	4.14	5.13	2.16	0.18	26.83
PFDoDA	98.2%	2.58	3.04	1.34	0.029	14.17
PFTriDA	98.2%	3.10	2.81	2.11	0.20	14.38
PFTeDA	100%	1.10	1.04	0.72	0.08	4.78
PFHxS	50.9%	0.04	0.07	0.02	0.01	0.30
PFOS	100%	5.58	7.44	2.56	0.14	36.16
6:2 Cl-PFESA	100%	10.47	19.84	3.85	0.13	119.05
8:2 Cl-PFESA	100%	1.23	3.04	0.33	0.01	14.89
HFPO-TA	7.14%	13.41	9.43	11.47	6.51	27.30

PFBA, PFPeA, PFHpA, and PFBS were not detected in any sample, and therefore are not presented. HFPO-TA was detected in all samples from Huantai (*n* = 4), but not in those from other locations.

Table S7. Recovery, limits of quantification (LOQs), measured PFAS levels (ng/L), and the proportion in ΣPFAS in waters from the four sampling sites.

	Recovery (%)	LOQ (ng/L)	Changshu		Huantai		Quzhou		Zhoushan	
			Level	Proportion	Level	Proportion	Level	Proportion	Level	Proportion
PFBA	104.0 ± 3.1	0.05	46.22	3.4%	2368	4.6%	3.10	14.6%	3.61	13.8%
PFPeA	103.5 ± 3.3	0.05	18.87	1.4%	1863	3.6%	1.06	5.0%	1.12	4.3%
PFHxA	104.5 ± 1.6	0.02	599.34	44.5%	1524	3.0%	0.93	4.3%	4.18	16.0%
PFHpA	104.7 ± 2.7	0.02	18.81	1.4%	1363	2.7%	2.73	12.8%	1.13	4.3%
PFOA	103.1 ± 3.3	0.02	648.36	48.2%	43936	86.0%	12.06	56.5%	9.69	37.1%
PFNA	105.7 ± 1.3	0.02	5.70	0.4%	15.22	0.0%	0.63	3.0%	1.68	6.4%
PFDA	105.4 ± 5.0	0.02	1.94	0.1%	1.63	0.0%	0.23	1.1%	0.72	2.7%
PFUnDA	106.1 ± 2.4	0.02	0.43	0.0%	0.03	0.0%	0.14	0.7%	0.50	1.9%
PFDoDA	99.0 ± 8.5	0.02	0.11	0.0%	0.02	0.0%	0.06	0.3%	0.08	0.3%
PFTriDA	93.3 ± 1.6	0.02	0.04	0.0%	0.01	0.0%	0.04	0.2%	0.01	0.0%
PFTeDA	104.5 ± 1.8	0.01	0.02	0.0%	0.01	0.0%	0.01	0.0%	0.01	0.0%
PFBS	104.3 ± 3.0	0.01	1.77	0.1%	11.82	0.0%	0.10	0.5%	0.56	2.1%
PFHxS	105.0 ± 3.5	0.05	0.71	0.1%	0.81	0.0%	0.03	0.1%	0.26	1.0%
PFOS	102.3 ± 3.7	0.02	2.12	0.2%	3.15	0.0%	0.10	0.5%	1.90	7.3%
6:2Cl-PFESA	104.9 ± 2.5	0.01	1.82	0.1%	0.93	0.0%	0.11	0.5%	0.69	2.6%
8:2Cl-PFESA	104.9 ± 4.3	0.01	0.02	0.0%	0.01	0.0%	0.01	0.1%	0.01	0.0%
HFPO-TA	100.3 ± 2.2	0.05	N.D.	0.0%	3660	7.2%	N.D.	0.0%	N.D.	0.0%
ΣPFAS			1346	100.0%	54747	100.0%	21.33	100.0%	26.14	100.0%

N.D., not detected

Table S8. Sex-related differences in tissue ΣPFAS levels (mean ± SD) for frogs from Changshu (n = 10).

Tissue	Male (n = 5)	Female (n = 5)
Kidney	91.69 ± 36.64	132.45 ± 27.85
Liver	51.91 ± 13.25	32.57 ± 8.82*
Gonad	30.86 ± 5.43	95.40 ± 45.58*
Skin	34.93 ± 15.80	17.81 ± 7.31*
Lung	29.00 ± 8.10	19.38 ± 2.84
Heart	22.37 ± 7.38	13.72 ± 2.50
Stomach	12.22 ± 3.63	9.58 ± 1.47
Intestine	10.94 ± 5.01	11.26 ± 2.31
Muscle	6.08 ± 1.92	3.20 ± 0.78*
Carcass	3.14 ± 0.86	2.01 ± 0.34

* represents significant differences between male and female tissues.

