Science of the Total Environment 876 (2023) 162828

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

PFAS levels in fish species in the Po River (Italy): New generation PFAS, fish ecological traits and parasitism in the foreground

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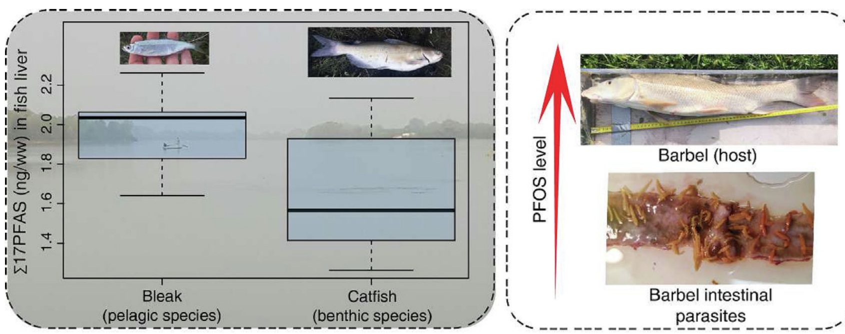
HIGHLIGHTS

- In this study, the level of 17 PFAS in fish fauna from the Po River was investigated.
- PFAS partitioning was studied for the first time in a parasite-fish system.
- PFOS was prevalent and found in all samples at the highest concentrations.
- PFOA and new generation PFAS (Gen X and C6O4) were not detected.
- No correlation was found between hepatic level of PFAS and fish size in *I. punctatus*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
C6O4
Ictalurus punctatus
Alburnus alburnus
Barbus barbus
Pomphorhynchus laevis (Acanthocephala)
Bioaccumulation

ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are resistant to breakdown and are now considered ubiquitous and concerning contaminants. Although scientific and legislative interest in these compounds has greatly increased in recent decades, our knowledge about their environmental fate and their effects on organisms is still incomplete, especially those of the new generation PFAS. In this study, we analysed the level of PFAS contamination in the fish fauna of the Po River, the most important waterway in Italy, to evaluate the influence of different factors (such as fish ecological traits and parasitism) on the accumulation of 17 PFAS. After solvent extraction and purification, hepatic or intestinal tissues from forty specimens of bleak, channel catfish, and barbel were analysed by liquid chromatography coupled with mass spectrometry (LOQ = 2.5 ng/g w.w.). The prevalent PFAS were perfluorooctane sulfonate (PFOS), present in all samples at the highest concentration (reaching a maximum of 126.4 ng/g and 114.4 ng/g in bleak and channel catfish, respectively), and long-chain perfluoroalkyl carboxylic acids (PFDA and PFUnDA). Perfluorooctanoic acid and new generation PFAS (Gen X and C6O4) were not detected. Comparison of the hepatic contamination between the benthic channel catfish and the pelagic bleak showed similar concentrations of PFOS ($p > 0.05$) but significantly higher concentrations of other individual PFAS and of the sum of all measured PFAS ($p < 0.05$) in bleak. No correlation was found between the hepatic level of PFAS and fish size in channel catfish.

For the first time, PFAS partitioning in a parasite-fish system was studied: intestinal acanthocephalans accumulated PFOS at lower levels than the intestinal tissue of their host (barbel), in contrast to what has been reported for other pollutants (e.g., metals). The infection state did not significantly alter the level of PFAS accumulation in fish, and acanthocephalans do not appear to be a good bioindicator of PFAS pollution.

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http://dx.doi.org/10.1016/j.scitotenv.2023.162828
Received 24 December 2022; Received in revised form 21 February 2023; Accepted 9 March 2023
Available online 15 March 2023

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are compounds that have become widespread worldwide since the 1950s and are used to make textiles, paper and coatings for food containers that are resistant to grease and water. They are also used in the production of photographic films, firefighting foams, and household cleaners (Giesy and Kannan, 2001; Glüge et al., 2020). However, their properties and chemical characteristics cause them to have negative effects on the environment. Because of their persistence and mobility, these compounds have been detected in significant concentrations in organisms and ecosystems, especially those of the riverine type, since they are affected by industrial waste (Giesy et al., 2010; Ding and Peijnenburg, 2013; Du et al., 2013; Renzi et al., 2013; Ahrens and Bundschuh, 2014; Evich et al., 2022).

PFAS form a very large generic group of substances, the best-known compounds of which are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). From a chemical point of view, PFAS consists of a hydrophilic functional group connected to carbon chains of various lengths in which hydrogen atoms have been partially or totally replaced by fluorine atoms (Giesy and Kannan, 2001). Compounds with carbon chains up to five atoms are considered short-chain, from six carbon atoms long-chain (Ankley et al., 2021).

As a result of restrictions and bans on the production of traditional PFASs, in particular PFOA and PFOS, due to the toxicological properties and widespread environmental occurrence of these molecules, perfluorinated substitutes were introduced to the market in the 2000s: short-chain (PFBA and PFBS, $n = 4$), GenX (HFPO-DA), ADONA, and C6O4 (Munoz et al., 2019; Semerád et al., 2020; Bernardini et al., 2021).

These new compounds, are different from the older PFAS homologues and have been modified in structure to include oxygen between the perfluorinated chains (ether group), making them more mobile and, therefore, causing greater adverse environmental effects (Munoz et al., 2019; Semerád et al., 2020; Fabrello et al., 2021). Although alternatives to long-chain PFAS have been in use for some years, little information is currently available on these substances, and in some cases, their chemical identity is unknown. In addition, to carry out selective and specific analyses, standards of many of these substances have not been made fully available, as for C6O4, making their identification not widely accessible or simple.

In 2013, the results of experimental research on potential "emerging" pollutants carried out by the National Research Council and the Ministry of the Environment in the Po River basin and other major Italian river basins indicated the presence of PFAS in groundwater, surface water and drinking water in Italy (Valsecchi et al., 2015). PFAS are included in the institutional monitoring of surface water in the Po River, and since the study carried out in 2019, the environmental agency of Veneto Region, northern Italy (ARPAV) has expanded the panel of perfluoroalkylcarboxylic and perfluoroalkylsulfonic acids, adding the perfluorinated substance C6O4, detected in water and in avian eggs from some Po River sampling points (ARPAV, 2019; Morganti et al., 2021). The compound belongs to the general category of PFAS, and no environmental limits have been established yet, probably also due to a crucial lack of information about its ecotoxicological effects (Bernardini et al., 2021). Since the 1980s, a fluoropolymer factory has produced and released several PFAS in the western area of the Po River valley and since 2015, C6O4 has replaced PFOA as a processing aid in the production of plastics and derivatives. As a result, in 2020, approximately 50 to 100 μg/L of C6O4 was found in this industrial wastewater, making it the main source of this new generation PFAS (Morganti et al., 2021).

