Assessment of perfluorooctane sulfonate and perfluorooctanoic acid exposure through fish consumption in Italy

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Abstract

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are pollutants of anthropic origin with possible side effects on human health. Diet, and in particular fish and seafood, is considered the major intake pathway for humans. The present study investigated the levels of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) contamination in twenty-five samples of fresh fillet of five widely consumed fish species purchased from large retailers in Italy, to be used for an estimation of the Italian population exposure to these contaminants. PFOS and PFOA were found in all samples, at concentrations up to 1896 (mean=627 ng/kg) and 487 ng/kg (mean=75 ng/kg), respectively, confirming the role of fish as high contributor to human exposure. However, a remarkable inter-species variability was observed, and multiple factors were suggested as potentially responsible for such differences, suggesting that the preferential consumption of certain species could likely increase the intake, and thus the exposure. The exposure estimates for both average and high fish consumers resulted far below the tolerable daily intakes for PFOS and PFOA in all age groups, confirming the outcomes of EFSA's scientific report. In particular, the calculated total dietary exposure for the 95th percentile consumers belonging to the toddler age class, the most exposed group, resulted equal to 9.72 ng/kg body weight (BW)/day for PFOS and 8.39 ng/kg BW/day for PFOA.

Introduction

Perfluoroalkyl substances (PFASs) is the collective name for a large group of synthetic compounds characterized by a hydrophobic fully fluorinated carbon chain with a hydrophilic terminal group. The presence of carbon-fluorine bonds gives these molecules a considerable physical and chemical stability, even in extreme conditions. Moreover, their amphiphilic character makes them water and oil repellent, and able to reduce surface tension (Buck et al., 2011). These useful properties lend PFASs an important commercial value, which resulted in their employment for over 60 years in a large number of industrial and consumer applications, including stain-resistant coatings, oil-resistant claddings applied to food packaging materials, firefighting foams, insecticides and detergents (Prevedouros et al., 2006; Lindstrom et al., 2011). The extended production of PFASs during the last 60 years, combined with their high resistance against thermal degradation, hydrolysis, photolysis and biodegradation, have resulted in their global distribution, persistence in the environment, even in areas far from anthropogenic activities, and accumulation in biota (Ahrens and Bundschuh, 2014; Eggers Pedersen et al., 2015). Their potential to accumulate is not fully understood yet, depending on each compound's chemical structure (Conder et al., 2008), however it is known that, unlike other persistent halogenated compounds, they have high affinity to proteins and are then easily found in human plasma, where they have a long half-life: Sundström et al. (2012) estimated a mean elimination half-life of 2665 days for perfluorohexanesulfonate. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the most investigated and frequently found molecules belonging to this family. A number of studies on their toxicity evidenced adverse health effects in experimental animals, such as hepatotoxicity, developmental and reproductive toxicity, neurobehavioral toxicity, immunotoxicity, lung toxicity and endocrine alterations; their effects on human health are not completely clear yet, therefore a certain public health concern raised recently towards these substances (OECD, 2002; EFSA, 2012). In 2009 PFOS and its salts have been included in the Annex B of the Stockholm Convention as persistent organic pollutants (POPs), because of their high persistency in the environment, tendency to bioaccumulate in organisms and potentially dangerous effects on human health, and their production was consequently restricted (Wang et al., 2009). There are many sources of exposure to PFASs for humans, including food, water, air and dust, but diet has been indicated as the most relevant route for the general population (Fromme et al., 2007; Vestergren and Cousins, 2009). For this reason, in 2010 the European Commission issued a document calling member states to monitor perfluoroalkylated substances including a variety of foodstuffs reflecting consumption habits, in order to enable an accurate estimation of exposure (Commission Recommendation 2010/161/EU; European Commission, 2010). Fish and seafood are considered the major contributors to human exposure (EFSA, 2012). Although the available data suggest that exposure estimates are well below the proposed tolerable daily intakes (TDIs) [150 and 1500 ng/kg body weight (BW)/day for PFOS and PFOA, respectively], the presence of PFASs in this food category is highly variable, being influenced by multiple factors, including species, habitat and food habits (Shi et al., 2012; Eriksson et al., 2013; Hlouskova et al., 2013). The aim of the present work was to measure PFOS and PFOA levels in samples of fresh fillets of five highly consumed fish species in Italy, purchased from large retailers, in order to assess the average contamination and to estimate the Italian population exposure to PFOS and PFOA due to consumption of fresh fish meat.