Table S9. Linear regression model between liver ΣPFAS concentration and frog age (n = 56).

Varibles	Unstandardized coefficients		Standardized coefficients	t	sig
	Beta	Std Error	Beta		
Crude model					
Constant	66.071	15.193		4.349	0.000
Age	-15.963	6.934	-0.301	-2.302	0.028
Adjusted model					
Constant	127.759	20.252		6.308	0.000
Age	-12.270	6.036	-0.232	-2.033	0.047
Sampling sites	-17.722	3.895	-0.510	-4.549	0.000
Sex	-20.597	9.871	-0.240	-2.087	0.042

Liver ΣPFAS concentration (continuous) was set as dependent variable while frog age (continuous) was set as independent variable. Sampling sites and frog sex (dummy variables) were further set as covariates in the adjusted model. The coefficients < 0 indicated negative associations of PFAS level with frog age, sex, and sampling sites. Liver ΣPFAS concentration tended to be lower in older, female frogs, and frogs lived in places without fluorochemical industry.

Table S10. Detection rate (%) of target PFASs in different tissues for selected frogs from Changshu and Huantai (*n* = 14).

	Kidney	Ovary	Liver	Testis	Skin	Lung	Heart	Stomach	Intestine	Muscle	Carcass
PFHxA	78.6	20.0	100	33.3	92.9	71.4	64.3	100	50.0	100	71.4
PFOA	100	100	100	100	100	100	78.6	100	100	100	92.9
PFNA	100	100	100	22.2	92.9	79	57.1	78.6	71.4	100	100
PFDA	100	100	100	44.4	100	100	100	100	100	100	100
PFUnDA	100	100	100	44.4	100	100	100	100	100	100	100
PFDoDA	100	100	100	100	100	100	100	100	100	100	100
PFTriDA	100	100	100	44.4	100	100	100	100	100	100	100
PFTeDA	100	100	100	44.4	100	100	100	100	92.9	100	100
PFHxS	71.4	100	71.4	0.00	28.5	28.5	14.3	28.5	57.1	0.00	71.4
PFOS	100	100	100	55.5	100	100	100	100	100	100	100
6:2 Cl-PFESA	100	100	100	100	100	100	100	100	100	100	100
8:2 Cl-PFESA	100	100	100	66.7	100	100	92.9	100	100	85.7	100
HFPO-TA	28.6	N.A.	28.6	21.4	28.6	28.6	28.6	28.6	28.6	28.6	28.6

Tissue samples were dissected from 14 individual frogs captured in Changshu (*n* = 10) and Huantai (*n* = 4)

Total number for ovaries and testes was 5 and 9, respectively.

HFPO-TA was only detected in samples from Huantai

N.A. not available, all frogs captured in Huantai were male.

Table S11. Mean concentration (ng/g ww) of PFASs measured in frog tissues separated by sex for selected samples from Changshu (*n* = 10).