Although PFAS monitoring in the Po water has become routine, very little data are available on the contamination of the biota of this river (Giari et al., 2015) and its delta area (Mazzoni et al., 2016; Parolini et al., 2021). Knowledge of the problem is therefore very limited, leading to critical issues, especially in relation to the toxicity of the compounds and their action as endocrine disruptors, characteristics that generate potentially important risks for exposed fish populations and consequent ecological imbalances (Renzi et al., 2013; Governini et al., 2015; Guerranti et al., 2013; Guerranti et al., 2016; Henning and Fuchsman, 2021; Manera et al., 2022). Due to their high bioaccumulation potential, even small levels of long-chain PFAS in water could be dangerous for aquatic organisms, posing an even greater risk to top predators (Loi et al., 2011; Ng and Hungerbühler, 2014; Semerád et al., 2022).

Among the aquatic organisms exposed to contaminants that could reveal the level of pollution are fish parasites. Several parasitic groups, such as Acanthocephalans, Cestodes, and Nematodes, show high pollutant accumulation capacity, as documented especially in aquatic ecosystems (Sures et al., 2017). A relatively recent discipline, called "Environmental Parasitology", explores this topic, among others (Sures, 2004). Some fish parasites are excellent bioaccumulators and, therefore, may be better sentinels of environmental contamination compared to other organisms (Nachev and Sures, 2016). They may be advantageous to host health by reducing the level of pollutants accumulated within the host tissues (Sures et al., 1999; Molbert et al., 2020). From this point of view, infection (i.e., occurrence and load of parasites) could be an important trait that can influence the interaction between fish and pollutants and, in some cases, limit the impact of pollution on fish (Sures and Nachev, 2022). Most available information has focused on the uptake of inorganic contaminants (i.e., metals) by aquatic parasites (Sures, 2003; Hursky and Pietrock, 2015; Paller et al., 2016; Leite et al., 2021). Fewer data refer to organic pollutants (Molbert et al., 2020) and none address PFAS.

The aims of this research are the following:

- to quantify the presence of 17 PFAS in the fish fauna of the Po River considering 3 target-species. Together with those in our previous paper (Giari et al., 2015), these results represent the very few available data on PFAS contamination of the Po fishes and the first record on C6O4 in fish in general.
- to assess accumulation levels in relation to factors, such as the following: 1) physicochemical characteristics of PFAS, including chain length and perfluorination (Labadie and Chevreuil, 2011; Wen et al., 2017; Lee et al., 2020; Macorps et al., 2022), and 2) ecological characteristics of fish, including PFAS levels compared between bleak and catfish as species with different habitus (pelagic and benthic), diet and trophic level (larger catfish eat bleak). The target organ is the liver, which has one of the largest accumulation capacities in the animal body;
- to determine whether there is a relationship between PFAS levels and catfish size;
- to study the PFAS distribution in a host-parasite system by comparing PFAS levels between the fish host (barbel) and its intestinal parasites (acanthocephalans). The present paper is the first to document the presence of this class of compounds in a host-parasite system.

2. Materials and methods

2.1. Study area and fish collection

A total of 40 fish specimens (bleak *Alburnus alburnus,* channel catfish *Ictalurus punctatus* and barbel *Barbus barbus*) were collected in the lower stretch of the Po River from two sampling stations in the Province of Ferrara, Pontelagoscuro (latitude 44.886502°; longitude 11.622340°) and Berra (latitude 44.979050°; longitude 11.998089°), in 2018 and 2021. The Po River represents the main Italian waterway in terms of length (652 km across all Northern Italy) and importance. It flows through the most productive area of the country and is consequently the sink of agricultural, industrial and urban discharge. An overview of fish data (number of specimens, total length, weight, number of parasites) is provided in the supporting information (Table S1). The fish were transported alive to the laboratory, euthanized by severing the spinal cord and, after necropsy, the following tissue samples were obtained, flash-frozen, and stored at −20 °C for chemical analysis:

- livers (5 pools) from bleak collected in September 2018;
- livers (16) from channel catfish caught in September 2018 and October 2021;

- intestines (9) and parasites (9 pools with approximately 40 parasites per pool) from barbel collected in October 2021. During fish dissection, the gut was isolated and opened longitudinally, and then all intestinal parasites were carefully removed.

2.2. Chemical analysis of fish samples

Table 1 reports the list of the compounds analysed in the fish samples. The analytical method was implemented following the protocol of the Istituto Superiore di Sanità (ISS) for aquatic biota, modified for the method for the matrix of water intended for human, mineral and natural consumption, according to ISO 25101: 2009.

2.3. Analytical standards

Chemical standards, in powder or crystal form, with certified purity >95 % for all analytes of interest were used. The stock standard solution was prepared for the internal standard (IS) at 50 μg/mL (± 2.5 μg/mL) with 4-atom ^{13}C perfluorooctane sulfonic acid (MPFOS) and 4-atom ^{13}C perfluorooctanoic acid (MPFOA) (producer certified solution by Wellington Laboratories). For PFOA and other fluorinated carboxylic acids was used MPFOA as internal standard, for PFOS and other sulphonated acids was used MPFOS.

2.4. Calibration

A six-point calibration curve was injected at each batch of analysis: 0.5 μg/L, 1 μg/L, 2.5 μg/L, 5 μg/L, 10 μg/L, and 25 μg/L of perfluorinated compounds added to 10 μg/L MPFOS and MPFOA.

2.5. Sample treatment, extraction and purification

Approximately 0.5 g of each sample was weighed and finely shredded before analysis. Then, 0.5 mL of IS 50 μg/L stock standard solution was added. The prepared samples were left to rest for 16 h at 4 °C before extraction.

Then, 4.5 mL of 0.05 N potassium hydroxide methanolic solution was added to the samples, they were shaken briefly in vortex, and then transferred for 8 h to an orbital shaker; subsequently, the samples were centrifuged.

