Environmental Science & Technology

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Legacy and Emerging Per- and Polyfluoroalkyl Substances Surveillance in *Bufo gargarizans* from Inlet Watersheds of Chaohu Lake, China: Tissue Distribution and Bioaccumulation Potential

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Cite This: *Environ. Sci. Technol.* 2023, 57, 13148−13160

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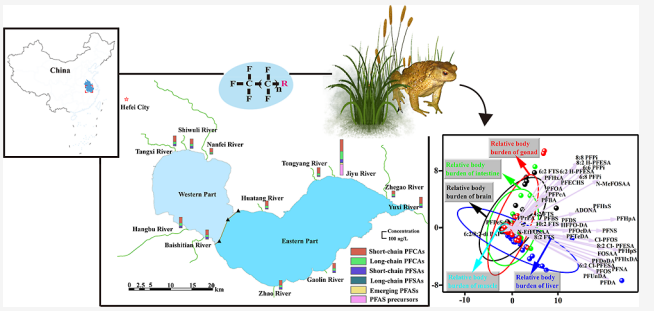
ABSTRACT: Amphibians are sensitive biomonitors of environmental pollutants but reports regarding per- and polyfluoroalkyl substances (PFAS), a class of synthetic organofluorine substances, are limited. In this study, samples of water and Chinese toads (*Bufo gargarizans*) were collected in Chaohu Lake, China. Tissue-specific bioaccumulation characteristics of 39 PFAS, including 19 perfluoroalkyl acids (PFAAs), 8 emerging PFAS, and 12 PFAA precursors, were investigated, and the levels of some biochemical indicators were determined. The highest PFAS concentrations were found in the liver [215.97 ng/g dry weight (dw)] of Chinese toads, followed by gonads (135.42 ng/g dw) and intestine (114.08 ng/g dw). A similar tissue distribution profile was found between legacy and emerging PFAS in the toads, and the occurrence of two emerging PFAS, 2,3,3,3-tetrafluoro-2-propanoate (HFPO-DA) and 6:2 hydrogen-substituted polyfluorooctane ether sulfonate (6:2 H-PFESA) in the amphibians were for the first time reported. Field-based bioaccumulation factors of HFPO-DA were higher than perfluorooctanoic acid, indicating the higher bioaccumulation potential of this emerging PFAS than the legacy C8 compound. Males had significantly higher gonad PFAS levels than females while estradiol levels in gonads increased with increasing concentrations of certain PFAS (e.g., 6:2 H-PFESA), implying that PFAS may trigger estrogenic effects in the toads, especially for male toads.



KEYWORDS: *amphibians, Cl-PFESA, H-PFESA, ADONA, PFAS precursor, gonad, sex hormone disorder, bioconcentration*

INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic fluorinated compounds that are commonly used in a variety of industrial and commercial products such as electroplating, textiles, and surface coatings.[1] Due to their stable structures and widespread from their production, processes, and products, these compounds are widely distributed and persistent in the environment and can be continuously recycled in the hydrosphere.[2,3] The new global boundary of PFAS has been exceeded if it is estimated according to the concept of the planetary boundary of "safe operation space for human beings in the earth system operation".[2]

One larger sub-category of PFAS, perfluoroalkyl acids (PFAAs), caused great public concern owing to their detrimental effects on wildlife and humans.[4,5] PFAAs can be divided into two major groups, perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonic acids (PFSAs). Some legacy PFAAs, such as perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid, their salts, and their related compounds, have been regulated or recommended to be eliminated or restricted in the world by the Stockholm convention.[6−8] Furthermore, in 2022, it was proposed that long-chain PFCAs with perfluorinated carbon chain lengths from 8 to 20 should be listed as persistent organic pollutants (POPs).[9] In the environment, PFAAs can also be derived from precursors by biological or abiotic transformation pathways, such as photooxidation of fluorotelomer alcohols (FTOHs), and biotransformation of perfluorooctyl sulfonamides (FOSAs) and sulfonamidoethanols (FOSEs).[10−12] In consideration of the disadvantages and restriction of legacy PFAAs, many alternative PFAS have been produced and used in industrial and commercial products. Such emerging PFAS have been observed in aquatic ecosystems,[3,13] such as the detection of hexafluoropropylene

Received: April 9, 2023
Revised: July 31, 2023
Accepted: August 1, 2023
Published: August 11, 2023

oxide-dimer acid (HFPO-DA) in fish,[14] and hexafluoropropylene oxide trimer acid in fish[14] and frogs.[15] However, concentrations of many emerging PFAS in amphibians remain largely unknown, while amphibians are regarded as one of the most threatened taxa with half of their species predicted to be at risk of extinction.[16]

The Chinese toad (*Bufo gargarizans*) is a kind of amphibian widely distributed throughout China, which is usually used for the extraction of Chinese medicine Venenum Bufonis.[17] Its wide distribution makes *B. gargarizans* a good biogeographic model for amphibian research. Over the years, due to the intensification of agricultural and industrial pollution, the Chinese toads have decreased dramatically.[18] As a species of conservation concern, it is important to investigate their tissue-specific bioaccumulation profile of emerging concern (e.g., PFAS) in their habitat, and assess the risks of these chemicals to their health and population fitness. Chaohu Lake is the fifth largest freshwater lake in China (total watershed area of 13,486 km^2), which suffers from complex water resource problems such as eutrophication.[19] Based on the ecosystem health evaluation model, pollution source identification, and lake trophic state evaluation, the Chaohu Lake watershed is divided into two areas: (1) the west lake area, which is dominated by industrial and domestic pollution, and (2) the east lake area, which is dominated by agricultural pollution.[19−21] Previous studies reported that many POPs, such as PFOA, PFOS, and other "legacy PFAS", were found in Chaohu Lake.[22,23] However, there is a lack of information on the bioaccumulation and biotransformation of PFAAs, especially novel PFAS, in the Chinese toads which are residents in Chaohu Lake.

In the present study, concentrations of PFAS, including PFAAs, their precursors or intermediates, and emerging PFAS, were investigated in water samples and various tissues of the Chinese toads collected from twelve major inlet watersheds of Chaohu Lake. Specifically, this study aimed to: (1) investigate the spatial distribution of PFAS in the inlet watersheds of Chaohu Lake and evaluate the influence of industrial and agricultural activities on the PFAS distribution; (2) reveal the tissue-specific bioaccumulation characteristics and potential mechanisms of PFAS, including some emerging PFAS and PFAS precursors, in the Chinese toads; (3) assess the ecological risk of PFAS, and (4) explore the potential relationship between tissue-specific PFAS concentrations and biochemical indicators (e.g., estradiol and testosterone) with a view to providing insights into their toxic mechanisms.