	Sex	Kidney	Liver	Gonad	Skin	Lung	Heart	Stomach	Intestine	Muscle	Carcass
PFHxA	M	0.65 ± 0.36	0.55 ± 0.30	0.75 ± 0.44	0.67 ± 0.53	0.50 ± 0.39	0.40 ± 0.31	0.43 ± 0.45	0.20 ± 0.26	0.78 ± 0.74	0.16 ± 0.13
	F	0.23 ± 0.15	0.32 ± 0.09	0.23 ± 0.21	0.63 ± 0.13	0.29 ± 0.15	0.25 ± 0.14	0.32 ± 0.15	0.21 ± 0.13	0.33 ± 0.19	0.09 ± 0.06
PFOA	M	0.55 ± 0.21	0.35 ± 0.25	1.08 ± 0.71	0.37 ± 0.24	0.40 ± 0.18	0.39 ± 0.26	0.17 ± 0.08	0.13 ± 0.05	0.10 ± 0.06	0.08 ± 0.01
	F	0.67 ± 0.20	0.15 ± 0.07	0.43 ± 0.21	0.35 ± 0.20	0.20 ± 0.07	0.21 ± 0.07	0.22 ± 0.12	0.16 ± 0.05	0.08 ± 0.03	0.06 ± 0.02
PFNA	M	0.51 ± 0.28	0.17 ± 0.04	0.47 ± 0.23	0.19 ± 0.08	0.35 ± 0.52	0.17 ± 0.10	0.06 ± 0.06	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
	F	1.02 ± 0.65	0.13 ± 0.06	0.32 ± 0.07	0.12 ± 0.06	0.11 ± 0.05	0.09 ± 0.02	0.20 ± 0.32	0.06 ± 0.03	0.03 ± 0.01	0.01 ± 0.00
PFDA	M	23.63 ± 13.86	5.16 ± 0.84	4.00 ± 2.16	2.32 ± 1.93	2.38 ± 1.01	2.44 ± 0.93	1.01 ± 0.53	1.04 ± 0.62	0.46 ± 0.23	0.24 ± 0.11
	F	40.91 ± 15.88	3.36 ± 0.74	9.15 ± 4.04	1.32 ± 0.97	1.90 ± 1.34	1.64 ± 1.22	0.69 ± 0.33	0.97 ± 0.60	0.22 ± 0.13	0.15 ± 0.08
PFUnDA	M	22.56 ± 12.39	7.79 ± 3.01	4.91 ± 2.33	4.44 ± 3.02	5.34 ± 2.30	4.67 ± 2.31	2.29 ± 1.28	2.03 ± 1.32	0.91 ± 0.46	0.45 ± 0.22
	F	24.69 ± 10.15	3.71 ± 1.05	16.25 ± 8.07	1.92 ± 1.16	2.93 ± 1.24	2.20 ± 1.03	1.22 ± 0.58	1.58 ± 0.71	0.33 ± 0.12	0.23 ± 0.10
PFDoDA	M	9.47 ± 3.81	4.88 ± 2.00	4.34 ± 1.43	3.48 ± 2.35	4.65 ± 2.29	3.44 ± 1.90	2.00 ± 1.22	1.76 ± 1.27	0.74 ± 0.38	0.34 ± 0.18
	F	8.83 ± 3.11	3.15 ± 0.75	13.48 ± 7.28	1.99 ± 0.87	3.23 ± 0.66	2.32 ± 0.66	1.48 ± 0.53	2.00 ± 0.74	0.36 ± 0.05	0.26 ± 0.09
PFTriDA	M	6.00 ± 1.99	4.09 ± 1.14	3.92 ± 0.95	4.40 ± 3.04	3.86 ± 1.01	2.28 ± 0.87	1.76 ± 0.70	1.59 ± 0.83	0.90 ± 0.40	0.50 ± 0.16
	F	5.36 ± 1.25	3.12 ± 0.44	8.91 ± 3.44	2.23 ± 0.62	2.87 ± 0.10	1.85 ± 0.37	1.47 ± 0.41	1.97 ± 0.56	0.53 ± 0.13	0.51 ± 0.15
PFTeDA	M	3.08 ± 0.93	1.64 ± 0.47	2.64 ± 1.30	1.27 ± 0.74	1.74 ± 0.53	1.09 ± 0.50	0.80 ± 0.39	0.83 ± 0.45	0.23 ± 0.08	0.13 ± 0.05
	F	3.72 ± 2.48	2.06 ± 1.04	4.91 ± 1.76	1.46 ± 0.58	2.34 ± 0.85	1.48 ± 0.70	1.24 ± 0.65	1.62 ± 0.69	0.29 ± 0.14	0.23 ± 0.12
PFHxS	M	0.07 ± 0.03	0.03 ± 0.03	0.50 ± 0.41	0.40 ± 0.20	N.D.	N.D.	N.D.	N.D.	0.13 ± 0.08	0.02 ± 0.02
	F	0.05 ± 0.02	0.04 ± 0.04	0.14 ± 0.08	0.52 ± 0.37	N.D.	N.D.	N.D.	N.D.	0.40 ± 0.65	0.04 ± 0.03
PFOS	M	13.56 ± 3.51	8.29 ± 2.09	3.32 ± 1.20	5.70 ± 4.67	3.14 ± 1.10	2.29 ± 0.93	1.30 ± 0.62	1.31 ± 0.83	0.62 ± 0.25	0.35 ± 0.07
	F	27.21 ± 5.60	7.67 ± 5.00	19.89 ± 10.98	2.68 ± 1.90	2.60 ± 1.01	1.73 ± 0.70	1.00 ± 0.32	1.46 ± 0.57	0.28 ± 0.08	0.23 ± 0.07