The extract was decanted, and 1 mL was taken from the decanted extract to which 100 mL of deionized water was added. The obtained extract was purified using the Strata-X-AW SPE cartridge, using the accredited method for the water matrix intended for human, mineral and natural consumption according to ISO 25101: 2009. The cartridge was conditioned first with 4 mL of 0.1 % ammonia in methanol, then with 4 mL of methanol, and finally with 4 mL of deionized water. After the conditioning step, the sample was loaded into the column. After sample loading, the column was washed with 4 mL of 25 mM acetate buffer (pH = 7) to elute some impurities, leaving the analytes in the column. The analytes were then eluted with 4 mL of methanol and 4 mL of 0.1 % ammonia in methanol. The collected fraction was evaporated and diluted to a final volume of 500 μL of 2 mM ammonium acetate in methanol.

The procedural blank was prepared using all the reagents used in the analytical procedure except the matrix (= 500 μL IS 50 μg/L + 4.5 mL KOH 0.05 N in MeOH).

Table 1
List of the analysed compounds, with their acronyms, full names, and chemical formulas, grouped by compound families.

Compound family	Acronym	PFAS name	Chemical formula
Perfluoroalkyl sulfonic acids (PFSA)	PFBS	Perfluorobutane sulfonic acid	$C_4HF_9O_3S$
	PFHxS	Perfluorohexane sulfonic acid	$C_6HF_{13}O_3S$
	PFHpS	Perfluoroheptane sulfonic acid	$C_7HF_{15}O_3S$
	PFOS	Perfluorooctane sulfonic acid	$C_8HF_{17}O_3S$
Perfluoroalkyl carboxylic acids (PFCA)	PFBA	Perfluorobutanoic acid	$C_4HF_7O_2$
	PFPeA	Perfluoropentanoic acid	$C_5HF_9O_2$
	PFHxA	Perfluorohexanoic acid	$C_6HF_{11}O_2$
	PFHpA	Perfluoroheptanoic acid	$C_7HF_{13}O_2$
	PFOA	Perfluorooctanoic acid	$C_8HF_{15}O_2$
	PFNA	Perfluorononanoic acid	$C_9HF_{17}O_2$
	PFDA	Perfluorodecanoic acid	$C_{10}HF_{19}O_2$
	PFUnDA	Perfluoroundecanoic acid	$C_{11}HF_{21}O_2$
	PFDoDA	Perfluorododecanoic acid	$C_{12}HF_{23}O_2$
	PFTrDA	Perfluorotridecanoic acid	$C_{13}HF_{25}O_2$
	PFTeDA	Perfluorotetradecanoic acid	$C_{14}HF_{27}O_2$
New generation compounds (PFECA)	P5MeODIOXOAc	C6O4	$C_6H_4F_9NO_6$
	HFPO	GenX	$C_6HF_{11}O_3$

2.6. Data quality assessment

Recovery of spiked internal standards: For the two ISs, recoveries in samples within the range from 50 to 120 % of the value obtained for one of the standards were considered acceptable. If a value was outside this range, the result was rejected and the analysis was repeated. Recoveries of native PFAS spiked in fish were tested, and data for RSD and recoveries were ≤ 20 % and 96 to 148 %, respectively.

Contamination due to interference in procedural blanks: each batch of samples was combined with a procedural blank whose contribution could affect the value of an analytical signal. The procedural blank signal was required to be lower than the first point of the line.

2.7. LOQ

The limit of quantification (LOQ) corresponded to the first point of the calibration line, which was 0.5 μg/L in the solution and 2.5 ng/g in the samples. Detected but nonquantifiable traces of analytes (below the LOQ) were considered equal to half the LOQ value.

2.8. Instrumental analysis

The analyses were performed with the liquid chromatography (HPLC) technique coupled with a triple quadrupole mass analyser with an electrospray ionization (ESI) system in negative mode.

By optimizing the parameters for precursor transmissions and fragmentation, transmissions of 299/80, 399/80, 449/80, 680/80, 213/169, 263/219, 313/269, 363/319, 413/369, 462/419, 512/468, 563/519, 613/568, 663/619, 713/669, 339/113, and 285/169 were selected for quantification of PFBS, PFHxS, PFHpS, PFOS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFTeDA, P5MeODIOXOAc, and HFPO, respectively. The other ion transmissions (299/99, 399/99, 449/90, 213/156, 263/169, 313/119, 362/169, 412/169, 463/219, 513/219, 563/219, 613/169, 663/169, 713/169, 339/179, 329/285) were selected for qualitative confirmation. Injection volume: 10 μL. Column: Kinetex 1.7 μm XB-C18 100 × 2.1 mm.

The analysis was performed in multiple reaction monitoring (MRM) mode.

The following chromatographic conditions were used:

- mobile phase: phase A consisted of 2 mM ammonium acetate in water; phase B consisted of 2 mM ammonium acetate in methanol.
- Flow: 0.2 mL/min.
- Column temperature: 30 °C.
- Gradient: phase B from 5 % increases up to 65 % in 2 min, up to 95 % from minute 2 to minute 10 and remains at this percentage until minute 15, then decreases up to 5 % in 0.5 min and remains such until the end of the run (20 min).

2.9. Statistical analyses

The assumptions of normality and homogeneity of variances were investigated using the *shapiro.test* and *leveneTest* functions in the 'car' R package (Fox and Weisberg, 2020). When the data did not meet these assumptions even after being log-transformed, nonparametric tests were applied.

The Mann–Whitney test was used to test for differences in liver concentrations of individual PFAS and total PFAS between catfish sampled in 2018 and 2021. Since there were no significant differences ($p > 0.05$) for any individual PFAS or for total PFAS between 2018 and 2021, the 2018 and 2021 catfish samples were combined for subsequent comparisons between species and correlation analysis with fish size.

The *t*-test was used to test the differences among PFAS concentrations in the livers of catfish and bleak after log-transformation of the data. The Kruskal–Wallis test, followed by the Wilcoxon test, was applied to test for differences in PFOS concentrations in the livers of catfish, bleak and eels.

To compare PFAS contamination levels between fish hosts and their parasites, the method of Sures et al. (1999) and Molbert et al. (2020) was adopted; the concentration ratio (r = C[Parasite]/C[barbel intestine]) was calculated for each PFAS and for the sum of PFAS ($r > 1$ means higher contamination of parasites compared to their host, and $r \leq 1$ means lower contamination of parasites compared to their host). Then, a *t*-test was used to verify whether the ratio differed from the threshold value of 1. Moreover, the correlation between PFAS levels in fish intestines and in parasites was tested through Spearman rank correlations.

