FIRST INVESTIGATION OF PER-AND POLY FLUOROALKYLSUBSTANCES (PFAS) IN STRIPED DOLPHIN *STENELLA COERULEOALBA* STRANDED ALONG TUSCANY COAST (NORTH WESTERN MEDITERRANEAN SEA)

Michele Mazzetti[1], Letizia Marsili[3,4], Sara Valsecchi[2], Claudio Roscioli[2], Stefano Polesello[2], Paolo Altemura[1], Alessandro Voliani[1], Cecilia Mancusi[1,3]

1 ARPAT, Via Marradi 114, 5716 Livorno (Italy),
phone +39 055 3206973, e-mail: m.mazzetti@arpat.toscana.it
2 IRSA-CNR, Brugherio MB, Italy
3 University of Siena, Siena, Italy
4 CIRCE, Siena, Italy

Abstract – Per- and poly fluoroalkylsubstances (PFAS) were measured in liver, muscle, blood and brain of 26 striped dolphins *Stenella coeruleoalba* stranded along Tuscany coasts (Italy, North Western Mediterranean Sea) from 2020 to 2022. Dolphins were collected thank to the Regional networking group. Morphometric parameters total length, weight, sex, age were collected when possible and also the condition of the stranded specimens and exact localization of stranding. Using high performance liquid chromatography coupled with high resolution mass spectrometry 18 target PFAS, including perfluoroalkyl-carboxylates (PFCA), -sulfonates (PFSA) and –sulfonamides (FASA), were quantified in each sample. Moreover, a suspect screening on the full scan analysis was carried out for identification of non-target PFAS. PFOS, PFHxS and FOSA were found in all samples with PFOS blood concentration level ranging from some tens to hundreds ppbs. The concentrations follow the trend PFOS>FOSA>PFHxS and the PFOS concentration appears to be inversely proportional to the weight of the animals, as reported in the most recent literature. No significant differences between sexes were registered.

The PFOS/FOSA blood concentration ratio has a threshold at about 20 kg, with two distinct numerical intervals related to the age of the animals (younger or older than 1 yr). PFNA, PFDA and PFUnDA are the PFCA with the highest concentrations in analysed samples, reaching with maximum concentrations of tens of µg/kg.

The presence of these high concern substances in striped dolphins underlines a remarkable impact of anthropic activities on wildlife, and prompts further researches about the impact of PFAS on marine mammal conservation and health.

Introduction

The striped dolphin (*Stenella coeruleoalba*) is the most common cetacean in the Mediterranean Sea and the most representative species of the continental slope of this semi-closed basin. For this reason there is a high frequency of strandings of this species along Italian coasts [14]. Sampling stranded specimens it has been possible to investigate levels of contaminants of different types and to carry out studies to assess health status of Mediterranean striped dolphin [10].

Referee List (DOI 10.36253/fup_referee_list)
FUP Best Practice in Scholarly Publishing (DOI 10.36253/fup_best_practice)

Michele Mazzetti, Letizia Marsili, Sara Valsecchi, Claudio Roscioli, Stefano Polesello, Paolo Altemura, Alessandro Voliani, Cecilia Mancusi, *First investigation of per-and poly fluoroalkylsubstances (PFAS) in striped dolphin* Stenella coeruleoalba *stranded along Tuscany coast (north western Mediterranean Sea),* pp. 729-737 10.36253/979-12-215-0030-1.69

Per- and poly fluoroalkylsubstances (PFAS) are a group of organic molecules synthetically produced and used in a wide range of commercial and industrial applications. Two well-studied groups include the perfluoroalkyl sulfonates (PFSA) and perfluoroalkyl carboxylates (PFCA). Perfluorooctanesulfonic acid (PFOS) is the most known PFAS. Due to its toxicity and bioaccumulability, in May 2009, PFOS and its related compounds were added to the Annex B "Restriction" of the Stockholm Convention on Persistent Organic Pollutants and they were also included in the list of priority hazardous substances which must be monitored in EU water bodies, including transitional and coastal waters, according to EQS Directive (2013/39/EU).