6:2 Cl-	M	10.44 ± 7.71	17.05 ± 9.7	4.21 ± 1.28	10.14 ± 7.47	5.96 ± 4.63	4.65 ± 2.89	2.11 ± 1.26	1.78 ± 1.47	1.06 ± 0.94	0.79 ± 0.22
PFESA	F	18.97 ± 14.35	8.41 ± 5.57	19.34 ± 20.46	4.07 ± 1.85	2.62 ± 1.15	1.75 ± 0.57	1.62 ± 1.70	1.08 ± 0.40	0.33 ± 0.15	0.19 ± 0.05
8:2 Cl-	M	1.17 ± 1.04	1.91 ± 1.79	0.73 ± 0.39	1.54 ± 1.24	0.65 ± 0.27	0.54 ± 0.25	0.27 ± 0.11	0.23 ± 0.11	0.09 ± 0.07	0.05 ± 0.03
PFESA	F	0.79 ± 0.55	0.45 ± 0.17	2.36 ± 1.90	0.51 ± 0.43	0.25 ± 0.07	0.18 ± 0.04	0.10 ± 0.04	0.14 ± 0.03	0.02 ± 0.01	0.02 ± 0.00
ΣPFCAs	M	66.45 ± 28.25	24.63 ± 7.30	22.11 ± 3.91	17.14 ± 11.08	19.23 ± 6.80	14.88 ± 6.49	8.52 ± 3.80	7.61 ± 4.30	4.16 ± 1.23	1.92 ± 0.66
	F	85.42 ± 25.3	160 ± 1.50	53.67 ± 23.33	10.03 ± 3.18	13.87 ± 2.46	10.05 ± 2.34	6.85 ± 1.19	8.57 ± 2.18	2.18 ± 0.21	1.53 ± 0.34
ΣPFSAs	M	13.63 ± 3.50	8.32 ± 2.08	3.81 ± 0.86	6.10 ± 4.83	3.16 ± 1.10	2.30 ± 0.93	1.31 ± 0.62	1.32 ± 0.83	0.76 ± 0.31	0.37 ± 0.07
	F	27.26 ± 5.59	7.71 ± 5.01	20.02 ± 11.04	3.20 ± 2.11	2.63 ± 1.02	1.74 ± 0.70	1.01 ± 0.32	1.48 ± 0.57	0.67 ± 0.63	0.27 ± 0.05
ΣPFESAs	M	11.61 ± 7.98	18.96 ± 10.03	4.94 ± 1.47	11.69 ± 8.60	6.62 ± 4.71	5.19 ± 3.02	2.38 ± 1.32	2.01 ± 1.56	1.16 ± 0.95	0.84 ± 0.24
	F	19.77 ± 14.85	8.86 ± 5.72	21.71 ± 22.27	4.58 ± 2.25	2.87 ± 1.22	1.94 ± 0.58	1.72 ± 1.71	1.22 ± 0.41	0.35 ± 0.16	0.21 ± 0.05
ΣPFASs	M	91.69 ± 36.64	51.91 ± 13.25	30.86 ± 5.43	34.93 ± 15.8	29.00 ± 8.10	22.37 ± 7.38	12.22 ± 3.63	10.94 ± 5.01	6.08 ± 1.92	3.14 ± 0.86
	F	132.45 ± 27.9	32.57 ± 8.82	95.4 ± 45.58	17.81 ± 7.31	19.38 ± 2.84	13.72 ± 2.50	9.58 ± 1.47	11.26 ± 2.31	3.20 ± 0.78	2.01 ± 0.34

M, male; F, female. N.D., not detected

Table S12. Mean concentration (ng/g ww) of PFASs measured in tissues of male frogs from Huantai (*n* = 4).