The relationship between PFOS levels in the liver and catfish size was investigated by Pearson correlation after log-transformation of total length, weight, and PFOS values.

The Kruskal–Wallis test, *t*-test and Wilcoxon test were performed in the FSA R package (Ogle et al., 2020). All analyses were performed using R software version 3.4.3.

3. Results

3.1. PFAS levels in samples

Table 2 shows a summary of the results obtained from the analysis of tissue samples. All fish species caught in the Po River were found to be contaminated with a range of PFAS, with many analytes never detected and some variability between values, as evidenced by high standard deviations (Table 2).

PFOS was detected in all the samples collected and at relatively high concentrations compared to the other PFAS analysed. In fact, on average, the PFOS concentration was at least ten times higher than that of the other analytes detected and was the most abundant PFAS in all samples analysed. The highest PFOS levels were determined in samples of bleak liver, both as median and as individual data (median: 57.4 ng/g w.w.; 126.4 ng/g w.w. in a single sample), followed by the liver of channel catfish (median: 27.7 ng/g w.w.; 114.4 ng/g w.w. in a single sample). The median PFOS concentration in the intestine of barbel was 19.3 ng/g w.w., while the intestinal parasite (*Pomphorhynchus laevis*) from barbel had a median PFOS concentration of 11.6 ng/g w.w. However, because perfluoroalkyl substances preferentially concentrate in liver tissue, a direct comparison cannot be made between liver and other tissues from different specimens (Kannan et al., 2005); generally, concentrations in muscle, gonads and intestine tissues are expected to be much lower than in liver (Kelly et al., 2009; Giari et al., 2015; Valdersnes et al., 2017; Hoa et al., 2022; Cao et al., 2023; Xin et al., 2023).

After PFOS, the highest concentrations were recorded for PFUnDA, PFDA, PFDoDA, and PFTrDA. Long chain perfluoroalkyl carboxylic acids (PFCAs) (i.e., PFDA and PFUnDA) showed similar contributions of 10 % and 10.5 %, respectively, with the highest PFDA and PFUnDA concentrations detected in the liver of the bleak. PFOS and PFDA showed the same trend in the different kinds of samples analysed (see Table 2, reporting mean data obtained from analyses and standard deviations).

The average contributions of the other PFAS studied here were <8 %. For example, PFHxS, a current POP candidate, was found below the LOQ in all fish samples. PFOA was detected in lower concentrations in only 3 out of 16 liver samples of channel catfish (mean PFOA: 0.4 ± 1.1 ng/g w.w., higher value: 4.3), in 5 in total intestine of barbel, and in intestinal parasites from barbel (nonquantifiable traces, i.e. below LOQ), in contrast to its ubiquitous presence in environmental matrices and in humans. PFBS was found in nonquantifiable traces in two bleak and one catfish liver samples. Some PFASs, namely, PFHxS, PFHpS, PFBA, PFHxA and PFHpA, were not detected in any of the samples; in addition, no new generation PFAS (GenX and C6O4) were detected.

3.2. Comparative and correlation studies on PFAS concentrations

The levels of PFUnDA, PFDoDA, PFTrDA, PFTeDA and the sum of all measured PFAS were significantly higher in the livers of bleaks than in those of catfish ($t = -7.18$, df = 19, $p < 0.01$; $t = -5.66$, df = 19, $p < 0.01$; $t = -5.01$, df = 19, $p < 0.01$; $t = -5.11$, df = 19, $p < 0.01$, $t = -2.20$, df = 19, $p < 0.05$, respectively, see also Fig. 1).

The hepatic concentrations of PFOA, PFDA and PFNA were similar in bleak and catfish (*t*-test, $p > 0.05$). Livers of bleak and catfish also did not significantly differ in PFOS concentration (t-test, $p > 0.05$), but they both had significantly higher PFOS levels than that reported by Giari et al. (2015) in the livers of European eels (*Anguilla anguilla*) sampled in the same stretch of the Po River in 2012 ($KW\chi^2 = 23.92$, df = 2, $p < 0.01$; Fig. 2).

The hepatic PFOS concentration was not correlated with the total length of the catfish ($r = -0.16$, $p > 0.05$) or their weight ($r = -0.09$, $p > 0.05$).

The level of PFAS contamination was compared among barbels and the acanthocephalans hosted in their intestines. The concentrations of PFOS and PFNA were higher in the fish intestines than in parasites ($t = -3.46$, df = 8, $p < 0.01$; $t = -4.45$, df = 3, $p < 0.05$; respectively), and for all the other PFAS, no differences in concentrations were found among hosts and parasites (*t*-test, $p > 0.05$). No correlation between PFAS levels (concentration of each PFAS and ΣPFAS) in fish intestines and parasites was significant ($p > 0.05$).

4. Discussion

Much of the literature on PFAS concerns PFOS and PFOA, with very limited data for the new generation PFAS (GenX and C6O4; Semerád et al., 2022). For the species considered here, there is very limited published data available (Babut et al., 2017 on barbel, and Lanza et al., 2017 on Ictaluridae, the same family to which *Ictalurus punctatus* belongs), so any comparisons must be made with due approximations and the results of comparisons with freshwater species with similar biological and ecological characteristics should be interpreted with caution. Furthermore, data reported for river fish are often limited to tissues such as muscle (as in Mazzoni et al., 2019, for instance) or liver, never intestine, making it difficult to find a reference for the results obtained for this type of organ.

The results agreed with previous works that reported that PFOS was the most frequently encountered PFAS and that it is predominant in fish. PFAS profiles in fish from the lower stretch of Po contained PFOS with a mean contribution of 62 % to ΣPFASs at the global level, which agreed with the findings of previous European studies (for instance: Hloušková et al., 2013; Svihlikova et al., 2015; Lorenzo et al., 2016; Pignotti et al., 2017; Ábalos et al., 2019; Junttila et al., 2019; Arinaitwe et al., 2020; Kumar et al., 2022).