MATERIALS AND METHODS

Chemicals. Except for 6:2 hydrogen-substituted polyfluorinated ether sulfonate (6:2 H-PFESA) and 8:2 H-PFESA (purities of >95%) provided by the Chinese Academy of Sciences (Beijing, China), all PFAS standard solutions were purchased from Wellington Laboratories Inc. (Guelph, ON) with chemical purities of >97%. Supporting Information Table S1 presents detailed information on all standards.

Locations and Sample Collection. From July to August 2021, a total of 36 Chinese toads were captured in 12 inlet wetlands of Chaohu Lake, with 3 toads in each sampling point (Supporting Information Table S2). At the same time, two water samples were collected at each sampling point and put into 500 mL polyethylene bottles prerinsed with methanol. The gender and weight information of toads were recorded. The age of each toad was identified based on skeletochronology (Supporting Information Figure S1 and Table S3). Toads were carefully dissected to obtain muscle, liver, intestine, brain, and gonad tissue samples. The obtained blood samples were used for hormone determination. Because the blood volume was not sufficient to meet the determination of hormones and PFAS at the same time, the PFAS level in the blood was not measured in this study. Tissues and water samples were treated according to the method of Wang et al. (2022).[24] In brief, tissue samples were freeze-dried, homogenized, and weighed. The samples were extracted with alkaline acetonitrile and purified using Supelco ENVI-Carb (Bellefonte, USA) and Oasis WAX cartridges (Watts, USA) after adding 2 ng mass-labeled surrogates. The water samples were extracted by Oasis WAX cartridges.[25] Specific details of the sample extraction are presented in the Supporting Information. All research protocols were approved by the Ethics Committee of Anhui Normal University.

Instrument Analysis. The instrumental methods applied to analyze the target PFAS were the same as those described in the previous study.[24] For the determination of PFAS concentrations, an Agilent 1290 Infinity ultra-performance liquid chromatograph (Agilent, Palo Alto, CA, USA) coupled with a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA) was used. All target compounds can be measured simultaneously in a multiple reaction monitoring mode. Details of the mass spectrometer conditions for the analysis of PFAS are presented in Supporting Information Table S4.

Quality Assurance and Quality Control. To minimize potential contamination, all labware and dissection tools were prescreened and cleaned with methanol before use. All compound-specific method quantitation limits (MQLs) and recoveries (n = 3) of individual native PFAS spiked to tissues and water (ranging from 54 to 108%) are listed in Supporting Information Tables S5 and S6, respectively. For quality assurance, a procedural blank and a reference standard were included in each batch of 10 samples. Limits of detection and limit of quantification (LOQs) were estimated based on an instrumental signal-to-noise ratio of 3:1 and 10:1, respectively. When the concentrations of PFAS were detected or not detected in the procedure blank, the MQL was defined as the average concentration of the substance in the procedure blank plus three times their standard deviation or equal to the LOQ, respectively. The relative standard deviations of replicate samples were <15%. Mass-labeled surrogate standards were spiked prior to sample extraction and quantification of individual PFAS was dependent on internal calibration with the corresponding surrogates.

Determination of Biochemical Indicators. According to the manufacturer's instructions, the contents of the total protein in corresponding tissues, and the activities of glutamic pyruvic transaminase (ALT) and glutamic oxaloacetic transaminase (AST) in blood were measured using the commercial colorimetric kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plasma concentrations of serum triiodothyronine (T3), thyroxine (T4), and phospholipid contents in tissues were determined with the amphibians-specific enzyme-linked immunosorbent assay kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China) following the manufacturer's instructions. Estradiol (E2) and testosterone (T) were measured according to the manufacturer's protocols of commercial immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA). All biochemical indices were normalized against the protein content. Experimental data

https://doi.org/10.1021/acs.est.3c02660
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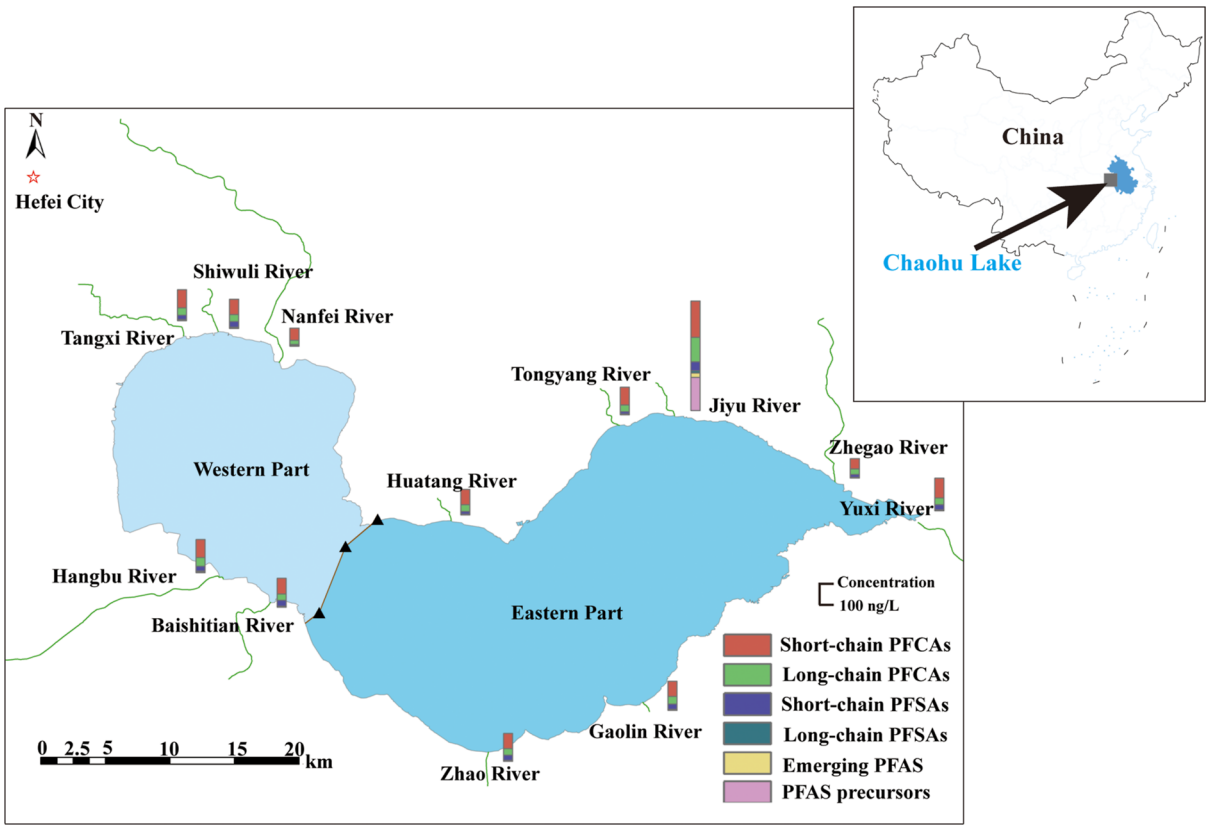

