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Phthalates and perfluorinated alkylated substances in Atlantic bluefin tuna (*Thunnus thynnus*) specimens from Mediterranean Sea (Sardinia, Italy): Levels and risks for human consumption

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ABSTRACT

Atlantic blue fin tuna (*Thunnus thynnus*) is a species of great importance for Mediterranean Sea area, from both ecological and commercial points of view. The scientific literature reports few data on the contamination of this fish by emerging organic compounds such as perfluorinated alkylated substances (PFASs) and phthalates, being the latter never been studied in tuna. This study therefore investigated the presence of the PFASs perfluorooctane sulphonate (PFOS) and perfluoroctanoic acid (PFOA) and the phthalate di-2-ethylhexyl phthalate (DEHP), also monitored by its metabolite mono-2-ethylhexyl phthalate (MEHP), to assess both the state of contamination of Atlantic bluefin tuna specimen and the risk due to the toxicity of these compounds for human consumption. While PFOA was never found, detectable levels of PFOS (0.4–1.88 ng/g), DEHP (9–14.62 ng/g) and MEHP (1.5–6.30 ng/g) were found. The results were elaborated relating the accumulation to the size and age of the individuals and showed a correlation between the levels of different pollutants investigated.

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KEYWORDS
Phthalates; perfluorinated alkylated substances; *Thunnus thynnus*; human consumption risks; plastic micro-litter; PFOS; PFOA; DEHP; MEHP

Introduction

The problem of contamination of the Mediterranean basin by xenobiotic chemicals is of great interest for environmental and human health. Large marine top predators, such as tuna fish, swordfish, and sharks, have the greatest capacities for bioaccumulation of chemicals, incurring high toxicological risk, and they can be used to check levels of contaminants in the marine environment.[1–3]

The probably segregation of a mediterranean population, their feeding practice, and the extensive employ of their fillets in human diet make the Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus 1758) from Mediterranean are markable species from both ecotoxicological and commercial point of view.[4] Since ancient times, *T. thynnus* represents an important food supply in many coastal communities, and nowadays it has become a basic part of the globalization of the fish market.[4] The Atlantic bluefin tuna is a fast growing, long-living and highly migratory species, highly tolerant to a wide ranges of environmental conditions, distributed throughout the Atlantic Ocean, Mediterranean basin, and Black Sea.[4–7] Bluefin tuna is also a fast swimming and voracious predator of all kinds of fishes, crustaceans and molluscs; with a longevity of more than 20 years.[8]

Studies made on specimens from the Mediterranean Sea are essential to assess the status of these organisms, since this basin receives a heavy contaminant input from the urban and industrial areas along its coasts, and from cultivated land through rivers. Its long turnover time and this narrow water exchange increases the chemical residence time and fosters bioaccumulation.

Emerging pollutants, like perfluorinated alkylated substances (PFASs) and phthalates, show bioaccumulation properties reported by many authors.[9–13] While it is well proved that the most important contribution to human PFASs intake is the diet, being fish and seafood the main contributors, [14,15] very limited data are available on phthalates concentration in foods and diet and in animals in general,[10,11,14] being inhalation and dermal contact the most important ways of human exposure.[15]

Studies on bioaccumulation and effects of some chemical contaminants on the Atlantic bluefin tuna, have already been published in scientific literature; several chlorinated pesticides, polychlorinated biphenyls, polychlorinated dibenzo–dioxins and –furans and PBDE have been detected in this species.[16,20] For what concerns perfluorinated alkylated substances, just Guerranti et al. report data on fresh tuna fillet, while nondata are available about phthalates in this species.[13]

The aim of this study was to assess the accumulation of two PFASs, perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA), and a phthalatedi-(2-ethylhexyl) phthalate (DEHP), measured also by its primary metabolite mono-(2-ethylhexyl) phthalate(MEHP) in the Atlantic bluefin tuna from the coasts of Sardinia (Tyrrhenian Sea). Furthermore, due to the importance of tuna for human consumption, the intake of PFASs and DEHP through tuna consumption was estimated and compared with safe thresholds.

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Materials and methods

Collection of samples

Twenty-three samples of muscle were excised from twenty-three Atlantic bluefin tuna (*Thunnust hynnus*) caught both by long lines and tuna traps over a period ranging from May to July 2012, off Western and Eastern coasts of Sardinia (CW Mediterranean Sea). The fork length (cm) and weight (kg) of each specimen were measured as soon as the fish were onboard the fishing vessel. After dissection, samples were wrapped in aluminium foil previously cleaned with solvents (acetone and *n*-hexane), stored in dry ice, and kept in the dark until transportation to the laboratory, where they were stored at −20°C until analysis. The data available for the specimens analysed are reported in Table 1.