Regarding the levels of PFOS in the liver samples of both bleak and channel catfish, the results obtained were lower than those reported in the literature. In particular, data were lower than the results reported for smallmouth bass liver from New York State lakes (10–140 ng/g, Sinclair et al., 2006), chinook salmon (30–170 ng/g) or whitefish (33–81 ng/g) of the Great Lakes (Houde et al., 2006), livers of eel, perch, roach from the river Main or Alz (15–4300 ng/g) (Federal Office for Environment, 2007) or in carp or gibel carp from Flanders (Belgium) (10–9030 ng/g, Houde et al., 2006). Higher concentrations than those found in the Po River fish liver samples were also highlighted by Becker et al. (2010), who reported PFOS and PFOA in chub liver from the Roter main river (Germany) values of PFOA from <0.5 ng/g w.w. to 3.6 ± 0.2 ng/g w.w. and PFOS ranging from 110 ± 12–152 ± 13 ng/g w.w. In contrast, the monitoring carried

Table 2
Results of PFAS analyses in fish samples from the Po River. The median, mean and standard deviation are reported for each group of samples in ng/g wet weight (w.w.). Nonquantifiable traces found are considered equal to half of the LOQ; nondetected analytes have a value of zero.

Species	Tissue	Number of samples		PFBS	PFHxS	PFHpS	PFOS	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA	GenX	C6O4	ΣPFAS
Bleak (*Alburnus alburnus*)	Liver	5	Median	0.0	<LOQ	<LOQ	57.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.0	12.3	11.03	5.6	4.1	1.3	<LOQ	<LOQ	107.5
			Mean	0.5	<LOQ	<LOQ	62.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.5	13.6	13.8	5.8	4.6	1.6	<LOQ	<LOQ	102.5
			Standard deviation	0.7	<LOQ	<LOQ	39.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.7	10.3	6.5	1.6	1.2	1.1	<LOQ	<LOQ	53.0
			% values >LOQ	40	0	0	100	0	0	0	0	0	40	80	100	100	100	80	0	0	
Channel catfish (*Ictalurus punctatus*)	Liver	16	Median	0.0	<LOQ	<LOQ	27.7	<LOQ	0.0	<LOQ	<LOQ	0.0	0.0	4.9	1.3	1.3	1.3	0.0	<LOQ	<LOQ	35.8
			Mean	0.1	<LOQ	<LOQ	45.0	<LOQ	0.1	<LOQ	<LOQ	0.4	0.2	4.9	2.1	1.3	1.0	0.1	<LOQ	<LOQ	55.1
			Standard deviation	0.3	<LOQ	<LOQ	34.9	<LOQ	0.4	<LOQ	<LOQ	1.1	0.4	3.4	1.5	1.0	1.0	0.3	<LOQ	<LOQ	38.1
			% values >LOQ	6	0	0	100	0	6	0	0	18	12	94	94	75	62	6	0	0	
Barbel (*Barbus barbus*)	Intestine	9	Median	<LOQ	<LOQ	<LOQ	19.3	<LOQ	0.6	<LOQ	<LOQ	0.0	<LOQ	2.9	8.0	5.8	4.7	1.3	<LOQ	<LOQ	43.7
			Mean	<LOQ	<LOQ	<LOQ	20.5	<LOQ	0.6	<LOQ	<LOQ	0.4	<LOQ	2.6	9.1	7.5	5.3	1.3	<LOQ	<LOQ	46.9
			Standard deviation	<LOQ	<LOQ	<LOQ	6.7	<LOQ	0.9	<LOQ	<LOQ	0.6	<LOQ	2.4	6.9	6.8	3.7	1.1	<LOQ	<LOQ	20.4
			% values >LOQ	0	0	0	100	0	11	0	0	33	0	66	78	89	100	78	0	0	
	Intestinal parasites (acantocephalan *Pomphorhynchus laevis*)	9	Median	<LOQ	<LOQ	<LOQ	11.6	<LOQ	2.0	<LOQ	<LOQ	0.0	<LOQ	0.0	4.2	7.3	3.2	0.0	<LOQ	<LOQ	26.5
			Mean	<LOQ	<LOQ	<LOQ	13.1	<LOQ	2.0	<LOQ	<LOQ	0.4	<LOQ	1.8	4.0	7.2	6.2	0.9	<LOQ	<LOQ	34.2
			Standard deviation	<LOQ	<LOQ	<LOQ	5.5	<LOQ	2.8	<LOQ	<LOQ	0.6	<LOQ	2.8	4.1	6.1	7.3	1.5	<LOQ	<LOQ	22.4
			% values >LOQ	0	0	0	100	0	14	0	0	28	0	42	60	100	70	42	0	0	

out by Orata et al. (2008) on Nile perch and tilapia liver samples from Lake Victoria shows mean values of PFOS (35.7 ng/g and 23.7 ng/g, respectively) slightly lower than those obtained for the liver of bleak and channel catfish. In the same study, the levels of PFOA were minimal, in line with the findings in the present study.

The monitoring carried out by Lam et al. (2014) on fish species liver samples (carp and mandarin) from freshwater environments in Korea provided interesting comparative data, always taking into consideration that they were from different species from those considered in the present study. The compounds analysed showed the same prevalence trend found in the present study in the two species (prevalent PFOS, followed by PFUnDA). The levels reported in the study were similar to those of Po River fish for PFOA, PFNA, PFUnDA and PFDoDA; on the other hand, the levels in the liver of fish sampled in the Po River were higher than in the samples from Korea for PFOS and for PFDA (6.2 ng/g and 19.4 ng/g, respectively, for carp and mandarin liver samples from Korea). A similar trend was also observed from the comparison with data reported by Valsecchi et al. (2021) for liver samples of L. *Iota* and *R. rutilus* from Geneva lake; the values of PFOA and PFNA were similar to those of the present study, while those of PFDA and PFOS, found in 100 % of the samples as found for the Po River fish, were slightly lower (single values between 0.6 and 4.8 ng/g w.w. and between 9.4 and 57.8 ng/g w.w.

A study carried out by Giari et al. (2015) on European eels in the same stretch of the Po showed the presence of PFOS (up to 6.3 ng/g w.w.) at lower concentrations than those detected in this work in both bleak and catfish. Albeit with caution given the different species studies and the different analysis methods used, the differences found can be attributed to interspecific variability or to environmental variations in PFOS levels in the Po River.

Following PFOS, in the present study, the next highest concentrations were recorded for PFUnDA, PFDA, PFDoDA and PFTrDA. Long chain perfluoroalkyl carboxylic acids (PFCAs) (i.e., PFDA and PFUnDA), showed similar contributions of 10 % and 10.5 %, respectively, with the highest PFDA and PFUnDA concentrations detected in the liver of the bleak. Previous studies also reported the relevance of these PFCAs after PFOS, with PFDA and PFUnDA having the greatest contributions (Svihlikova et al., 2015; Pignotti et al., 2017).