Figure 1. Concentration and composition of PFAS in the inlet watersheds of Chaohu Lake in China. (The map is produced based on the standard maps publicly available on the standard map base map service website of the Ministry of Natural Resources of China (http://bzdt.ch.mnr.gov.cn). The review number is GS (2016) 1554 and the base map has not been modified).

determination was performed using a Tecan Spark 10M microplate reader (Tecan Trading AG, Switzerland).

Calculation. To assess the contribution of specific tissues to the bioaccumulation potential of the whole body, the relative body burden (RBB; %) was calculated based on the following formula equation (eq 1)

$$\mathrm{RBB}_{\mathrm{tissue}} = 100 \times \frac{C_{\mathrm{tissue}} \times m_{\mathrm{tissue}}}{\sum_{n=1}^{i} C_{\mathrm{tissue}} \times m_{\mathrm{tissue},n}} \quad (1)$$

where c_{tissue} is the PFAS concentration in a given tissue (ng/g DW) and m_{tissue} is the weight of the tissue (g). The bioaccumulation factor (BAF) was calculated as the PFAS concentration in the whole body of the toad over the PFAS concentration in the corresponding water sample, which was calculated by the following equation (eq 2)

$$\mathrm{BAF}_{\mathrm{whole\ body}} = \frac{\sum_{n=1}^{i} c_{\mathrm{tissue},n} \times f_{\mathrm{tissue},n}}{c_{\mathrm{water}}} \quad (2)$$

where f_{tissue} (unitless) is the mass fraction of tissue relative to the total body weight and c_{water} is the concentration of PFAS in water. The above calculation method refers to the methods of Shi et al. (2018).[26] The Kolmogorov−Smirnov test (K−S test) was used to test the normality of the data. Student's *t*-test was used to test the significance of differences between groups, if the data passed the tests of normality and equal variance; otherwise, nonparametric Mann−Whitney *U* test was used instead of the *t*-test. Clustering correlation heatmap and Correlation Network based on Spearman's test were performed separately using pheatmap software (1.0.12) and igraph software (1.2.6) in R (version 3.6.3) on the OmicStudio tools at https://www.omicstudio.cn/tool. Statistical significance was set at $p < 0.05$ and all procedures were performed using SPSS software (version 24, IBM Corporation, New York, NY, USA).

RESULTS AND DISCUSSION

Concentration and Distribution of PFAS in Water Samples. The total concentrations of PFAS in water samples collected from the 12 inlet watersheds of Chaohu Lake ranged from 86.41 to 524.38 ng/L (mean: 164.29 ng/L). 39 PFAS were detected, including 12 PFCAs, 7 PFSAs, 8 emerging PFAS, and 12 PFAS precursors (Supporting Information Table S7). PFCAs were the most abundant compounds, contributing 74.5% of the total PFAS, followed by PFSAs, PFAS precursors, and emerging PFAS which contributed 15, 9, and 1.5%, respectively. Short-chain PFCAs ($C_nF2_{n+1}COOH$, $n \leq 6$) contributed 50.7% of the ∑PFAS, which was higher than that of long-chain PFCAs (23.8%). Similarly, the proportion of short-chain PFSAs ($C_nF2_{n+1}SO_3H$, $n \leq 5$) to the ∑PFAS (13%) was higher than that of long-chain PFSAs (2%) (Figure 1 and Supporting Information Table S7). The increase in short-chain PFCAs in Chaohu Lake watersheds may be due to the controlled use of long-chain PFCAs, leading to the production and use of short-chain alternatives. For example, PFOA, one of the most prominent long-chain PFCAs detected in the environment, is now being replaced by short-chain carbon compounds including perfluorohexanoic acid (PFHxA) and perfluorobutyric acid (PFBA).[27] Moreover, short-chain PFAS have been shown to be less trapped in soil or sediment

https://doi.org/10.1021/acs.est.3c02660
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and more present in the aqueous phase than long-chain PFAS.[28] The flux of short-chain PFAS in this study was higher than that of traditional long-chain PFAS, hinting that the threat of these short-chain PFAS to water ecological safety needs to be taken seriously. Comparison with data from previous surveys on PFAS in river waters entering Chaohu Lake shows that the total PFAA concentrations in the rivers had decreased (from 1866 ng/L in 2015[23] to 344 ng/L in 2021 for the maximum value). The decreasing trend of PFAAs in the river water of Chaohu Lake was possibly be attributed to the positive effect of ecological restoration projects implemented by the government in recent years to intercept the pollution along the lake,[29] caused by enforcement of the Stockholm Convention on PFAAs or due to their gradual phasing out in China. However, the exact cause needs to be thoroughly investigated in conjunction with the water source entering the lake.

To further understand the spatial distribution and sources of PFAA in the watershed, the watersheds of Chaohu Lake were divided into two parts: the eastern and the western areas. The division was based on the pressure-state-response model commonly used to assess the ecological health and safety of Chaohu Lake.[21] A previous study on the distribution of PFAAs in Chaohu Lake has shown that the rivers entering the western areas, such as the Nanfei River and Shiwuli River, receive wastewater from industrial parks.[22] The levels of PFAAs could be closely related to the discharge of industrial and domestic pollution in the western areas. The rivers entering the eastern areas of Chaohu Lake, mainly flow through rural areas, so the levels of PFAAs could be correlated with agricultural pollution in the eastern areas.[22] In this study, PFAS composition patterns varied among different sampling sites (Figure 1). The content of PFAS in the aqueous phase was significantly less in the inlet rivers from the western urban industrial area (201.02 ng/L) than that from the eastern farming area (127.57 ng/L; $p < 0.05$). In particular, Jiyu (524.38 ng/L) river in the eastern area presented the highest concentration. Therefore, PFAS has significant regional differences and isolated pollution "hot spots" in the inlet watersheds of Chaohu Lake. The rivers in the adjacent western areas of Hefei City have undergone ecological restoration projects.[22] The current results implied that the source control in the western urban industrial area might have provided a more effective pollutant removal. However, agricultural nonpoint source pollution in the eastern area might have contributed more to PFAS contamination in the rivers. The total contribution of livestock farming, poultry breeding, and rural household waste among the sources of agricultural nonpoint source pollution is up to more than 80% of organic pollutants.[30,31] Therefore, the high concentrations of PFAS caused by agricultural nonpoint source pollution deserve attention.