Materials and Methods

Reagents and chemicals

Sodium perfluoro-1-octanesulfonate (PFOS), perfluoro-n-octanoic acid (PFOA) and the relative ^{13}C-labeled isotopes (purity greater than 98%) were purchased from Wellington Laboratories (Guelph, Ontario, Canada); taurochenodeoxycholic acid (TCDCA), with a purity grade >97%, was bought from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile was from VWR (Radnor, PA, USA), while formic acid, anhydrous magnesium sulfate, sodium

chloride and Supelclean ENVI-Carb sorbent (120-400 mesh) were purchased from Sigma-Aldrich. Bondesil-C18 sorbent (40 μm) was purchased from Agilent (Santa Clara, CA, USA). Ultrapure water (18.2 M /cm) was obtained from a Human Power I lab water purifier system (Seoul, South Korea). Methanol and ammonium acetate, employed as mobile phases, were of LC-MS grade and were purchased from Sigma Aldrich.

Sample collection

Twenty-five fish, purchased from local markets in the area of Bologna (Italy), were employed for this investigation. Five samples of five different species, fished in the Mediterranean Sea and collected from large retailers in late 2012, were considered. In more details, Atlantic mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), European hake (*Merluccius merluccius*), European sea bass (*Dicentrarchus labrax*) and Flathead mullet (*Mugil cephalus*) were included in the study, being among the most consumed species in Italy. For each fish, all the muscle tissue was collected, chunked and homogenized at 4°C with an electric blender, then stored in polypropylene tubes in the dark at -20°C the same day of purchase. Prior to analysis, samples were thawed overnight at 4°C.

Sample preparation

Muscle samples were extracted in accordance with the protocol described by Lacina *et al*. (2011). Seven and a half g of homogenized tissue were weighted into a polypropylene tube, then 25 μL of a methanol solution containing both the isotopically labeled internal standards at a concentration of 25 μg/L were added, followed by 10 mL ultrapure water, and the tube was shaken for 1 min. Then 15 mL of acetonitrile and 0.2 mL of formic acid were added and, after shaking vigorously, 6 g of $MgSO_4$ and 1.5 g of NaCl were also placed in the tube, which was again intensively shaken and centrifuged for 5 min at 8,500 $\times g$ (Hettich, Germany). In the next step 12 mL of the upper acetonitrile layer were transferred to another polypropylene tube, previously prepared with 1.8 g of $MgSO_4$, 0.18 g of C18 sorbent and 0.09 g of ENVI-Carb sorbent, and then shaken and centrifuged for another 5 min at 8500 $\times g$. Eight mL of the extract were then placed into a new polypropylene tube and evaporated under nitrogen stream. The dried extract was then reconstituted in 0.5 mL of methanol and filtered through a 0.2 μm PVDF filter (Whatman, USA) to a polypropylene vial prior to analysis in LC-MS/MS.

UPLC-MS/MS analysis

Analysis was conducted on a UPLC-MS/MS system. The LC instrument was an Acquity UPLC binary pump, equipped with built-in vacuum degasser, thermostated autosampler and column heater by Waters Corporation (Milford, MA, USA). Chromatographic separation was obtained using an Acquity UPLC HSS T3 reversed-phase column (50×2.1 mm, 1.8 μm), fitted with a Waters VanGuard guard column with the same packing (Waters Corporation). The column was kept at 45 °C in order to limit backpressure. The mobile phase was 5 mM ammonium acetate aqueous solution (solvent A) and methanol (solvent B); the flow rate was set at 0.5 mL/min under programmed conditions. After 1 min at 100% A, the gradient switched to 40% A and 60% B over 1.5 min, then to 5% A and 95% B in 1.5 min, went back to the initial conditions over 1 min and finally equilibrated for 3 min. The autosampler maintained vials at 5°C, and 10 μL were injected in the system in *full loop* mode.

The absence of background contaminations deriving from the equipment and solvents used was verified by specific tests performed before the beginning of the experiment, and constantly monitored through all the analysis sessions by injecting a mobile phase sample every five fish samples, ascertaining the absence of chromatographic signals corresponding to any of the target analytes. A specific transition was also monitored for taurochenodeoxycholic acid (TCDCA), a naturally occurring endogenous biliary acid that can interfere with PFOS determination, potentially causing overestimation.