For C6O4, which was not detected in any of the samples analysed in this study and may represent not only an environmental hazard but also a potential risk for human health (Bernardini et al., 2021), no data are currently available in the literature for fish species. A recent monitoring program carried out by the Regional Environmental Protection Agency of Veneto (Italy) detected relatively high levels of C6O4, up to 3200 ng/L in groundwater and approximately 300 ng/L in the Po River (ARPAV, 2019), creating the rationale for the interest in analysing the levels of the contaminant in organisms from the same environment in the present study. Another study revealed the presence of C6O4 in the surface water and eggs of wild birds collected near the Solvay production area of Spinetta Marengo (Alessandria, Piedmont, Italy) (Morganti et al., 2021); other publications reported results of experiments regarding exposure to C6O4 that indicated adverse effects on the organisms studied (Bernardini et al., 2021). The Regional Environmental Protection Agency of Emilia Romagna analysed water from the Po River in Pontelagoscuro and Berra in 2021 and found C6O4 in quantifiable levels in two out of six samples performed (54.5 and 77.2 ng/L and 32.7 and 76.1 ng/L, respectively). Having detected C6O4 in the two sampling stations of the Po River where we took our fish samples means that although fish tissues were not contaminated by this compound, the fish were certainly exposed. The absence of C6O4 in fish from the Po River supports the lower bioaccumulative potential of this compound and of the other new generation PFAS, as stated by publications from the Stockholm Convention (Bernardini et al., 2021).

The catfish, a benthic species, was equally or less contaminated than the pelagic bleak. This result agreed with the findings of Lanza et al. (2017) on ictalurids. This paper reported that PFOS concentrations in Ictaluridae specimens were lower than those in the pelagic fish families analysed (Centrarchidae, Cyprinidae, and Poeciliidae) and similar to the

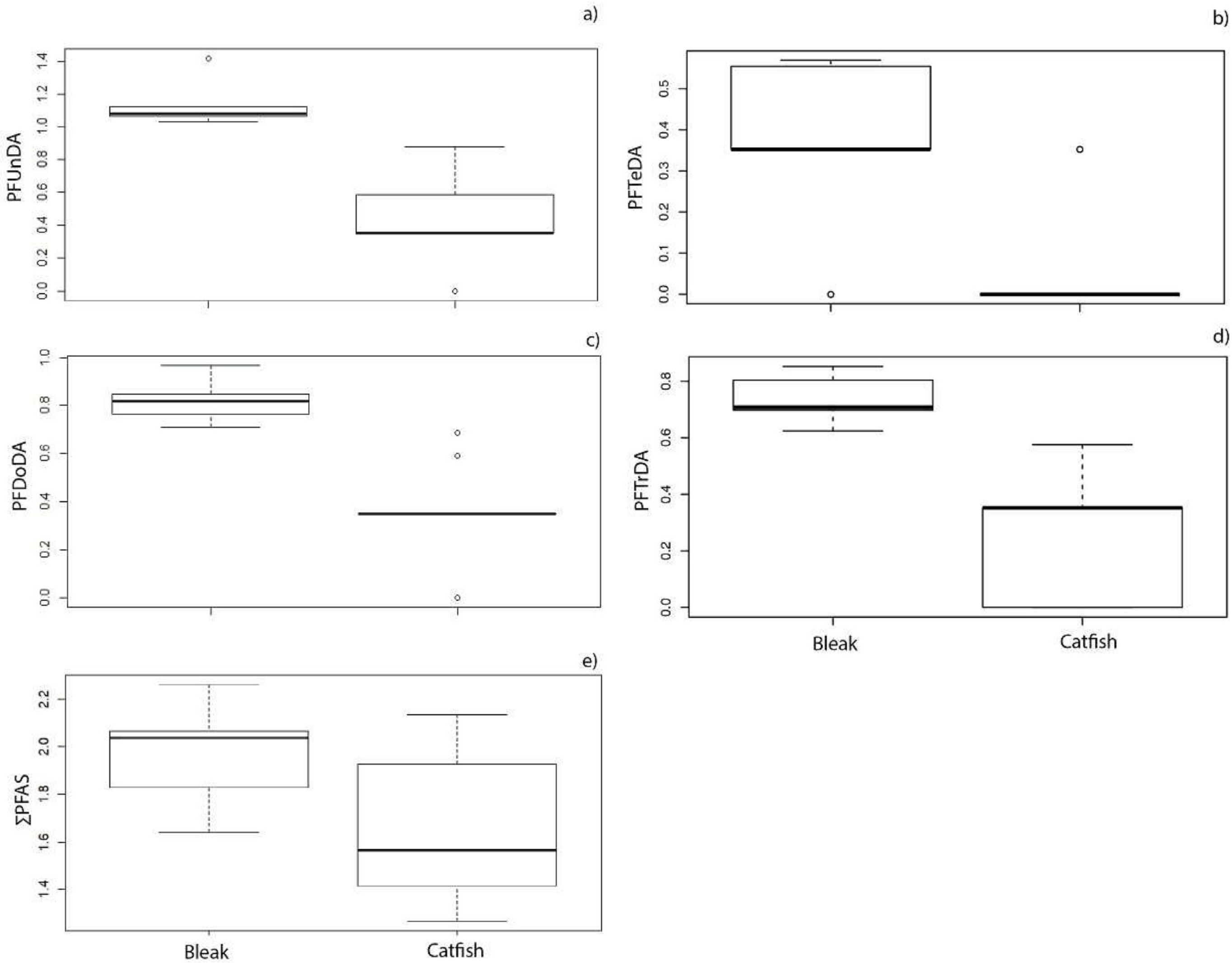

Fig. 1. Significantly different hepatic PFAS concentrations between bleak and catfish: a) PFUnDA, b) PFTeDA, c) PFDoDA, d) PFTrDA, e) sum of all PFAS concentrations. Values (ng/g w.w.) of PFAS were log-transformed. The horizontal bars in the boxes represent the median, the boxes' hinges represent the first and third quartiles, and the notches represent the 95 % confidence interval of the median.

concentrations observed in crayfish (Cambaridae), indicating less PFAS accumulation in catfish and benthic invertebrates compared with colocated pelagic species. The authors attributed this result to differences in metabolism or prey items or to the ability of catfish to tolerate pollution, resulting in reduced assimilation or amplified excretion of these compounds. Limited contamination in benthic individuals was also reported by de Solla et al. (2012). In Nania et al. (2009), a relationship between fish habitats and the levels of PFOA and PFOS accumulation was found, but contrary to the results achieved on samples from the Po River and in the paper by Lanza et al. (2017), benthic fish showed PFOA and PFOS levels, on average, higher than pelagic fish. The authors hypothesized that benthic fish can absorb contaminants both from seawater and from sediments, as also stated by several studies that reported the presence of PFOA and PFOS in riverine and marine sediments (e.g., Berger et al., 2004; Nakata et al., 2006).