PFAS Concentrations in Chinese Toad Tissues. The PFAS species that occur in water samples from the inflowing river were also distributed in the Chinese toads inhabiting this habitat (Figure 2 and Table S7). PFCAs were found in higher concentrations in all tissues compared to PFSAs, emerging PFAS, and PFAS precursors. PFUnDA and PFOS were the dominating PFCA and PFSA in all Chinese toad tissues, respectively. The half-lives of PFUnDA and PFOS in humans are up to 9.7 and 5.4 years, respectively. Fish samples from Chaohu Lake also showed high levels of PFUnDA and PFOS,[23] and their long-term accumulation in tissues or organs may have toxic effects on aquatic animals in the watersheds of

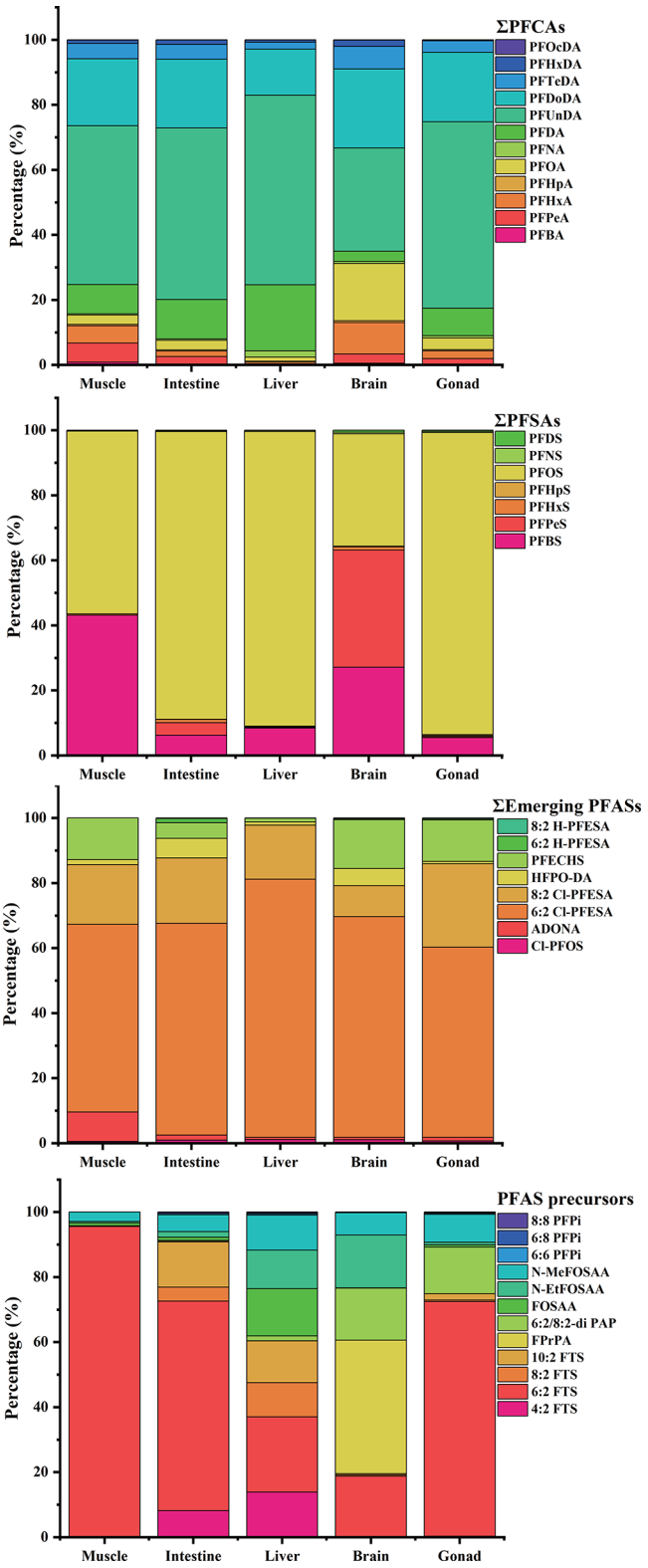

Figure 2. Percent compositions of ∑PFCAs, ∑PFASs, ∑Emerging PFAS, and PFAS precursors within each tissue in the Chinese toads collected from the inlet watersheds of Chaohu Lake in China.

Chaohu Lake. 6:2 Cl-PFESA and HFPO-DA were the most predominant emerging PFAS and 6:2 fluorotelomer sulfonic acid (FTS) was the predominant PFAS precursor in Chinese toad tissues. As far as we know, HFPO-DA and 6:2 FTS were for the first time detected in amphibians. 6:2 Cl-PFESA (trade