Analytical methods

All the samples were extracted for the determination of PFOS, PFOA, DEHP, and MEHP. All chemicals were analysed following widely tested methods and described elsewhere.[11,13,21–26]

The procedure for PFOS and PFOA analysis was that described by Guerranti et al.[13] Briefly PFOS and PFOA were extracted using an ion-pairing extraction procedure and measured using high-performance liquid chromatography (HPLC) with electrospray ionization (ESI) tandem mass spectrometry (MS). Standards for the five-point calibration curve were prepared by progressive dilution with methanol from a neat standard and analytes concentrations were evaluated in comparison to this unextracted standard curve and were not corrected for the recoveries or for the purity of the standards (more than 98%). Individual stock solutions of the target analytes were prepared in methanol and stored in polypropylene bottles at −20°C. LOD, determined as three times the signal-to-noise (S/N) ratio, was 0.4 ng/g wet weight (w.w.). Teflon coated labware were avoided during the whole process of sampling, pretreatment, and analysis to minimize contamination of the samples. Data quality assurance and quality control protocols included matrix spikes, laboratory blanks, and continuing calibration verification. Blanks were analysed with each set of five tissue samples as a check for possible laboratory contamination and interferences; recoveries, assessed using spiked matrix with a concentration of 5 ng/g of each analyte, were over 92%, for both the two analytes.

Table 1. Biometric parameters of the *T. Thynnus* specimens analysed.

Specimen ID	Fork length (cm)	Sampling site	Weight (kg)	Age (yr)
1	252	Porto Scuso (39°14′/08°22′E)	258.8	15–20
2	137	Porto Scuso (39°14′/08°22′E)	46.5	0–5
3	215	Porto Scuso (39°14′/08°22′E)	164.2	10–15
7	207	Isola Piana (39° 11′/08° 18′E)	148.2	10–15
11	193	Isola Piana (39° 11′/08° 18′E)	121.6	5–10
16	206	Isola Piana (39° 11′/08° 18′E)	146.2	10–15
22	207	Isola Piana (39° 11′/08° 18′E)	148.2	10–15
52	206	Isola Piana (39° 11′/08° 18′E)	146.2	10–15
53	148	Isola Piana (39° 11′/08° 18′E)	57.6	5–10
55	166	Isola Piana (39° 11′/08° 18′E)	79.6	5–10
57	204	Isola Piana (39° 11′/08° 18′E)	142.2	10–15
60	213	Isola Piana (39° 11′/08° 18′E)	160.6	10–15
61	226	Isola Piana (39° 11′/08° 18′E)	189.7	10–15
64	214	Isola Piana (39° 11′/08° 18′E)	162.7	10–15
66	211	Isola Piana (39° 11′/08° 18′E)	156.4	10–15
67	210	Isola Piana (39° 11′/08° 18′E)	154.3	10–15
68	168	Isola Piana (39° 11′/08° 18′E)	82.3	5–10
69	208	Isola Piana (39° 11′/08° 18′E)	150.2	10–15
75	164	Isola Piana (39° 11′/08° 18′E)	76.9	5–10
76	192	Isola Piana (39° 11′/08° 18′E)	119.9	5–10
79	209	Isola Piana (39° 11′/08° 18′E)	152.2	10–15
30	196	Villa Putzu (39° 25′/09° 44′E)	120.0	5–10
31	208	Villa Putzu (39° 25′/09° 44′E)	137.0	10–15

The procedure for DEHP and MEHP analysis followed the method described elsewhere.[9,21] DEHP and MEHP were extracted using an ion pairing procedure comprising also several sonication steps and determined through HPLC-ESI-MS.