On the other hand, in the work of Chen et al. (2021), PFAS concentrations were generally positively correlated with trophic levels. The profiles of PFAS were significantly different among bitterling, crucian and other fish, which might be related to their different metabolic capacities. A direct relationship between PFAS accumulation and fish species fed different diets was also revealed by Semerád et al. (2022) in a study that evidenced a correlation between molecular mass and PFAS concentrations in fish tissue, suggesting greater accumulation of longer-chain PFAS compounds. Furthermore, as reported by Macorps et al. (2022) in a study on specimens of European chub, PFAS levels in fish were dependent on the sampling site, but trophic ecology significantly explained interindividual variations. In the Po, bleak occupies the middle part of the water column and is a typically planktivorous species. This distinguishes it considerably from catfish, a bottom benthivorous species. However, to understand the possible pathways of propagation and thus PFAS contamination, the main systematic groups that make up the diet of the three species must also be considered. Bleak feed mainly on crustaceans and on various developmental stages of aquatic insects (Froese and Pauly, 2022). Similarly, channel catfish also feed on benthic invertebrates, crustaceans, insects and molluscs, but shows an omnivorous diet that also comprises small fish, for a fraction that increases with age and size (Froese and Pauly, 2022). Therefore, the similar contamination found in bleak and catfish for some PFAS might be attributed to a similar diet, consisting of invertebrates such as crustaceans, insect larvae and molluscs, which have high PFAS contents (Koch et al., 2021). Specific investigations on the diet of these species in lower stretches of Po and isotope analyses could be performed to ascertain the influence of feed composition and trophic position.

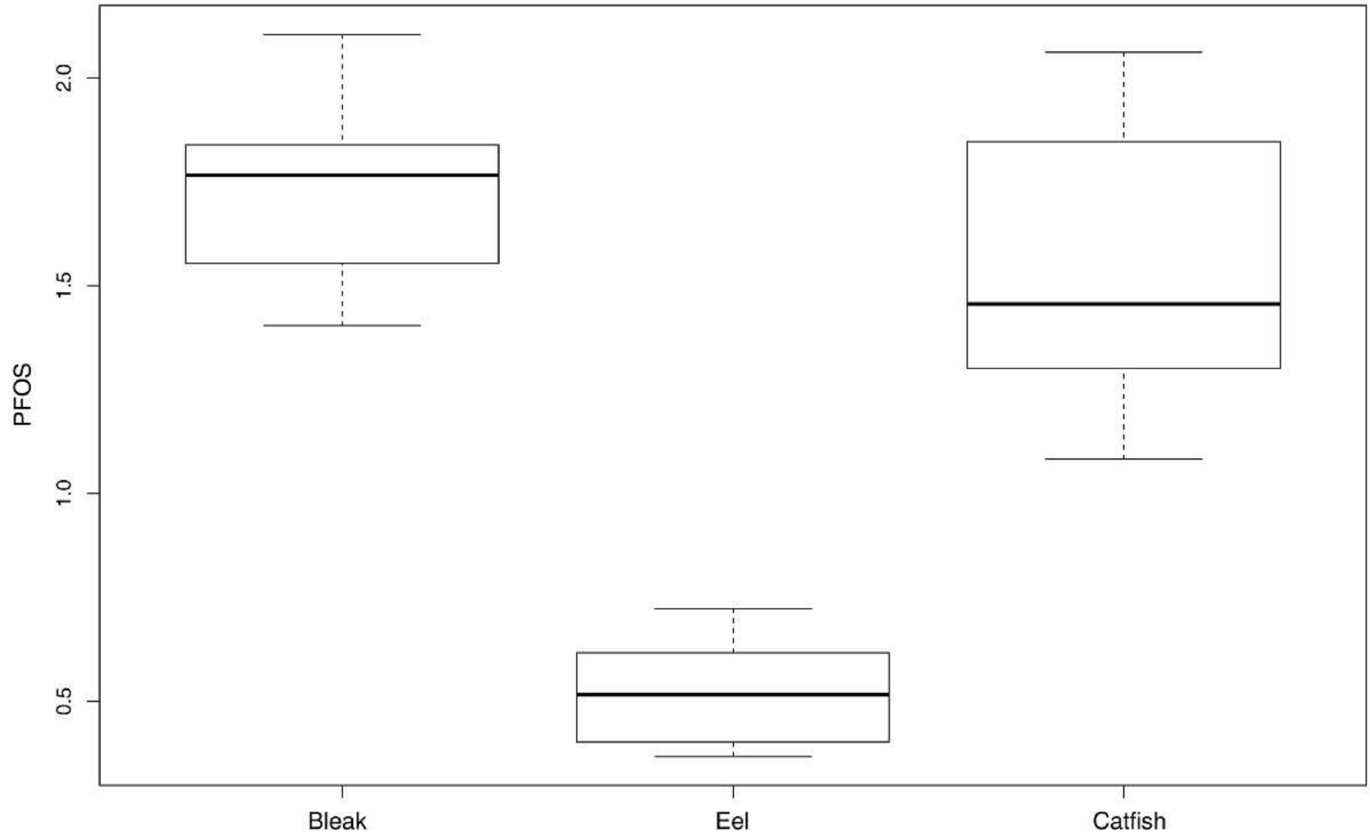

Fig. 2. Log-transformed PFOS concentrations in the livers of bleak, eel and catfish. The horizontal bars in the boxes represent the median, the boxes' hinges represent the first and third quartiles, and the notches represent the 95 % confidence interval of the median.