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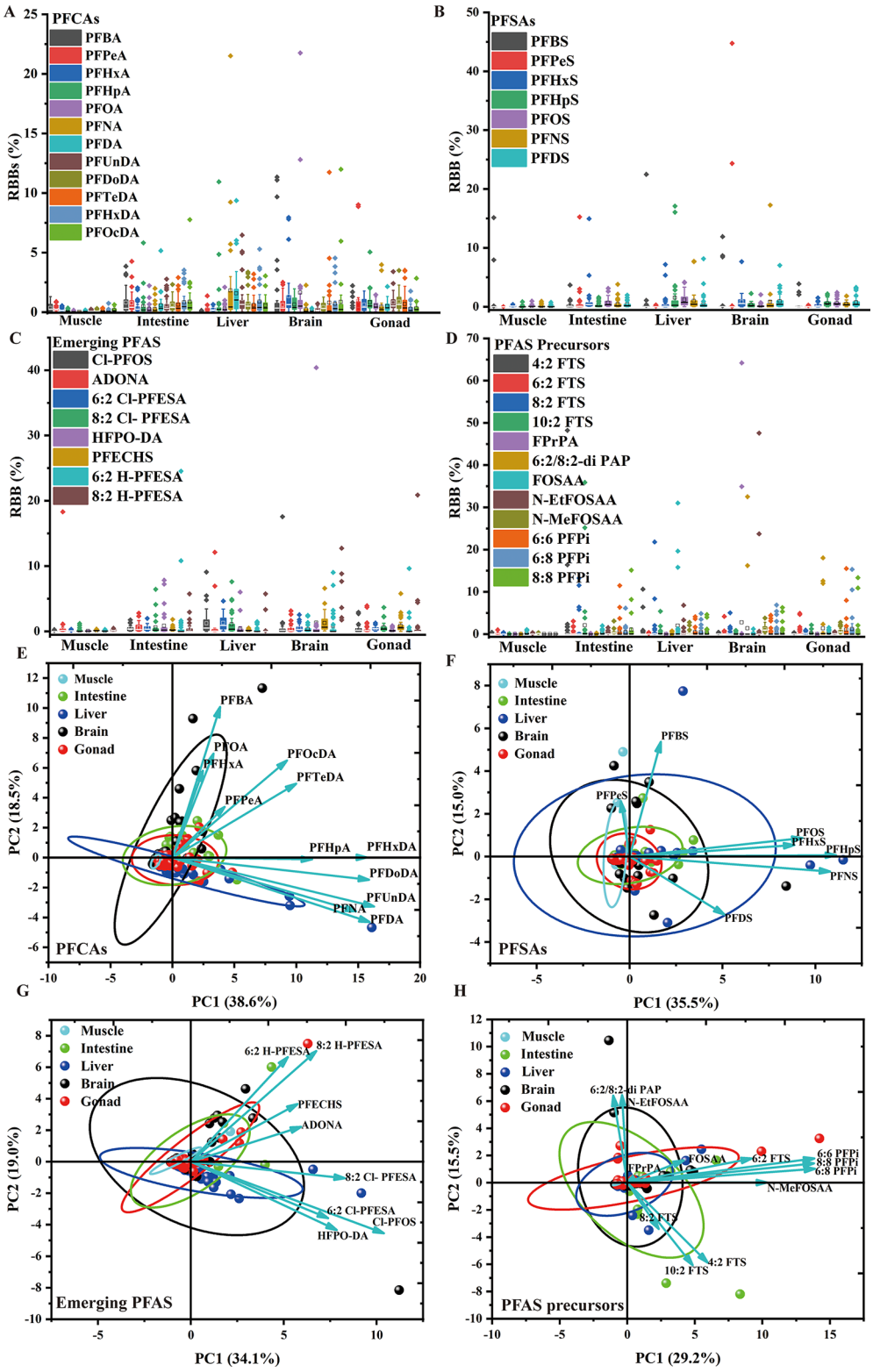

Figure 3. RBBs of PFCAs (A), PFSAs (B), Emerging PFASs (C), and PFAS precursors (D). Boxes indicate 25th to 75th percentiles and whiskers indicate 5th and 95th percentiles. Extreme values in the data sets are indicated as a rhombus. PCA plots for RBBs of PFCAs (E), PFSAs (F), emerging PFAS (G), and PFAS precursors (H); arrows indicate the vector matrix of individual PFASs while circles indicate significantly separated groupings.

name: F-53 B) has been used as a mist suppressant in the metal plating industry since the 1970s, considering its lower cost and simplified production process.[32] It was presumed to be the most persistent PFAS in humans reported to date, with the longest estimated half-life for renal clearance (median 280 years) and total elimination period (median 15.3 years).[33] Its exposure of black-spotted frogs (*Rana nigromaculata*) induced liver damage, causing liver vacuolation and inflammation.[34]

https://doi.org/10.1021/acs.est.3c02660
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HFPO-DA (trade name: GenX) is a replacement PFAS for PFOA and one of the components of specific processing aids. Its toxic effects have not been as well studied as PFOA, but limited data suggest that its exposure could alter lipid metabolism and liver dysfunction, and changed neonatal and maternal physiological parameters in rodents.[35,36] 6:2 FTS was a novel PFOS alternative used globally in aqueous film-forming foams, which exhibited bioaccumulation potential and slow elimination and caused moderate hepatotoxicity in mice.[37] In this study, the elevated concentrations of certain emerging and precursor PFAS detected in tissues of the Chinese toads may cause their physiological toxicity. Moreover, the concentrations of PFAS in the Chinese toads were found to be the highest in their liver (215.97 ng/g dw), followed by their gonads (135.42 ng/g dw) and intestine (114.08 ng/g dw), whereas the lowest concentrations were detected in their brain (65.74 ng/g dw) and muscle (20.98 ng/g dw). The present results were in good agreement with the previous studies which also found the highest concentration of PFAS occurring in the liver of wild animal.[38,39] This may be due to the fact that the liver is a highly perfused organ with high blood flow, and the liver fatty acid binding protein has high affinity for PFAS.[22,40] Additionally, the liver is the main site of metabolism and detoxification.[41] The above characteristics of the liver may confer high fugacity of such compounds to the Chinese toads. The second highest concentrations of PFAS (Figure 2, Supporting Information Table S7) were found in the gonad of the toad, which was rich in phospholipids (Supporting Information Figure S2). Large quantities of phospholipids in the gonad may facilitate their binding with PFAS in the gonad.[40,42] Moreover, the presence of higher concentrations of PFAS in the gonad may result in reproductive impairment of the Chinese toads.[43,44]

A trend of sex differences in ∑PFAS concentrations was observed in the Chinese toads' gonads at the inlet watersheds of Chaohu Lake (Supporting Information Figure S3), where the mean ∑PFAS level in male toads was significantly higher than that in their female counterparts ($p < 0.05$). Similar sex-specific differences in PFAS concentrations (males > females) were observed in birds,[45] fish,[46] and mammals[47] in previous studies. These variations may be ascribed to factors such as maternal elimination of eggs,[48] sex hormone expression affecting substance half-life,[49] and sex-related differences in organic anion transporter protein expression.[50] These mechanisms jointly accelerate the elimination of PFAS in female organisms. However, an opposite sex difference in PFSA concentration was reported in the black-spotted frog.[15] The reason for this difference may be due to whether or not sampling was done during the breeding season. In this case, the black-spotted frogs were collected during the breeding season when the female frogs carried a large number of eggs,[15] whereas the Chinese toads in this study were collected during the nonbreeding or post-spawning season. However, in contrast to the gonad, PFAS levels in the Chinese toads showed no gender differences in the intestine, muscle, brain, and liver ($p > 0.05$).