Standards of MEHP (≥99.5%) and DEHP (≥98.5%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany); for the quantitative analysis, a five-point calibration curve, prepared by the progressive dilution with acetonitrile of a solution of the two analytes of interest, was used, evaluating the concentrations in comparison to this unextracted standard curve and not correcting for the recoveries or for the purity of the standards. Recoveries, assessed using matrix spiked with a concentration of 20 ng/g for DEHP and 10 ng/g of MEHP, were over 93% in blood, and over 90% in tissues for both the two analytes. Three procedural blanks were analysed with each set of five samples as a check for possible laboratory contamination and interference.[27,28] When the concentrations of DEHP in the three procedural blanks varied widely, and if the difference in concentrations among the blanks exceeded 30 ng, then all the data were discarded and samples were reanalysed. Mean blank values were subtracted from sample values for each batch. The data quality assurance and quality control protocols also included daily calibration verification. Plastic labware was avoided during the whole process of sampling, pretreatment, and analysis to minimize contamination of the samples. The limits of detection (LODs) for the compounds analysed were the values of the compound in the blanks +3 SD. The LODs were 1, 5, and 9 ng/g, respectively, for MEHP and DEHP.

Both for PFASs and phthalates, chemicals and reagents were analytical grade and glassware was carefully washed to avoid sample crossover contamination.

Estimated daily ingestion (EDI)

The EDI of DEHP and PFASs via fish consumption was calculated by multiplying the contaminant concentrations in fish fillets by the estimated daily intake for the general population (38.8 g/day) and fish consumers (71.0 g/day) in Italy;[29] the resulting values were then used to evaluate the daily intake per kg of body weight, considering an average body weight of 50 kg for women and 70 kg for men. The results were compared with the tolerable daily intakes (TDIs) of 150 ng/kg b.w., 1500 ng/kg b.w., and 0.05 mg/kg b.w. established, respectively, for PFOS, PFOA, and DEHP.[10,28,30] When the contaminant concentrations were under the respective LODs, daily intakes were calculated assuming that respective values would be equal to one-half of the LOD.

Statistical analysis

The multivariate analysis was developed using Primer E Software package version 6.0 (Plymouth Marine Laboratory, UK, Routines In Multivariate Ecological Research). This analysis was applied to

Table 2. Results of chemical analysis on tuna muscle samples (in ng/g w.w.).

	PFOS	PFOA	DEHP	MEHP
Mean	0.60	0.20	9.14	2.13
Standard deviation	0.51	0.00	3.27	1.52
Median	0.20	0.20	9.43	1.73
Range of values >LOD (LOD)	0.84–1.88 (0.4)	−(0.4)	9.27–13.93 (9)	1.58–6.30 (1.5)
Values>LOD (%)	43	0	74	74

evaluate the statistical significance of observed distributions in a multivariate dimension.[31,32] The Euclidean distances resemblance matrix was calculated on pollution levels, considering pollutants as variables and sampling replicates (fish tissues) as samples, after the application of the log(x+1) and successively the square root ($\sqrt{}$) transformations of collected data.[33] Principal Component Analyses (PCA) and non-metric multi-dimensional scaling (*nm*-MDS) routines were performed and superimposed to the cluster-analysis results to visualized data segregations. Multivariate ANOSIM (ANalysis Of SIMilarity) one-way test was performed to calculate significances running 9,999 permutations and imposing fish age (calculated on the basis of length and weight, according to Santamaria et al.,) or fish provenience as factors in pollution levels segregation.[34]

Values below the LOD were considered equal to one-half of the LOD.

Results and discussion

The presence of PFOS, DEHP, and its metabolite MEHP was detected in the tuna muscle samples analysed and the results are shown in Table 2. All the chemicals were detected in most of the specimens, except PFOA which was always below the LOD, with the prevalence of DEHP. Pearson's correlation coefficients between all pairs of variables were calculated among variables of interest. Results reported in a Draftsman plot evidenced that pollutants are not related to biometrics (length; weight). The strongest relationship was observed between the couple PFOS–MEHP (0.80), whereas relationships between DEHP–MEHP (0.76) and PFOS–DEHP (0.73) were lower (Fig. 1).

Principal Component Analyses (PCA) performed on PFOS, MEHP, and DEHP levels evidenced that the first three axes accounted for the 100% of the total variance (85.1; 10.8; 4.1). The main contribute to the first axis is due to MEHP (−0.598), whereas a strong positive contribute to the second one is due to PFOS (0.801).

Nonmetric multi-dimensional scaling (*nm*-MDS) was performed on the Euclidean distance matrix, results from the Cluster Analysis were superimposed on *nm*-MDS evidencing both factors of interest: fish provenience (three levels) and fish age (year, four levels). Observed data segregation was related neither to fish age nor to fish provenience. The ANOSIM test performed on the factor fish age evidenced no significance (Global R of 0.062, significance level of sample statistic of 18,6%, and a number of permuted statistics greater than or equal to Global R of 185) even if the pair-wise test performed on age couples (years: 0–5; 5–10; 10–15; 15–20) evidenced significant differences between the couple 10–15 and 15–20 (Global R of 0.451, significance level of sample statistic of 6.7%) (Fig. 2).