The absence of correlation between the size of the catfish and the degree of contamination by PFOS, although the increase in size (approximately 300 g upwards) involves a change in the diet of the catfish, which become piscivorous, can be explained by the fact that PFOS uptake depends on equilibrium partitioning between gills and water rather than by food intake. The lack of a relationship between the weight/length of the fish and PFAS concentration was also confirmed by other studies (Arinaitwe et al., 2020; Semerád et al., 2022). In contrast, Babut et al. (2017) reported that PFAS bioaccumulation in fish tissues (barbel) was influenced by size to various extents depending on the compound.

The ability of aquatic parasites, especially intestinal helminths, to accumulate inorganic and organic substances/compounds has been documented both in field and laboratory exposure conditions (see Sures et al., 2017; Sures and Nachev, 2022), and sometimes the pollutant loads are orders of magnitude higher than those in the host tissues (Sures and Siddall, 1999). In freshwater fish infected by acanthocephalans, significantly higher concentrations of contaminants were measured in the parasites than in the host tissue (Sures et al., 1999; Thielen et al., 2004; Brázová et al., 2012; Molbert et al., 2020). In this study, the uptake of several PFAS by the acanthocephalan *P. laevis* was observed for the first time, but the concentration reached by parasites was similar to or lower than that reached by the barbel host. This result was contrary to that of Molbert et al. (2020), who considered organic pollutants such as phthalates, PAHs, and PCBs, which have hydrophobic/lipophilic natures and quite different characteristics from PFAS. Yen Le et al. (2014) underlined that the POP distribution between parasites and hosts is unpredictable and varies depending on several biotic and abiotic factors, including the chemical properties of the compound, the temperature, and the trophic relation in the host-parasite system. Acanthocephalans lack a digestive tract and uptake nutrients across the tegument directly from the host intestine (Sayyaf Dezfuli and Giari, 2022); this is likely the way by which contaminants are transferred from fish to intestinal parasites (Molbert et al., 2020). The low concentration of PFAS found in acanthocephalans could also be linked to and reflect the main route of exposure of barbels, mainly through water (with uptake by gills) rather than through food webs (with intestinal uptake). The factors affecting the ability of acanthocephalans to take up, accumulate and metabolize PFAS still need to be elucidated. In addition, the absence of a preferential accumulation of PFAS in parasites compared to their host suggests that *P. laevis* is not a reliable bioindicator of PFAS pollution or a potential advantage for infected exposed fish.

5. Conclusions

Monitoring and research on the bioaccumulation of PFAS are necessary for a valid environmental risk assessment of these contaminants (Ankley et al., 2021). This study provided among the first essential information on the level of contamination of fish fauna due to 17 PFAS in the Po River, a system crucial for the importance and history of PFAS pollution (Loos et al., 2008; Valsecchi et al., 2015; Morganti et al., 2021). PFOS was predominant in all three fish species examined, followed by some perfluoroalkyl carboxylic acids, such as PFDA and PFUnDA. These results confirm the greater bioaccumulative potential of long-chain PFASs and highlight that PFOS, despite being regulated since 2009 by the Stockholm Convention, is still present and could represent an environmental concern (Semerád et al., 2022). The knowledge gap on the occurrence and effects of new generation PFAS on fish fauna needs to be filled, and the present research makes a contribution. Although C6O4 was not detected in fish tissues, supporting the lower bioaccumulative capacity of new PFAS (Bernardini et al., 2021), fish of the Po River are surely exposed to this contaminant with unexplored consequences.

Despite the information provided by a number of recent studies, the fate of PFAS, especially their accumulation in aquatic biota, remains incompletely understood, probably due to the numerous driving factors that could vary depending on the specific hydrosystem (Ahrens and Bundschuh, 2014; Chen et al., 2021; Macorps et al., 2022; Semerád et al., 2022). The examination of two fish species of the Po River with different ecological traits shows similar or lower levels (depending on the specific PFAS) of contamination in channel catfish, a benthic, omnivore and potentially piscivorous species, compared to bleak, a pelagic nonpiscivorous species. This might suggest a limited influence of the habitat, trophic position and/or food as routes of exposure but a possible interspecific difference in metabolic capacity (Lanza et al., 2017). Additionally, the lack of a relationship between catfish size (which increases imply a higher fraction of fish prey in the diet) and the degree of PFOS contamination seems to indicate that accumulation depends mostly on respiratory surface uptake and only partially on food intake. The role of feed composition and biomagnification in determining specific patterns of contamination in different fish species remains an open issue that needs further insight and the support of isotope analysis to obtain solid conclusions (Babut et al., 2017; Semerád et al., 2022).

A consistent body of literature documents the role of parasites of fish as accumulation bioindicators, with many case studies from freshwater ecosystems (Sures et al., 2017; Molbert et al., 2020). For the first time, the uptake capacity of PFAS, in particular of PFOS, was demonstrated by intestinal fish parasites belonging to Acanthocephala. These organisms do not seem to be effective bioaccumulators of PFAS and they are not better environmental sentinels than their host fish, as they are known to be for other pollutants, mainly metals (Sures et al., 1999, 2017). The data must certainly be expanded with a greater number of samples and analysed in other host-parasite systems.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162828.

CRediT authorship contribution statement

Luisa Giari: Conceptualization, Data Curation, Writing – Review &Editing. Cristiana Guerranti: Methodology, Writing – Original Draft Preparation, Writing – Review &Editing. Guido Perra: Data Curation, Writing – Original Draft Preparation. Alessandra Cincinelli: Validation, Funding Acquisition, Writing – Review &Editing. Anna Gavioli: Sampling, Data Curation. Mattia Lanzoni: Sampling. Giuseppe Castaldelli: Validation, Project Administration, Funding Acquisition.

Data availability

No data was used for the research described in the article.

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.

Acknowledgements

The authors thank Dr. Cristian Marinelli, Prof. Tania Martellini and Jenny Vetralla for PFAS analysis, Floriana Costante for data organization, and Dr. Katalin Patonai and AJE for English language editing of the first and revised versions of the paper, respectively. The authors are also grateful to the Fisheries Bureau of the Emilia-Romagna Region (Italy) for the constructive long-lasting support of ecological research on fish communities. This research was partly financed by the Bureau within the grant for activities of common interest, under article 15 of law No. 241/90, aimed at sharing and use of ichthyological data and knowledge.

Ethics statement

Fish handling and sacrifice were conducted in accordance with Italian regulations on animal research to minimize pain, stress and discomfort. The authorization for scientific fish capture was delivered by local administration authorities (Regione Emilia-Romagna).

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