The PFAS concentrations were further analyzed in various tissues of the Chinese toads at different ages. The results showed that ∑PFAS concentrations were higher in 3 year olds than in 4 year olds in each tissue examined, with such significant differences in muscle ($p < 0.05$) and gonads ($p < 0.01$) (Supporting Information Figure S3). The findings suggested that there were possible age-related differences in PFAS accumulation in the toads, where PFAS levels were lower at an older age. Similar findings have been reported in studies of the black-spotted frog,[15] the Yangtze crocodile,[51] and the bottlenose dolphin,[52] where PFAS residues in internal tissues decreased with age in adults, possibly as a consequence of clearance and dilution processes with cell growth.[53] However, there were also opposite or nonsignificant trends, such as the increase of PFAS accumulation with age in Chinese sturgeon[54] and the similarity of PFAS concentrations in seabirds at different ages.[55] Therefore, the bioaccumulation process of PFAS in wildlife is likely species specific and thus it is essential to explore the species-specific bioaccumulation profile of PFAS.

RBBs of PFAS. Conversion of concentration data into RBBs showed that the ratio of liver to body burdens was high in PFSAs, PFCAs, and emerging PFAS (Figure 3A−D). The liver accounted for more than 26% of the total body burden, although the relative importance of the liver showed some variation across the different PFAS. The highest median RBB values were observed in PFSAs, PFCAs, and emerging PFAS for PFOS (0.76%), PFDA (1.01%), and 6:2 Cl-PFESA (0.90%) in the liver, respectively. Therefore, the liver is relatively more important as a PFSA reservoir in the Chinese toads. The PCA plots shown in Figure 3E−H indicate the high variability in the PFAS concentrations in the liver and brain when compared to other tissues. Without considering the concentration of PFAS in blood, these two tissues may be an important reservoir. Moreover, the concentration of individual PFAS was found to be dependent on the tissue type (i.e., tissue specific) in the Chinese toads. The present result was different from a previous study that the concentration of individual PFAS in the fish Crucian Carp.[26] This may be due to differences in tissue composition between species.

By assessing RBBs, it was obvious that the liver had the greatest effect on the whole-body bioaccumulation potential in the Chinese toads compared to other tissues (Figure 3A−D). The adsorption mechanisms of these tissues play a crucial role in determining the whole-body bioaccumulation potential. Some PFAS are structurally similar to endogenous fatty acids in the liver with fluorine instead of hydrogen.[56] PFAS excreted into the intestine can also be reabsorbed into the shared membrane transport pathway of the enterohepatic circulation, and thus PFAS may be enriched in the liver in both ways.[56,57] Furthermore, PFAS have been shown to bind to liver fatty acid binding proteins, with binding affinity increasing with the chain length of PFAS.[58,59] High levels of protein were also observed in the liver of the Chinese toads (Supporting Information Figure S2). Therefore, molecular adsorption and transport organs may be responsible for the higher enrichment and distribution of PFAS in the liver of the Chinese toads. PFAS can invade blood circulation and finally reach the brain by destroying the brain barrier, such as the blood−brain barrier and the blood−cerebrospinal fluid barrier.[60] Many PFAS are ionic. Ions readily penetrate the brain barrier, which makes it easy for them to penetrate the brain barrier and enter the brain.[61−64] In addition, a large amount of phospholipid (Supporting Information Figure S2) was also observed in the brain tissue of the Chinese toads. PFAA has a hydrophilic head and hydrophobic tail similar to phospholipids, making it easy to distribute and enrich on phospholipid-rich cell membranes. Therefore, the brain of the Chinese toads rich in phospholipids may be more exposed to PFAS when it continuously receives the freshest blood supply, which eventually leads to the accumulation of PFAS in its brain tissue.

https://doi.org/10.1021/acs.est.3c02660
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Bioaccumulation Characteristic. For PFAS detected in at least four samples of the biota and water samples, BAF was calculated to assess their bioaccumulation potential. BAFs were mostly >1 indicating various degrees of bioaccumulation potential.[65] Although adult toads do not only inhabit water, which may be somewhat biased, the calculation of BAF values based on water sample concentrations is still a useful method for assessing the bioaccumulation potential of contaminants and has been extensively used in studies on amphibians.[17,65−67] The log BAF ranged from 1.11 (PFBS) to 5.12 (PFDoDA). The log BAF for PFCAs with seven to eleven perfluorocarbons and PFSAs linearly increased with the carbon chain length (Figure 4). The occurrence of PFAS in the Chinese toads is

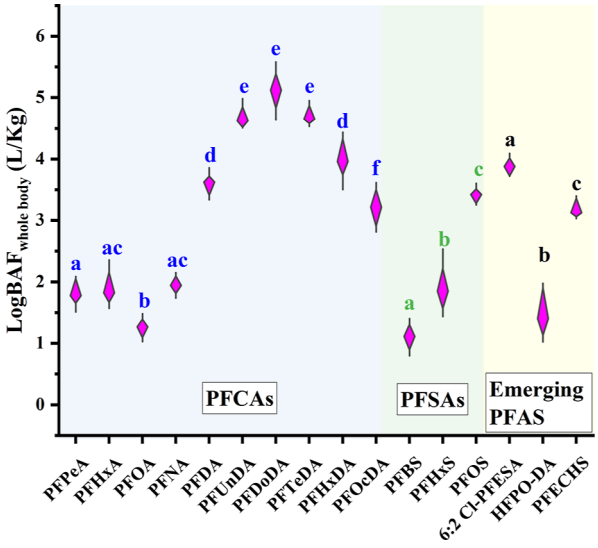

Figure 4. Whole-body BAFs with PFCAs, PFSAs, and emerging PFAS. Different letters in the same color indicate statistically significant differences for PFCAs (blue letters), PFSAs (green letters), and emerging PFAS (black letters) in BAFs.