To the authors' knowledge, this is the first time that phthalates have been detected in Atlantic bluefin tuna. Guerranti et al. report PFASs data on four samples of fresh tuna fillet

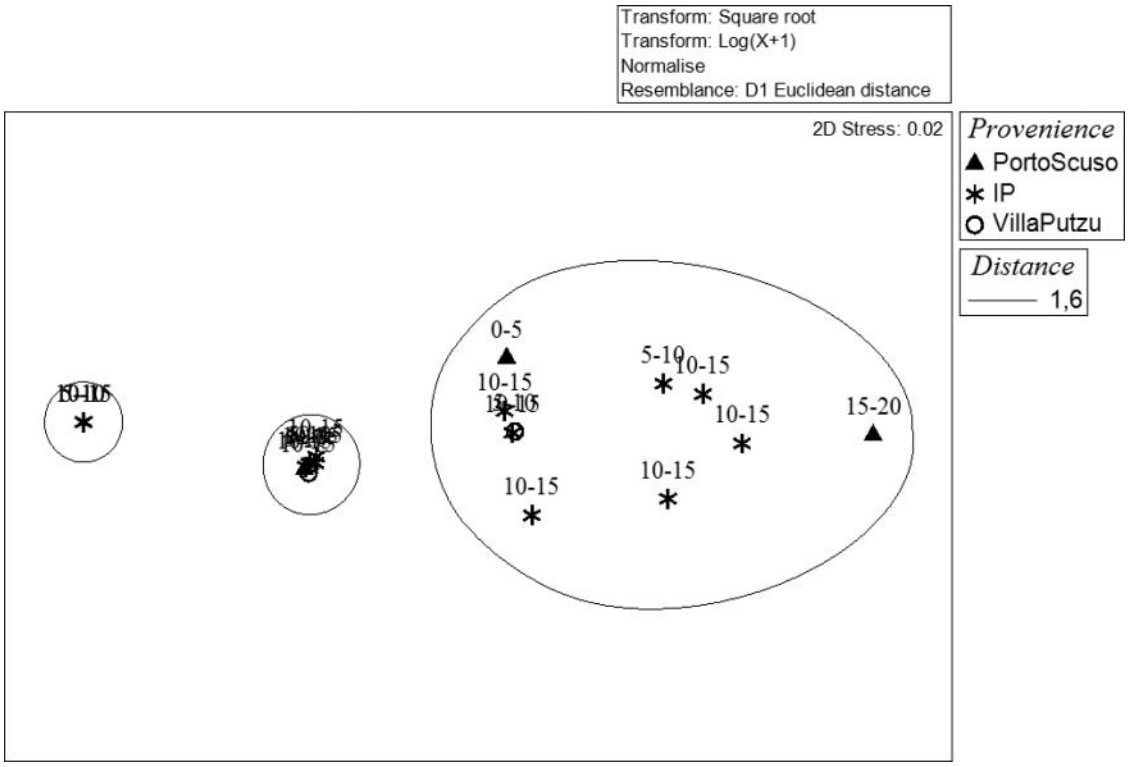

Figure 1. Multivariate analysis performed on collected data. Nonmetric multidimensional scaling (nm-MDS) performed is superimposed to the cluster analysis. Two different factor of interest are evidenced (sampling stations and fish age).