The LC system was coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters Corporation) operating in negative electrospray ionization (ESI–) mode and monitoring two transitions for each analyte and each internal standard (MRM - multiple reaction monitoring mode); one specific transition was also observed for the biliary acid (interfering compound). The followed transitions (and their relative optimized values of cone voltage and collision energy, respectively) were: 413>369 *m/z* (14 V, 10 eV) and 413>169 *m/z* (14 V, 18 eV) for PFOA; 499>80 *m/z* (62 V, 44 eV) and 499>99 *m/z* (62 V, 38 eV) for PFOS; 417>372 *m/z* (15 V, 10 eV) and 417>169 *m/z* (15 V, 18 eV) for ^{13}C-PFOA; 503>80 *m/z* (55 V, 41 eV) and 503>99 *m/z* (55 V, 38 eV) for ^{13}C-PFOS; 499>124 *m/z* (95 V, 52 eV) for TCDCA. The following instrumental settings were applied: capillary voltage was set at 2.0 kV, temperature was 150 °C for the source and 450 °C for desolvation, nitrogen flows on the cone was 100 L/h, while for desolvation it was 800 L/h. Argon was used as collision gas with a flow of 0.35 mL/min. MassLynx 4.1 software (Waters Corporation) was employed for data acquisition and processing.

Method and validation

The protocol was validated in accordance with current European guidelines (Commission Decision 2002/657/EC). Since it is quite difficult to find fish samples not presenting measurable PFAS contamination, in order to ensure a reliable quantification of the analytes, two sea breams (*Sparus aurata*) bought at a local market, which had proved during preliminary tests to be PFOS and PFOA free, were homogenized, stored at -20°C, and 7.5 g aliquots were subsequently employed for the preparation of all the calibration curves and quality control samples. Matrix-matched calibration curves (9 points: 5, 10, 50, 100, 500, 1000, 2000, 5000, 10,000 ng/kg) were prepared each day of analysis by addition of appropriate amounts of methanol standard solutions to blank samples at the beginning of the procedure, and analysed before each series of samples. A linear regression model was applied, proving the satisfying linearity of the method, with R^2 values always >0.99 for both compounds. Four replicate samples at three different concentrations (100, 500 and 5,000 ng/kg) were prepared to verify precision and accuracy: relative standard deviation to the mean (CV%) was always lower than 13%, while accuracy, expressed as relative difference between the mean value detected and the expected concentration, was always below 14%. Lower limits of quantification (LLOQs) of the method, which are the concentrations that give a chromatographic signal with a signal-to-noise (S/N) ratio of 10, extrapolated from the analysis of the matrix-matched calibration curves, were 9 ng/kg for PFOA and 6 ng/kg for PFOS. Specificity of the method was proved by the analysis of multiple non-contaminated sea bream samples, in order to assess that no interferences were present around the retention times of the analytes. A blank sea bream sample was also prepared together with each calibration curve. Moreover, one specific transition of taurochenodeoxycholic acid, which may interfere with PFOS quantification, was monitored in each analysis.

Dietary exposure assessment

In general, dietary chemical exposure is calculated by multiplying the amount of food consumed by the concentration of the substance in the food item divided by the consumer's body weight. The Italian population food consumption data by age class were thus extracted from the EFSA Comprehensive Food Consumption Database (EFSA, 2010). From the food consumption database, within the category *Fish and other seafood (including amphibians, reptiles, snails and insects)*, the subcategory *fish meat* was selected. The mean concentration of PFOS and PFOA in the 25 samples of fish purchased for this work was used as representative of PFOS and PFOA concentrations in all types of fish present in the Italian market. The average concentration of PFOS and PFOA was multiplied by the amount of fish consumed (g/kg BW/day) in Italy from

the different age groups. In particular, the exposure was calculated for the *average consumers* by using the mean fish consumption and for the so-called *extreme consumers* using the 95th percentile. Finally, the percentage of TDI of PFOS and PFOA attributable to the consumption of fish was calculated.

Results and Discussion

Method optimization

It is quite common, measuring PFASs levels, to assess background interferences deriving from solvents and laboratory equipment. These can significantly affect the results when monitoring food matrices, where PFASs contamination is usually in the ng/kg range. For this reason, from the beginning of the present study great attention has been paid to limit and control as much as possible such phenomenon as previously described, with successful results.