	Kidney	Liver	Testis	Skin	Lung	Heart	Stomach	Intestine	Muscle	Carcass
PFHxA	5.81 ± 2.44	1.01 ± 0.64	0.55 ± 0.36	0.38 ± 0.13	0.22 ± 0.08	0.29 ± 0.13	0.14 ± 0.09	0.07 ± 0.03	0.28 ± 0.10	0.14 ± 0.04
PFOA	43.6 ± 7.18	5.95 ± 4.50	2.54 ± 0.39	2.47 ± 1.46	1.74 ± 0.14	2.30 ± 0.18	0.94 ± 0.43	0.63 ± 0.45	1.06 ± 0.36	1.06 ± 0.02
PFNA	1.45 ± 0.63	0.17 ± 0.05	0.54 ± 0.18	0.11 ± 0.06	0.12 ± 0.03	0.23 ± 0.08	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
PFDA	7.06 ± 3.07	0.87 ± 0.23	1.07 ± 0.36	0.19 ± 0.08	0.42 ± 0.06	0.45 ± 0.11	0.15 ± 0.05	0.09 ± 0.04	0.10 ± 0.04	0.08 ± 0.01
PFUnDA	4.14 ± 1.88	1.22 ± 0.40	1.28 ± 0.43	0.32 ± 0.13	0.83 ± 0.14	0.73 ± 0.16	0.29 ± 0.10	0.17 ± 0.09	0.14 ± 0.04	0.13 ± 0.03
PFDoDA	1.11 ± 0.44	0.65 ± 0.22	1.82 ± 0.83	0.52 ± 0.29	0.66 ± 0.13	0.49 ± 0.18	0.28 ± 0.13	0.18 ± 0.13	0.11 ± 0.04	0.10 ± 0.02
PFTriDA	0.93 ± 0.25	0.59 ± 0.21	1.28 ± 0.43	0.41 ± 0.28	0.96 ± 0.27	0.52 ± 0.19	0.35 ± 0.13	0.18 ± 0.10	0.14 ± 0.04	0.21 ± 0.06
PFTeDA	0.34 ± 0.11	0.21 ± 0.07	1.28 ± 0.43	0.12 ± 0.07	0.31 ± 0.08	0.18 ± 0.08	0.11 ± 0.04	0.08 ± 0.04	0.05 ± 0.02	0.05 ± 0.01
PFHxS	1.25 ± 0.24	0.23 ± 0.05	N.D.	0.04 ± 0.03	0.08 ± 0.02	0.10 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	N.D.	0.19 ± 0.13
PFOS	6.62 ± 1.44	2.34 ± 0.38	0.77 ± 0.26	0.57 ± 0.32	0.78 ± 0.05	0.62 ± 0.19	0.34 ± 0.15	0.21 ± 0.12	0.25 ± 0.08	0.26 ± 0.02
6:2 Cl-PFESA	7.92 ± 1.00	4.02 ± 0.75	1.20 ± 0.20	1.67 ± 1.22	1.53 ± 0.20	1.88 ± 0.56	0.80 ± 0.48	0.43 ± 0.24	0.40 ± 0.10	0.55 ± 0.14
8:2 Cl-PFESA	0.50 ± 0.11	0.55 ± 0.08	0.33 ± 0.05	0.17 ± 0.10	0.45 ± 0.08	0.39 ± 0.12	0.15 ± 0.07	0.09 ± 0.06	0.06 ± 0.01	0.05 ± 0.01
HFPO-TA	59.3 ± 21.9	13.4 ± 8.16	3.24 ± 1.49	6.10 ± 4.17	3.93 ± 1.70	7.39 ± 4.35	1.96 ± 1.02	0.93 ± 0.36	1.76 ± 0.63	1.08 ± 0.56
∑PFCAs	64.4 ± 10.6	10.7 ± 5.36	10.4 ± 1.97	4.51 ± 2.01	5.25 ± 0.72	5.18 ± 0.97	2.30± 0.93	1.42 ± 0.80	1.92 ± 0.55	0.14 ± 0.04
∑PFSAs	7.87 ± 1.35	2.57 ± 0.42	0.77 ± 0.26	0.62 ± 0.32	0.90 ± 0.04	0.85 ± 0.24	0.38 ± 0.17	0.23 ± 0.14	0.25 ± 0.08	1.06 ± 0.20
∑PFESAs	8.42 ± 1.02	4.57 ± 0.72	1.53 ± 0.25	1.84 ± 1.30	1.97 ± 0.28	2.27 ± 0.67	0.96 ± 0.54	0.51 ± 0.30	0.45 ± 0.10	0.03 ± 0.01
∑PFASs	140 ± 24.0	31.2 ± 8.34	15.9 ± 4.13	13.1 ± 5.64	12.1 ± 1.40	15.7 ± 5.04	5.60 ± 2.20	3.09 ± 1.26	4.38 ± 0.85	0.08 ± 0.01

N.D., not detected