Global distribution of PFAS in waters [12] and in aquatic organisms [7,6], have been documented in many studies, demonstrating their persistence in the environment and their bioaccumulation and biomagnification. According to the scenario reported above, PFAS represent emerging chemicals that are of environmental concern for marine mammals [3]. At the same time, marine mammals share the coastal environment with humans and consume similar food, thus they may also serve as indicators for environmental change and ecosystem health [13]. Moreover, factors controlling the bioaccumulation and tissue distribution of different PFASs are not fully understood. Two mechanisms for PFAS accumulation have been proposed: partitioning to phospholipids and binding to specific proteins. The study of the bioaccumulation in multiple organ tissues characterised by different phospholipid and protein content can help to better understand factors affecting PFAS accumulation and partitioning among tissues [5].

In this study, we investigated the PFAS occurrence in 4 tissues (liver, blood, muscle and brain) of striped dolphins (*Stenella coeruleoalba*, Meyen 1833) of different ages stranded along Tuscany coast between 2020 and 2022.

Materials and Methods

Tissue samples (blood, liver, brain, and muscle) were sampled from stranded striped dolphins along Tuscany coasts during 2020 and 2022.

For each specimen, date, exact localization of stranding, status of conservation of the dolphin and, when possible, total length, weight and sex were recorded. Stranded animals were classified into 5 categories on the degree of post-mortem autolysis: 1. live stranded and subsequently died on the beach; 2. fresh carcass, no external signs of decomposition; 3. early signs of decomposition (skin decolouration, sloughing); 4. strong decomposition; 5. mummification. No animals beyond code 4 were analysed. Tissue sampling was conducted during the pathological examination. If not registered, weight was estimated according to exponential curve specifically created for this species (W=2.1839 exp0.0175 L; R^2=0.95) with data recorded in Tuscany. Age was assigned according to Marsili et al. [10]. Individuals below 16 months and less than 20 kg weight were considered unweaned. All the sample were stored frozen at -20 °C until the chemical analysis.

Samples of striped dolphin's tissue were extracted by QuEChERS protocol, and the extracts were analysed by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS Thermo Fisher Scientific Orbitrap, Waltham, MS, USA).

The whole method used in ARPAT-AVL Laboratory is accredited according to UNI EN ISO 17025 [11] for analysis of PFOS in whole fish. 2 g test portion of frozen tissue was put in a 50 ml glass tube with a glass stopper and spiked with a 100 µl solution of PFAC-ILS at 200 ng/ml. The tubes were allowed to stand overnight at -18 °C. The next day, one ceramic homogenizer was introduced and, subsequently, 10.0 mL of water and 10.0 mL of acetonitrile were added in two separate steps. Then a QuEChERS pouche was added and the tube was stirring again in the same mode and centrifuged at 3500 rpm for 10 minutes. Surnatant was transferred in a polypropylene tube and stored overnight at -20 ºC. The frozen mixture was centrifuged at 10000 rpm for 5 minutes at -10 ºC (SL Plus series Centrifuge, Thermo Fisher, Waltham, MS, USA) and, finally, 1 ml of the surnatant was put in a polypropylene vial suitable for direct injection in LC-MS analysis. 18 target PFAS (perfluorobutanoate PFBA, perfluoropentanoate PFPeA, perfluorohexanoate PFHxA, perfluoroheptanoate PFHpA, perfluorooctanoate PFOA, perfluorononanoate PFNA, perfluorodecanoate PFDA, perfluoroundecanoate PFUnDA, perfluorododecanoate PFDoDA, perfluorotridecanoate PFTrDA, perfluorotetradecanoate PFTeDA, perfluorobutansulfonate PFBS, perfluoro-pentanesulfonate PFPeS, perfluorohexanesulfonate PFHxS, perfluoropentanesulfonate PFHpS, PFOS, perfluorohexanesulfonate PFDS, perfluorooctane sulfonamide FOSA) were quantified.

Data acquisition and analysis was performed by Xcalibur software platform (Thermo Fisher, Waltham, MS, USA). The PFAS compounds were identified by full scan mode (FS) and retention time matching with the calibration standards. A further confirmation was performed by parallel reaction monitoring (PRM). The concentration of each PFAS was determined using the response ratio of the PFAS quantitation ion to that of the relevant labelled. Analytes lacking an analogous labelled standard were quantified using the internal standard with the closest retention time. HRMS raw files were also processed using Compound Discoverer 3.1 (Thermo Scientific, USA) in order to identify the presence of non-target PFAS by comparison with the PFAS lists submitted to the NORMAN Suspect List Exchange Database.