Besides this instrument related contamination, another relevant aspect which must be taken in consideration when measuring PFOS in fish samples is the analytical interference caused by the taurochenodeoxycholic acid (TCDCA). This is one of four cholic acids isomers, which can be found in various biological matrices and interferes with PFOS 499>80 transition, generating a potentially relevant overestimation of the chromatographic signal (Kadar *et al.*, 2011). To prevent this inconvenience, the transition 499>99, which is specific for PFOS, can be employed for quantification, although it generally provides a slightly worse sensitivity. Otherwise, it can be considered that some chromatographic columns seem more suitable than others to efficiently separate this compound from its interferent. In more detail, after some test with different gradients using a C18 column (Waters Acquity UPLC BEH C18), which is the common choice for PFASs analysis in liquid chromatography but does not allow a good separation of PFOS and taurochenodeoxycholic acid, we transferred the method to an Acquity UPLC HSS T3 column managing to obtain, after some gradient adjustment, an optimal peak resolution. In order to ensure a sensitive but still selective quantification of this target analyte, we therefore decided to use the more abundant 499>80 as primary transition for PFOS quantification, 499>99 for confirmatory purposes and to monitor also a taurochenodeoxycholic acid specific transition (499>124), which permitted to constantly assess the different retention times of the two molecules (Figure 1).

Sample analysis

The present survey evidenced once more how this matrix is affected by PFASs contamination, having both target analytes been found in all samples. As reported in Table 1, PFOS was measured in all considered species with concentrations between 54 and 1896 ng/kg, while PFOA ranged from trace levels up to 487 ng/kg.

If on the one hand these results confirm the role of PFOS as frequent contaminant in fish, on the other hand a certain variability is evident, suggesting that the role of this food category as major source of exposure for humans can vary significantly. In more detail, interspecies differences are relevant for both PFOS and PFOA but, although the number of samples for each species is rather limited and intraspecies standard deviations are not negligible, similar trends can be observed: European sea bass, flathead mullet and European hake resulted the most contaminated species by both compounds, while Atlantic mackerel presented lower levels of PFOS and often only traces of PFOA.

The reasons for such differences can be multiple. An important factor that has been proved to affect the found contamination in fish tissues is the habitat of each species. Rivers are generally more contaminated than
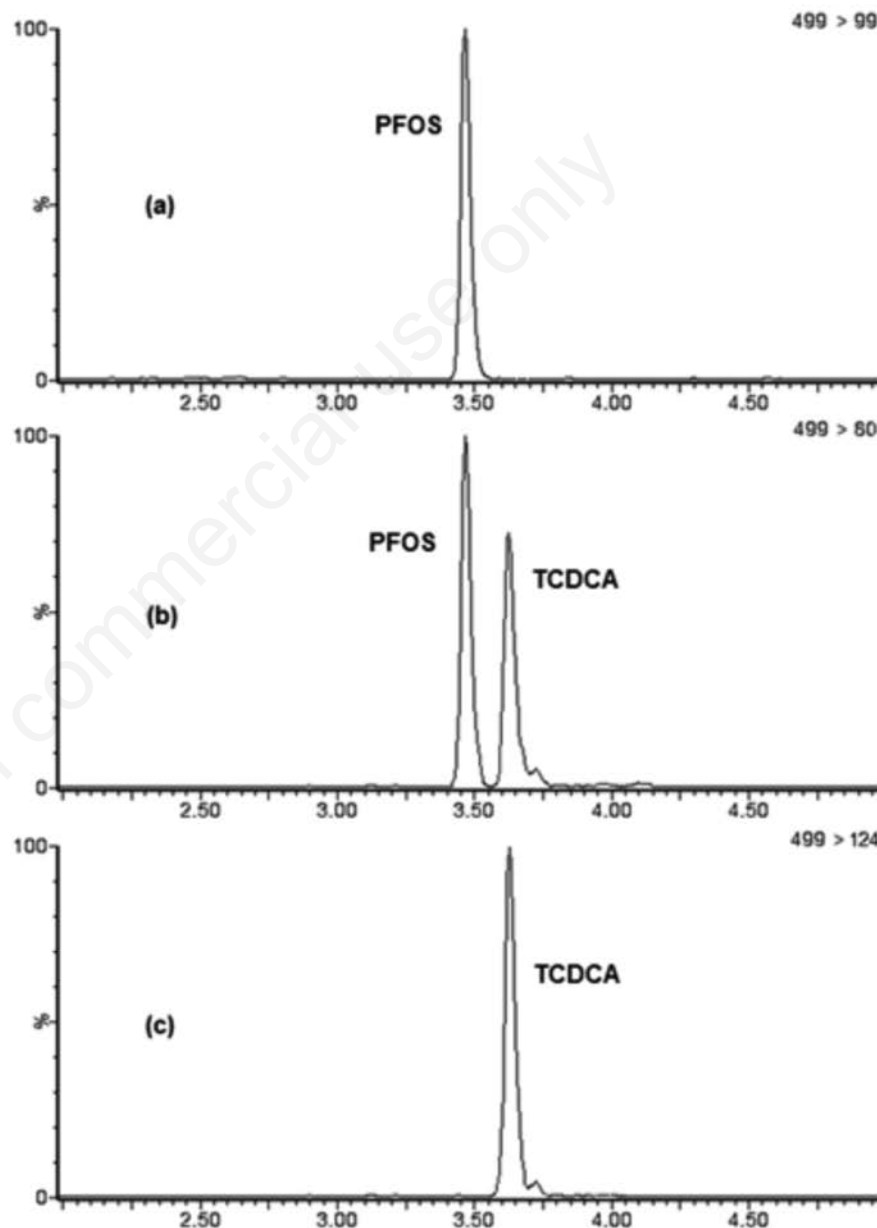