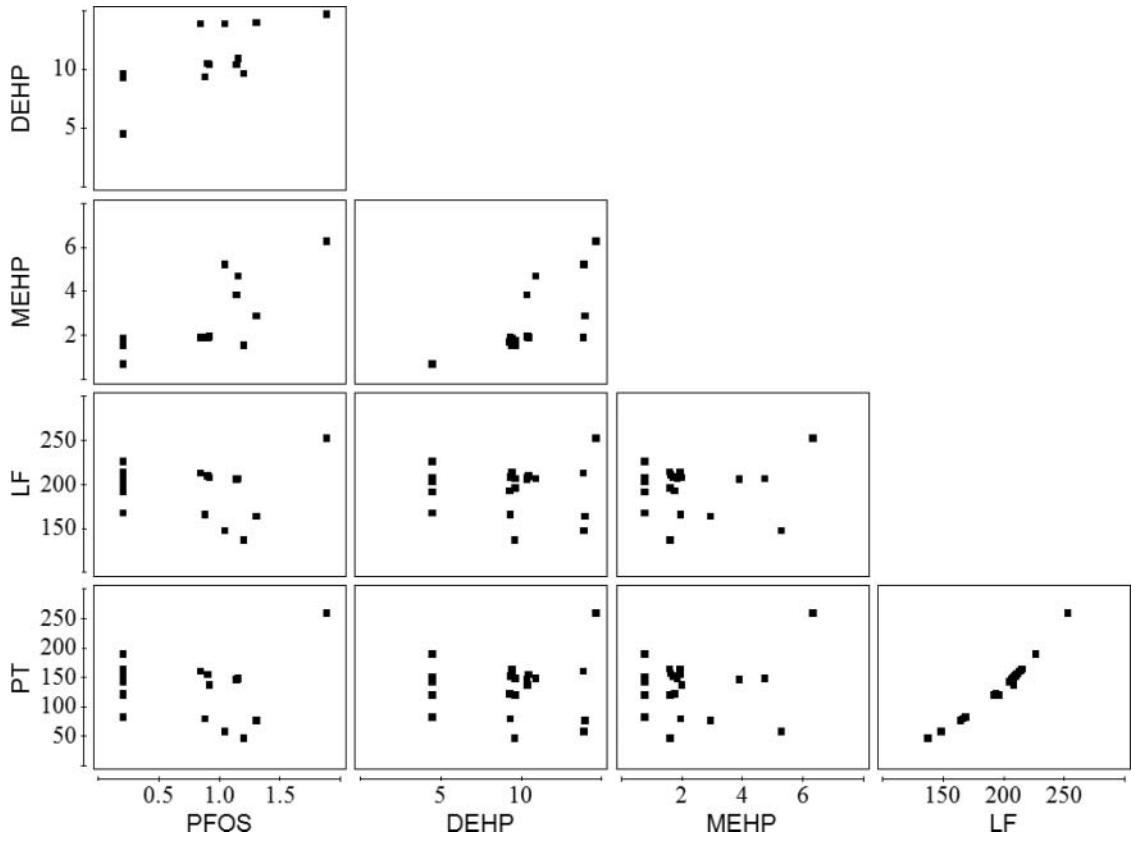

Figure 2. Univariate correlations between couples of variables and fish biometrics. Notes: PT = fish weight, LF = fork length, MHEP = mono-2-ethylhexyl phthalate, PFOS = perfluorooctane sulphonate, DEHP = di-2-ethylhexyl phthalate. Perfluoroctanoic acid (PFOA) resulted always <LOD.

purchased from a supermarket in central Italy: as well as in this study, the PFOA was always < LOD, while the PFOS had an average of 30.2 ± 36.5 ng/g w.w., a median of 19.92 ng/g w.w.[13] Although we do not know the origin and the exact species of tuna fillets purchased at the supermarket and analysed in the study cited, by this comparison the tuna muscle samples from Sardinia analysed in this study are much less contaminated with PFOS also considering the percentage of samples with detectable concentrations (75% in fresh tuna fillet from supermarket and 45% in Atlantic bluefin fillet from Sardinia). Kannan et al. reported levels of the contamination by PFASs in the liver of tuna sampled along the coasts of the Mediterranean (Calabria, Southern Italy); PFOS was present at concentrations between 21 and 87 ng/g w.w., while PFOA was never detected, as in the present study.[9]

Since no data are available about phthalates in tuna, the comparison of results can be done with the only other study on animal species present in the scientific literature.[11] In the mentioned study, just MEHP was detected in stranded fin whale blubber samples (mean 57.97 ng/g), while both DEHP and MEHP were found in superficial neustonic/planktonic samples (mean 20.36 and 53.47 ng/g, respectively).