Results & Discussion

Twenty-six striped dolphins (16 females, 9 males and 1 not identified) stranded along Tuscany coasts during the period 2020-2022 were dissected to analyse PFAS content in the different tissues (Tab. 1). Total lengths ranged from 91.5 to 216 cm, registered weight from 9 to 89 kg, and age of specimens from 6 months to 22 years. The stomach content was scrutinized for 23 dolphin specimens in order to classify the unweaned (RT144, 158, 159, 162, 170) because their stomach was completely empty.

PFAS concentrations and profiles in the tissues of stranded striped dolphins are summarized in Figures 2 and 3. Among the 18 target PFAS analysed in the present work and the 2 confirmed suspects, PFBA, PFPeA, PFHxA, PFBS, PFPeS and PFDS were always below detection limits and are excluded by any further discussion, while PFNA, PFDA, PFUnDA, PFHxS, PFOS, perfluorobutane sulfonamide FBSA, perfluorohexane sulfonamide FHxSA and FOSA were found in all samples.

Table 1 – Specimen detail of the stranded striped dolphins analysed in this study; ID= code of the animal, TL=total length cm, W=weight kg, F=female, M=male. *weight estimation.

ID	Year	Stranding site	TL	W	Sex	Age
RT144Sc	2020	Forte dei Marmi (LU)	114	14.8	F	1
RT149Sc	2020	M. del Boccale (LI)	195	70	F	12
RT150Sc	2020	M. del Boccale (LI)	120	25	F	1
RT151Sc	2020	Calambrone (PI)	210	86*	F	18
RT152Sc	2020	Rio Marina (LI)	199	62	F	13
RT154Sc	2020	M.Grosseto (GR)	192	74	F	11
RT156Sc	2020	Capoliveri (LI)	200	84	M	14
RT157Sc	2020	Rosignano (LI)	216	89	M	22
RT158Sc	2020	Porto Azzurro	100	9.5	F	8 months
RT159Sc	2020	Porto Azzurro	91.5	9	F	6 months
RT160Sc	2020	Capoliveri (LI)	191	65.2	F	11
RT161Sc	2020	Piombino (LI)	205	72	F	16
RT162Sc	2020	Camaiore (LU)	103	13	M	9 months
RT164Sc	2020	Campo nell'Elba	197	54	M	13
RT165Sc	2020	Capalbio (GR)	190	60*		10
RT166SC	2021	Lacona (LI)	204	77*	F	15
RT167Sc	2021	Portoferraio (LI)	149	32.2	F	14
RT169Sc	2021	Bibbona (LI)	163	47.6	M	14
RT170Sc	2021	Castag. Carducci (LI)	112	18	M	3
RT171Sc	2021	Pisa (PI)	193	62.3	M	9
RT175Sc	2021	Pisa (PI)	204	67	F	4
RT177Sc	2021	Forte dei Marmi (LU)	200	71.9	F	n/a
RT178Sc	2021	Piombino (LI)	200	68.5	M	n/a
RT181Sc	2021	Capoliveri (LI)	148	29*	M	n/a
RT187Sc	2021	Roccamare (GR)	183	68.3	F	n/a
RT188Sc	2022	Rocchette (GR)	152	30.6	F	n/a

The PFAS profile in liver, blood and muscle showed a similar pattern (Figure 3) and was generally composed of the same six dominant PFAS: PFOS>FOSA>PFNA>PFUnDA>PFTrDA>PFDA. Otherwise, PFTrDA was detected at higher concentrations than PFOS in brain with a different PFAS profile: PFTrDA>PFOS>PFTeDA>PFUnDA>FOSA>PFDoDA. PFCA in blood and muscle tissue showed a clear pattern with higher concentrations of the odd-chained PFCA than either the next and the previous even-chained PFCA supporting the hypothesis that the degradation of atmospheric polyfluorinated precursors is a relevant source for PFCA in the marine environment [8]. Suspect screening analysis allowed identifying 2 novel FBSA and FHxSA) in all the analysed tissues. Only few studies reported these compounds in biota. FBSA has previously been detected in fish tissues [4,1], FBSA and FHxSA in the blood serum of turtles [2] and to our knowledge, this is the first study to report FBSA and FHxSA in cetacean tissues. FOSA was the most abundant perfluorosulfonamide followed by FHxSA and FBSA in all the tissues.