likely the result of several different processes acting together. At least four processes may be involved: (1) biomagnification by predation on terrestrial organisms; (2) biomagnification by predation on aquatic organisms; (3) inhalation exposure on land (including direct and indirect sources, i.e., degradation of gaseous precursors); and (4) bioconcentration by dermal exposure in water. With the increase in carbon chain length, the raised *n*-octanol/water partition coefficient (log K_{OW}) of PFAAs could promote their accumulation through pathways 1, 2, and 4, which is consistent with previous laboratory experiments and field-based investigations on aquatic organisms.[54,68−70] Nevertheless, for PFCAs with more than eleven perfluorocarbons, a decreasing trend was found between BAF and carbon chain length, which is also observed in previous studies, and the most possible reason is the relatively large molecular size could inhibit the bioaccumulation of PFCAs with perfluorocarbon chain longer larger than eleven.[54,70−72] Notably, the BAF of two short-chain PFCAs, PFPeA and PFHxA, exhibited higher BAF (1.77 and 1.82, respectively) compared to PFOA (1.27). This result indicated the high bioaccumulation potential of the short-chain PFCAs in the Chinese toads, and the bioaccumulation potential of PFPeA and PFHxA is even higher than the C7 compound. Short-chain PFCAs were conventionally believed to exhibit negligible biomagnification (pathways 1 and 2) and bioconcentration potential (pathway 4).[70,73] The frequent detection and high BAF of short-chain PFCAs in the Chinese toads could be attributed to: inhalation exposure of short-chain PFCAs and their gaseous precursors (e.g., FTOHs) and the bioaccumulation/bioconcentration of short-chain PFCA precursors (e.g., FTSs). Accumulation of PFASs may increase the risk of triggering toxicity in the Chinese toads.[74] Therefore, more analytical approaches (e.g., nontarget screening and total oxidizable precursor assay) should be applied in future investigations to comprehensively identify and quantify various PFASs and their precursors, in order to better evaluate the bioaccumulation and ecological risk of PFASs on amphibians.

In addition, the median BAF of PFAS alternatives was higher than that of the prototype compounds PFOS and PFOA [i.e., 6:2 Cl-PFESA (3.89) > PFOS (3.42); HFPO-DA (1.40) > PFOA (1.27)], indicating that this emerging PFAS has a stronger cumulative potential in the Chinese toads compared with their predecessors. 6:2 Cl-PFESA exhibited the highest BAF values among the studied PFSAs. With the insertion of oxygen atoms and the substitution of fluorine by chlorine, the estimated log K_{OW} values of Cl-PFESAs are higher than those of certain detected legacy PFAS.[75] This means that 6:2 Cl-PFESA is more likely to bind to animal tissues and has higher tissue enrichment properties.[76] HFPO-DA was found to exhibited low bioaccumulation potential in the teleost through laboratory experiments. Nevertheless, in recent years, HFPO-DA has been detected in wild animals such as carp, bivalves, striped bass, and marine cetaceans.[39,77−79] The bioaccumulation mechanism of HFPO-DA is still not clear, which could be potentially attributed to inhalation exposure and/or biomagnification. It should be noted that the field-based log BAF value for HFPO-DA (1.4) is higher compared to the legacy C8 substance, PFOA (1.27), in the investigated Chinese toads. Currently, there is still lack of reports on the adverse effects of HFPO-DA on amphibian, and more toxicology studies regarding HFPO-DA on amphibians is needed considering the increasing environmental concentrations of HFPO-DA after phasing out of PFOA.[7,39] Furthermore, an emerging PFAS, perfluoroethylcyclohexane sulfonate (PFECHS) also presented a higher bioaccumulation potential in the Chinese toads with a median BAF of 3.13. It has been confirmed to induce oxidative stress with potential endocrine disruptor effects in aquatic invertebrates.[80] The log K_{OW} (predicted value, 3.19−5.92) of PFECHS was similar with PFOS (log K_{OW} = 4.9),[81] and there was no significant difference in BAF values between PFECHS and PFOS ($p > 0.05$) in the Chinese toads, implying the close bioaccumulation potential between these two compounds. Therefore, we have demonstrated for the first time that some emerging PFAS presented close or even higher cumulative potential in the Chinese toads and the mechanisms causing the bioaccumulation pathways may differ.

Associations between Levels of Single PFAS and Blood Biochemical Indicators. In order to evaluate the relationship between levels of individual PFAS and blood biochemical indicators in the Chinese toads at environmental concentrations, their brain, liver, and gonads were used as target tissues for testing the correlation between tissue concentration of PFAS with thyroid hormone parameters (T4 and T3), liver function parameters (ALT and AST) and sex hormone parameters (E2 and T), respectively. The results showed no significant correlation between PFAS in brain tissue and thyroid hormones (T4 and T3) and between PFAS in liver tissue and liver function indicators (ALT and AST) (Figure 5).

https://doi.org/10.1021/acs.est.3c02660
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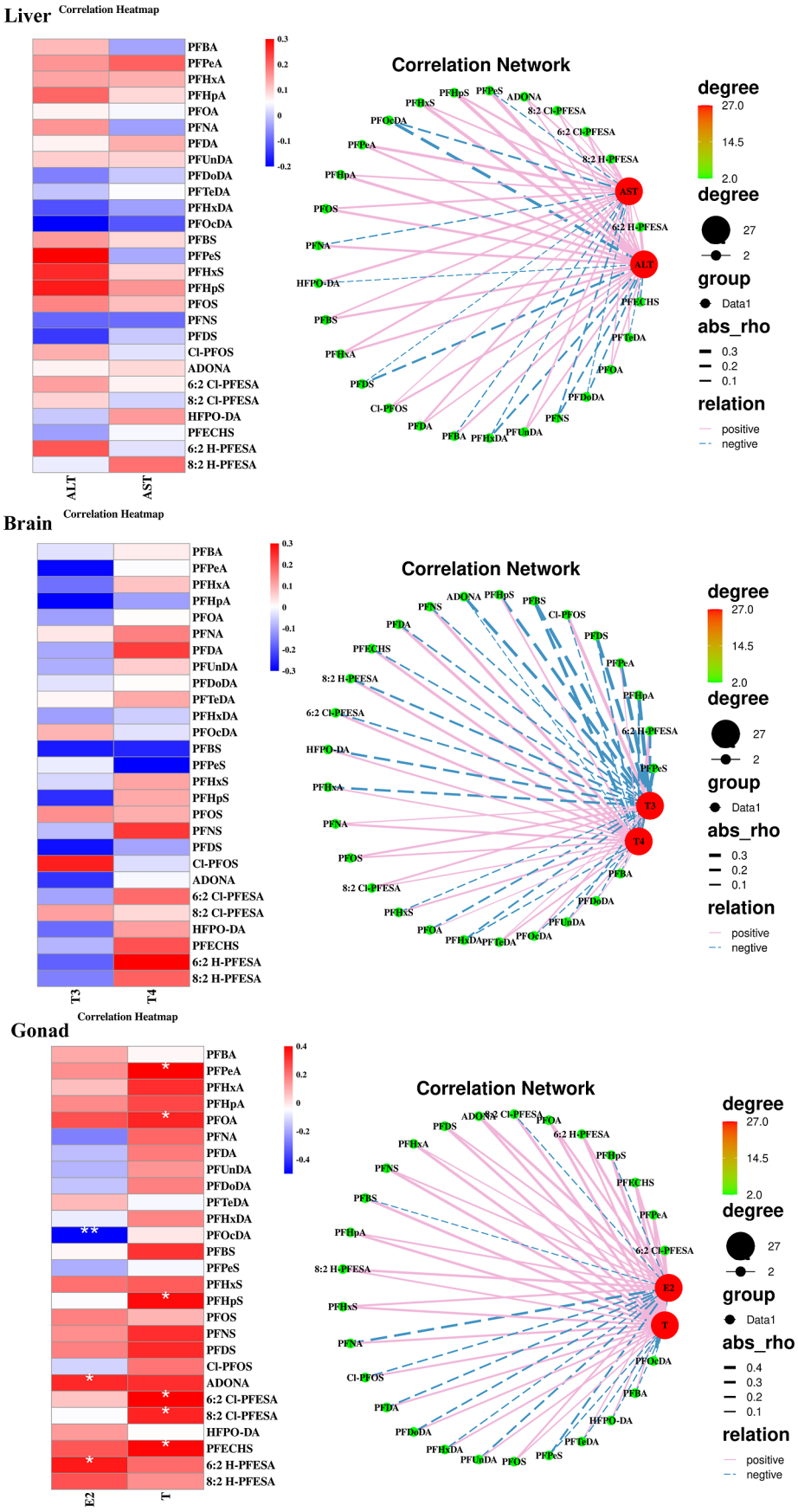