All samples of Atlantic bluefin tuna muscle with detectable levels of phthalates showed the simultaneous presence of both DEHP and its primary metabolite MEHP, with ratios DEHP/MEHP approximately between 2 and 7, even if without a strong statistical correlation. Considering that in human studies the absence of the original phthalate molecule (DEHP) is considered normal, due to the rapid metabolization to its primary metabolite MEHP it can be assumed that these tuna have had a recent exposure to DEHP, which, thus may not have been completely metabolized.[35] However, this hypothesis requires further study, since the metabolism of phthalates in wild animals, and fish in particular, is much less known than human one. At the same time, the presence of DEHP and MEHP may be due to the ingestion of plastic debris, since previous studies suggest that phthalates, among the principal constituents of plastics are not covalently bound to plastic and migrate from the products to the environment and can serve as a tracer of the intake of microplastics resulting from the ingestion of micro-litter.[10,11,24,25]

Moreover, results presented in this work could be extended to the Atlantic bluefin tuna stock in the Mediterranean. Many studies were conducted in order to investigate the presence of subpopulations of Atlantic bluefin Tuna (e.g., chemical signatures in hard structures, genetic markers) within Mediterranean waters but results remained controversial; however, two subpopulations could be recognized from the previously cited literature: the Western Mediterranean and the Eastern Mediterranean.[36–39] Because of this we could extend our results, at least, to the Western Mediterranean population, confirming the absence of correlation between the contaminants concentration and the capture site in our samples.

All samples were collected during Atlantic bluefin Tuna fishing period, comprised between May and July; fishing activity targeting this species are limited to the pre-spawning and spawning periods when bluefin tuna swim in surface waters after the reproductive migration.[40] Such period, consequently,

Table 3. EDIs (ng), calculated on half-bound averages of contaminants concentrations, for general population and fish consumers. Percents of TDIs with tuna fillet consumption are reported between parenthesis for men and women.

	DEHP EDI		DEHP+MEHP EDI		PFOS EDI		PFOA EDI	
	Man	Woman	Man	Woman	Man	Woman	Man	Woman
General population	355 (0.01%)	355 (0.01%)	437 (0.01%)	437 (0.02%)	23 (0.2%)	23 (0.3%)	8 (0.01%)	8 (0.01%)
Fish consumers	649 (0.02%)	649 (0.02%)	800 (0.02%)	800 (0.03%)	43 (0.4%)	43 (0.6%)	14 (0.01%)	14 (0.02%)

matches the maximum gonadal development observable in Mediterranean specimens of bluefin tuna, as demonstrated by previous studies on gonadosomatic index.[40,41]

Since both phthalates and PFASs show toxicity and prolonged exposure can affect endocrine function and cause neurodevelopmental disorders and infertility we have calculated the estimated daily ingestion of the contaminants of interest through tuna consumption.[42–49] The tolerable daily intake (TDI) established for DEHP is 0.05 mg/kg, a value that was used in this work as a basis of comparison for the intake of DEHP itself and for the total amount of DEHP and MEHP, being the latter a direct metabolite of DEHP.[12]

We calculated the EDI for the general Italian population and for those who claimed to be fish consumers, assuming that their daily fish intake consisted solely of Atlantic bluefin tuna fillets (Table 3).[29] In all cases considered, the estimated ingestion of toxic contaminants through tuna fillet consumption seems very far from posing any risk for humans, as the intakes of PFASs and phthalates (also considered as the sum of DEHP and MEHP) are very low.

Conclusions

The presence of harmful chemicals, such as phthalates, in Atlantic bluefin tuna was demonstrated for the first time by the results of this study, which documented the presence of relevant concentrations of DEHP and MEHP in the muscle of this species. Also PFOS was found, at low levels, while PFOA was never detected, confirming previous studies; although the prevalence of PFOS on PFOA in animal, human and environmental samples is very diffuse, it is interesting to notice that this compound has never been detected in tuna (muscle or liver) and this leads to hypothesize a rapid metabolization of this compound and/or a very limited exposure or assumption.[9,13]

Assuming that the contamination from phthalates may be due to the ingestion of plastic debris, demonstrated in recent studies on tuna, and considering that in this study levels of PFOS and MEHP resulted to be strongly correlated, further studies are to be conducted in order to establish if also no lipophilic compounds, such as PFASs, are carried by micro-debris, which have yet demonstrated to be a significant source of lipophilic chemical.[10,11,25,50] The estimated ingestion of the contaminants considered through tuna fillet consumption resulted very far from posing any risk for humans, confirming the great value of tuna fillet as food. On the other hand, concerning the health of the Atlantic bluefin tuna, particular attention should be paid to the accumulation of contaminants, because evidence of possible reproductive impairment due to the endocrine disrupting activity may be causes of concern for this species, listed in the International Union of Conservation of Nature (IUCN) RED list as "endangered species."[5]

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