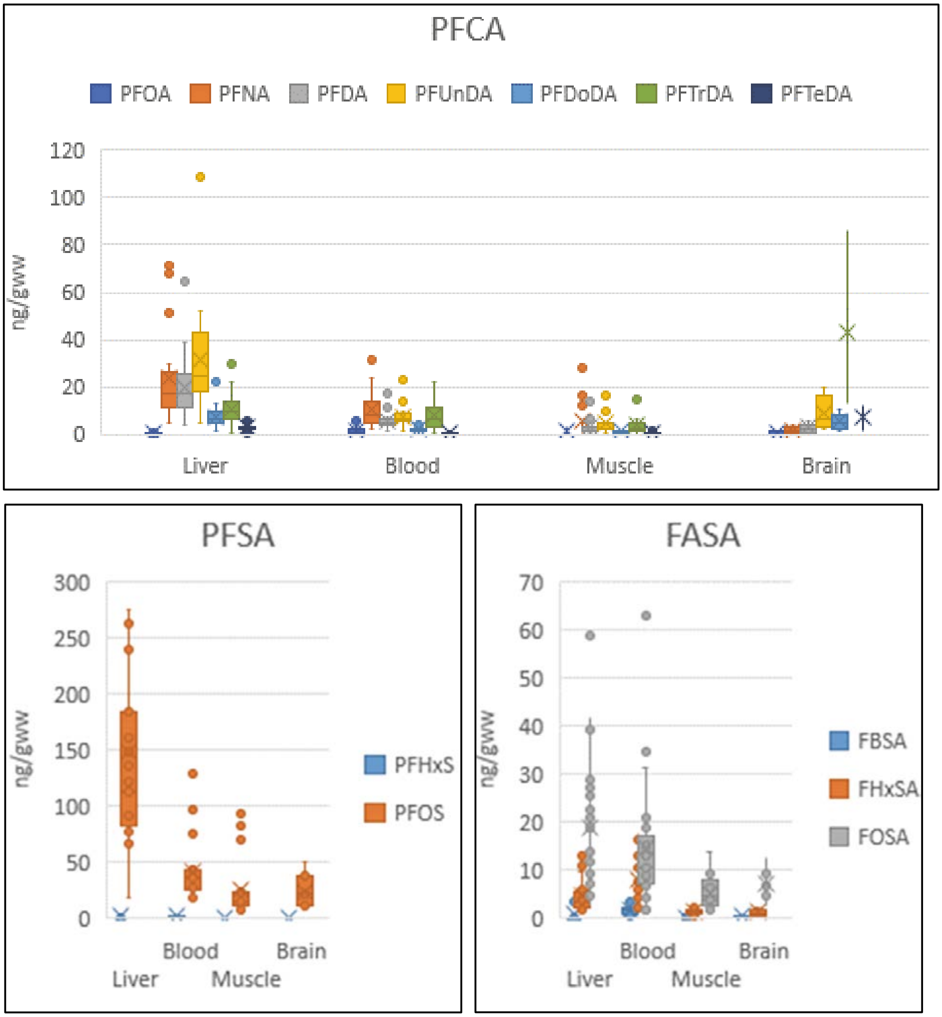

Figure 2 – Concentration of the PFAS detected above LOQ in the tissue of stranded striped dolphins.

ΣFASA concentrations were slightly lower than corresponding-tissue ΣPFCA and ΣPFSA ranging from 1.8 to 77 ng/g wet weight (ww). PFOS concentration level ranged from 4.2 ng/g ww in a muscle sample to 501 ng/g ww in a liver sample. PFTrDA ranged from 13 to 87 ng/g ww in the brain of the analysed striped dolphins.

No differences between sexes for all the investigated compounds and the total PFAS concentrations were evidenced.