Figure 1. Chromatographic separation of the endogenous contaminant taurochenodeoxycholic acid (TCDCA) from perfluorooctane sulfonate (PFOS) in a fish meat sample: a) PFOS specific transition; b) common transition of PFOS and TCDCA; c) TCDCA specific transition. The optimized conditions allowed unambiguously identifying and quantifying target compound.

seas, but it must also be considered that coastal environment, in particular depending on the proximity of river outflows and human activities, is a more polluted ecosystem compared to the open sea (Sánchez-Avila *et al*., 2010). As a consequence, higher contaminations have been described in benthic compared to pelagic fish by some authors (Nania *et al*., 2009; Miniero *et al*., 2014); similarly, in the present monitoring, PFASs levels were higher in European sea bass and flathead mullet, both species living in coastal waters. Food habits are another species-specific factor potentially related to the level of contamination: various surveys on different fish species suggested that the contamination extent in carnivorous species is generally higher compared to omnivorous species (Van Leeuwen *et al*., 2009; Shi *et al*., 2012; Hlouskova *et al*., 2013; Xu *et al*., 2014). The relatively high concentrations that we measured in certain species, as European sea bass and European hake, might therefore reflect the trophic magnification potential of

PFOS. Moreover, these latter two and flathead mullet are leaner species compared to the others considered in the present investigation, suggesting that fat fish is likely to be a lower contributor to consumers' exposure, as also reported by Noorlander *et al*. (2011). Having all that said, it must however be also considered that fish age can play a role too. For example, although it has been observed that farmed fish is less contaminated by PFASs than wild fish (Van Leeuwen *et al*., 2009; Paiano *et al*., 2012; Shi *et al*., 2012), the outcomes of a recent study by Miniero *et al*. (2014) on multiple persistent pollutants suggest that small size wild fish seems to have a similar contamination profile to farmed fish. This means that the found level of contamination of a wild fish can significantly vary depending also on its age at the moment it was caught.

The data obtained in the present study are comparable with those from similar recent monitoring projects on PFASs presence in fish (Haug *et al*., 2010; Hradkova *et al*., 2010; Shi *et*

al., 2010; Domingo *et al*., 2012; Zhang *et al*., 2011; Guo *et al*., 2012; Hlouskova *et al*., 2013), although variability is rather evident in some cases. It has been discussed how this can be related to multiple factors; however, talking about PFOS, the possibility that some results were affected by the previously mentioned TCDCA-related interference is a further aspect to consider.

Exposure estimates

The overall mean concentration of PFOS was 627 ng/kg (SD 489), and 75 ng/kg (SD 106) for PFOA. The mean and the 95th percentile of exposure to PFOS and PFOA in the different age groups and the relative percentage of TDI due to fish meat consumption are shown in Table 2.