Figure 5. Heatmap and correlation network illustrating the relationship between target tissues and biochemical indicators (brain vs T4 and T3; liver vs ALT and AST; gonad vs E2 and T).

https://doi.org/10.1021/acs.est.3c02660
Environ. Sci. Technol. 2023, 57, 13148−13160

However, the concentrations of PFPeA, PFOA, PFHpS, 6:2 Cl-PFESA, 8:2 Cl-PFESA, and PFECHS in gonads all had a significant positive correlation with testosterone concentrations. Both ADONA and 6:2 H-PFESA had a significant positive correlation with E2, while PFOcDA had a significant negative correlation with E2. The above results implied that sex hormone levels might be related to the concentration of certain PFAS enriched in gonadal tissue. PFAS may regulate androgen levels through several pathways. First, PFAS competes for androgen receptors, leading to increased testosterone.[82] Second, PFAS itself has the function of activating steroidogenic enzymes, increasing serum testosterone concentrations.[83] In addition, PFAS can regulate luteinizing hormone levels, which further regulate the hypothalamic pituitary-gonadal axis, leading to increased androgen secretion.[84,85] The results of correlation analysis showed a significant positive correlation between ADONA (r = 0.34, p = 0.04) and 6:2 H-PFESA (r = 0.36, p = 0.03) and E2. It is possible that these PFAS have estrogenic effects in the Chinese toads. A similar finding was observed in zebrafish.[86,87] It has been demonstrated that 6:2 Cl-PFESA can bind to estrogen receptors and exhibit stronger estrogenic effects than PFOS, increasing estrogen level.[87] However, the relationship between 6:2 H-PFESA, as a dechlorinated reduction product of 6:2 Cl-PFESA, and estrogen levels has not been reported, which is mainly due the lack of its commercial standards. ADONA cannot activate estrogen or androgen receptors at noncytotoxic concentrations.[88] However, ADONA and E2 showed a significant positive correlation in this study, which may imply that the effect of ADONA on amphibian reproduction deserves extra attention. The mechanisms underlying the correlation between specific PFASs and estrogens and androgens are currently unknown, and whether these mechanisms are valid in the Chinese toads deserves further study.

Environmental Implications. In this study, we investigated the occurrence and distribution of 39 PFAS in samples collected from the Chinese toads and surrounding waters in the inlet watersheds of Chaohu Lake in China. To our knowledge, this is the first simultaneous study of the tissue distribution of PFAS precursors and substitutes in amphibians, including 6 emerging PFAS (e.g., HFPO-DA, PFECHS, and 6:2 H-PFESA) and 12 PFAA precursors (e.g., 6:2 FTS, FPrPA, and N-EtFOSAA). The spatial distribution of PFAS in river waters indicated agricultural nonpoint sources as an important PFAS pollution source that would require special attention in terms of the formation of pollution control measures. The highest PFSA concentrations were found in the liver of the Chinese toads followed by gonads, implying the potential hepatotoxicity and reproductive toxicity. In addition, significantly higher PFAS levels were found in the gonads of male toads compared to females. PFAS levels were higher in younger than in older toads in the adult stage. These results suggest that PFAS concentrations in the Chinese toads may show a trend of sex and age differences. Considerable short-chain PFCAs were found in the Chinese toads which could be attributed to PFAS precursor degradation and inhalation exposure. Some emerging PFAS alternatives (e.g., ADONA and 6:2 Cl-PFESA) exhibit higher BAF values than legacy PFAS (e.g., PFOA and PFOS), triggering a concern about the potential risk of growth and developmental impairment caused by these PFAS alternatives to the Chinese toads and other species. Notably, the concentrations of certain PFAS enriched in gonadal tissue may affect sex hormone levels, leading to endocrine disruption. However, more detailed toxicokinetic and toxicodynamic data on emerging PFAS are needed to comprehensively assess the exposure risks associated with these widespread chemical contaminants of emerging concern. The current results also support the use of the Chinese toads as a sensitive biomonitor for environmental risk assessment of PFAS and other contaminants of emerging concern.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c02660.

Details for age determination of adult toads, standards and reagents, sample treatment, instrumental analysis, sampling information, and matrix-spiked MQLs and recoveries; PFAS concentrations in water and tissue samples; protein and phospholipid contents, and sex- and age-related PFAS concentrations in different tissues (PDF)

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https://doi.org/10.1021/acs.est.3c02660
Environ. Sci. Technol. 2023, 57, 13148−13160

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was financially supported by the National Natural Science Foundation of China (no. 31971236 and 32071498), and the University Synergy Innovation Program of Anhui Province (no: GXXT-2020-075). The visit and research work of Pei Hong at the City University of Hong Kong were partially supported by the State Key Laboratory of Marine Pollution which received regular research funding from the Innovation and Technology Commission (ITC) of the Hong Kong SAR Government. However, any opinions, findings, conclusions, or recommendations expressed in this publication do not reflect the views of the Hong Kong SAR Government or the ITC. The authors also thank staff at the forestry bureau of Anhui Province in China for their help with sampling.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on August 11, 2023, with a spelling error in the Abstract. The corrected version was reposted on September 5, 2023.

https://doi.org/10.1021/acs.est.3c02660
Environ. Sci. Technol. 2023, 57, 13148−13160