Few studies have investigated the contamination by PFAS in cetacean specimens of the Mediterranean Sea. Kannan et al. [8] measured PFOS levels in livers of striped dolphins stranded in North Tyrrhenian Sea and South Adriatic Sea in 1991 and Lopez-Berenguer et al. [9] measured the content of PFCA and PFSA in liver and muscle samples of stranded striped dolphins in the south-eastern coast of Spain during the period 2009-2018. The median of PFOS concentrations measured in liver tissue collected from stranded dead animals along the coasts of Tuscany in this study (37 ng/g ww, N=23) was similar to the level measured in 1991 (22 ng/g ww, N=1) [8] and 3.5 times lower than the median value measured in the western Mediterranean sea in 2009-2018 (118 ng/g ww, N=29) [5]. However, currently a clear geographical comparison is difficult since other PFAS in the liver of the stranded dolphins in the coastline of Spain [9] confirm higher contamination of the Spanish specimens than the Italian ones but the PFAS levels in muscle tissue of the Italian and Spanish striped dolphins were very similar.

Though there are only few data and have to be confirmed with further monitoring data, however we might conclude that no decreasing trend in PFOS or PFOS-precursor occurrence has happened since 1990s, but a difference between the Western and the Eastern basins of the Mediterranean sea in PFAS sources and occurrence is likely present.

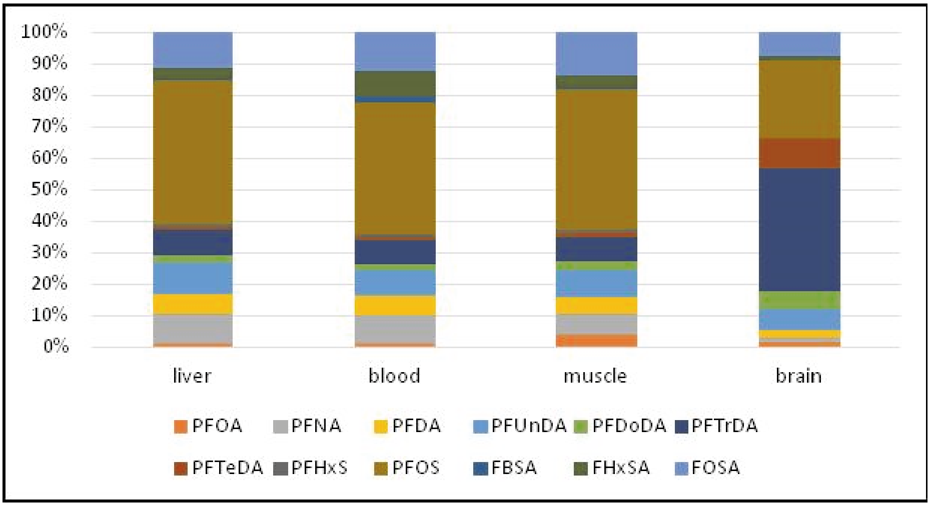

Figure 3 – Percentage distribution pattern of the median concentration of the PFAS detected above LOQ in the tissue of stranded striped dolphins.

The PFSA and PFCA concentrations in calves were significantly higher than in older individuals. This pattern, already detected in other cetacean monitoring study [13], can be explained by multiple processes: the maternal transfer of PFAS from the mother to the offspring or a marked difference in diet composition between the calves and adults. Calves with less than 16 months mainly feed on their mothers' milk [9], so the maternal transfer by lactation can cause a higher PFAS body burden in calves while, as they grow, they feed on various preys of different trophic levels. Furthermore, an increasing ability of metabolism and elimination of PFAS in juvenile and adult dolphins or a grow dilution effect cannot be excluded. Otherwise, no significant differences were found between adults and calves for perfluorosulfonamides but sulfonamides are the only neutral compounds considered in the present study which are known to have different distribution mechanisms in the organism respect to the ionic PFAS.