When estimating dietary exposure due to fish meat consumption, toddlers were the age group with the highest exposure both for PFOS and for PFOA, with 1.41 ng/kg BW/day (95th percentiles: 4.13 ng/kg BW/day) and 0.17 ng/kg BW/day (95th percentiles: 0.50 ng/kg BW/day),

Table 1. Measured concentrations of perfluorooctane sulfonate and perfluorooctanoic acid (range, mean and standard deviation) for each fish species (n=5).

Species	PFOS (ng/kg)		PFOA (ng/kg)	
	Range	Mean±SD	Range	Mean±SD
European sea bass (*Dicentrarchus labrax*)	703-1243	1026±205	93-487	231±138
Flathead mullet (*Mugil cephalus*)	487-1896	926±508	12-113	47±35
European hake (*Merluccius merluccius*)	91-1292	716±476	Traces-127	63±52
European plaice *(Pleuronectes platessa)*	240-510	378±99	10-36	23±9
Atlantic mackerel (*Scomber scombrus*)	54-120	87±24	Traces-22	9±7

PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; SD, standard deviation. When the target compound was found at trace level, the concentration was assumed to be equal to the lower limits of quantification (9 ng/kg) for estimating the mean±SD.

Table 2. Mean and the 95th percentile of exposure to perfluorooctane sulfonate and perfluorooctanoic acid in the different age groups and relative percentage of tolerable daily intake related to fish meat consumption.

Age group	PFOS			
	Mean exposure	%TDI (150 ng/kg BW/day)	95th percentile exposure	%TDI (150 ng/kg BW/day)
Infants (<1 year)	0.16	0.09	1.32	0.88
Toddlers (1 to <3 years)	1.41	0.94	4.13	2.75
Other children (3 to <10 years)	0.56	0.38	1.90	1.27
Adolescents (10 to <18 years)	0.32	0.21	1.11	0.74
Adults (18 to <65 years)	0.29	0.19	0.96	0.64
Elderly (65 to <75 years)	0.30	0.20	1.07	0.71
	PFOA			
	Mean exposure	%TDI (150 ng/kg BW/day)	95th percentile exposure	%TDI (150 ng/kg BW/day)
Infants (<1 year)	0.02	0.00	0.16	0.01
Toddlers (1 to <3 years)	0.17	0.01	0.50	0.03
Other children (3 to <10 years)	0.07	0.01	0.23	0.02
Adolescents (10 to <18 years)	0.04	0.00	0.13	0.01
Adults (18 to <65 years)	0.04	0.00	0.12	0.01
Elderly (65 to <75 years)	0.04	0.00	0.13	0.01

PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; TDI, tolerable daily intake; BW, body weight.

respectively. The mean exposure was the lowest in the infants group: for PFOS it was equal to 0.14 ng/kg BW/day and for PFOA to 0.02 ng/kg BW/day, but the 95th percentile increased to 1.32 ng/kg BW/day for PFOS and to 0.16 ng/kg BW/day for PFOA. In the other age groups, mean values ranged from 0.29 to 0.56 ng/kg BW/day for PFOS and from 0.04 to 0.07 ng/kg BW/day for PFOA, while the 95th percentile ranged from 0.96 to 1.90 ng/kg BW/day for PFOS and from 0.12 to 0.23 ng/kg BW/day for PFOA. The exposure estimates in all age groups and categories of consumers of fish meat (average and extreme) eventually resulted far below the TDIs for both PFOS (150 ng/kg BW/day) and PFOA (1500 ng/kg BW/day). EFSA estimated that consumption of *Fish and other seafood* justifies 50-80% of PFOS and 7.6-27% of PFOA total dietary exposure, and that *Fish meat* represents more than 80% of the *Fish and other seafood* category. Consequently, based on our results, the total dietary exposure for the 95th percentile consumers of the toddler age group, representing the most exposed group, would be 9.72 and 8.39 ng/kg BW/day for PFOS and PFOA, respectively.

Conclusions

The present monitoring evidenced the presence of both PFOS and PFOA in some of the most consumed fish species available on the Italian market. The estimates of Italian consumers' exposure based on the obtained data suggest that the risks related to fish consumption are unlikely, even for high consumers. However, the observed inter-species and inter-studies variability suggests that such risk cannot be generalized and can depend on multiple factors. As a consequence, further surveys focused on certain species, possibly considering also samples place of origin, and including other perfluoroalkyl acids which are likely to be found as well, are needed to better understand the entity of the health risks related to this class of pollutants.

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