Unlike most legacy POPs, PFAS do not follow lipophilic pathways. They bind to blood proteins and through the bloodstream they displace to different tissues according to their affinity for the tissue constituents (phospholipids or/and specific proteins) and their capacity to pass the blood/tissue barriers. Both the functional group and carbon-chain length can affect the PFAS distribution. Moreover, structural proteins and storage lipids could also act as significant sorption compartments for neutral or semi-neutral PFAS. The propensity of the various PFAS in distributing in the different tissues can be assessed by calculating tissue/blood ratios (Figure 4).

The muscle-blood ratios (Figure 4, left) were generally below one for most of the dolphin indicating a low affinity of all the PFAS for the muscle tissue. Part of the PFAS detected in muscle can be due to the blood embedded into the muscle fibres. However, there is a weak but significant correlation (y = 0.05x - 0.13; R^2 = 0.81) between the median of the muscle-blood ratios and the number of fluorinated carbon atoms for all the PFAS homologues and groups, except PFOA. This indicates a slightly higher muscle accumulation of the long-chain PFAS than of the short-chain ones, probably due to the affinity of the fluorinated tail with the membrane phospholipid bilayer.

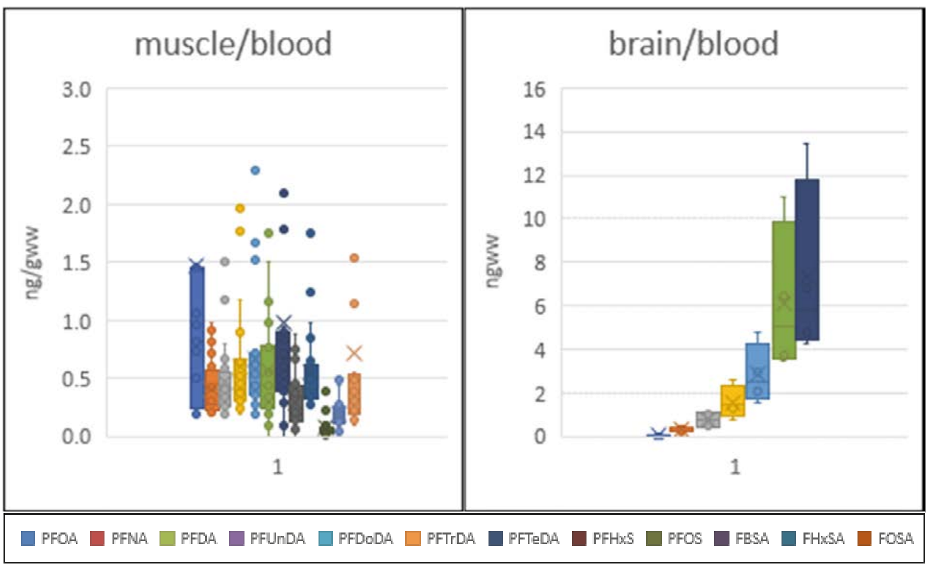

Figure 4 – Muscle-blood and brain-blood ratios of the concentration of PFAS detected above LOQ in the stranded striped dolphins.

The calculated PFAS brain-blood ratios increased with chain length (Figure 4, right), and PFCA (>C9) showed brain-blood ratios higher than one and up to 13 for PFTeDA, the longest congener. This trend is consistent with most of the published data for wildlife species. The long-chain PFAS may cross cerebral barriers more effectively than short-chain PFAS and in addition they have stronger association with phospholipids that can enhance the bioaccumulation in phospholipids rich tissue such as the brain [2].

The liver contains high concentrations of fatty acid-binding protein that may also bind PFAS; moreover, the liver is the primary site of metabolism, and elevated levels of PFCA and PFCA in this tissue may represent a high level of exposure to precursor compounds. Unlike muscle and brain, the liver-blood ratios were extremely variable depending on the specific compound. The liver-blood ratio was always above one for PFCA except for PFOA. PFHxS and FOSA have ratios around the value of one and sulfonamides showed a clear increase of the liver-blood ratio according to the chain length. It has been hypothesized that cetaceans may lack totally or partially the ability of transforming PFAS precursors such as FOSA to PFOS within their tissues because generally they have the FOSA levels of the same order or even higher than those of PFOS [9]. On the contrary, the FOSA/PFOS ratios calculated in the present study were generally around 0.25, indicating lower concentrations of FOSA than PFOS for all tissues and suggesting a biological degradation within the liver followed by a redistribution of the PFOS into the other tissues. An opposite behaviour is instead detected for FHxSA: the FHxSA/PFHxS ratios were above the value of one with median values >4.5 for all the tissue, and FHxSA reached the highest concentrations in blood tissue. This finding suggests for this semi-neutral precursor a slow rate of metabolic transformation, and accumulation into bloodstream of the striped dolphin.

Conclusions

The study of the accumulation of hazardous substances in stranded mammals can help to understand the health status of individuals and their behaviour. It is evident from the present study that longer PFAS accumulate in the different tissues, with a similar distribution pattern in liver, blood and muscle, but different from that found in brain, where the longest perfluoroalkylcarboxylates prevail. The highest concentrations have been determined in liver, where the precursors partially undergo oxidation to perfluoroalkylacids.

The PFSA and PFCA concentrations in calves were significantly higher than in the older individuals. This behaviour is in contrast to what is only due to bioaccumulation and biomagnification through preys but it might be attributed to the maternal transfer of PFAS during the pregnancy period or the following lactation, showing that younger and more vulnerable individuals are subject to a higher pressure by these very persistent compounds.

The presence of these high concern substances in striped dolphins underlines a remarkable impact of anthropic activities on wildlife, and prompts further researches about the impact of PFAS on marine mammal conservation and health.

References

[1] Baygi, S.F., S. Fernando, P.K. Hopke, T.M. Holsen, B.S. Crimmins (2021) - *Nontargeted Discovery of Novel Contaminants in the Great Lakes Region: A Comparison of Fish Fillets and Fish Consumers,* Environ. Sci. Technol. 55, 3765–3774. doi:10.1021/acs.est.0c08507

[2] Beale, D.J., K. Hillyer, S. Nilsson, D., Limpus, U. Bose, J.A. Broadbent, S. Vardy (2022) - *Bioaccumulation and metabolic response of PFAS mixtures in wild-caught freshwater turtles (*Emydura macquarii*) using omics-based ecosurveillance techniques*, Sci. Total. Environ. 806, 151264. doi:10.1016/j.scitotenv.2021.151264

[3] Bossart G. D. (2011) - *Marine Mammals as Sentinel Species for Oceans and Human Health*, Veterinary Pathology 48(3) 676 - 690.

[4] Chu, S., R.J. Letcher, D.J. McGoldrick, S.M. Backus (2016) - *A New Fluorinated Surfactant Contaminant in Biota: Perfluorobutane Sulfonamide in Several Fish Species*, Environ. Sci. Technol. 50, 669-675. doi:10.1021/acs.est.5b05058

[5] Dassuncao, C., H. Pickard, M. Pfohl, A.K. Tokranov, M. Li, B. Mikkelsen, A. Slitt, E.M. Sunderland (2019) - *Phospholipid Levels Predict the Tissue Distribution of Poly- and Perfluoroalkyl Substances in a Marine Mammal*, Environ Sci Technol Lett 6, 119-125. doi:10.1021/acs.estlett.9b0003

[6] Fossi M.C., C. Panti (2018) - *Marine mammal ecotoxicology: Impacts of Multiple Stressors on Population Health*, Chapter 5, Academic Press, Elsevier

[7] Houde M., A.O. De Silva, D.C.G. Muir, R.J. Letcher (2011) - *Monitoring of Perfluorinated Compounds in Aquatic Biota: An Updated Review*, Environ. Sci. Technol. 45, 7962 - 7973.

[8] Kannan, K., S. Corsolini, J. Falandysz, G. Oehme, S. Focardi, J.P. Giesy (2002) - *Perfluorooctanesulfonate and Related Fluorinated Hydrocarbons in Marine Mammals, Fishes, and Birds from Coasts of the Baltic and the Mediterranean Seas*, Environ. Sci. Technol. 36, 3210-3216.

[9] Lopez-Berenguer, G., R. Bossi, I. Eulaers, R. Dietz, J. Penalver, R. Schulz, J. Zubrod, C. Sonne, E. Martinez-Lopez (2020) - *Stranded cetaceans warn of high perfluoroalkyl substance pollution in the western Mediterranean Sea*, Environ Pollut 267, 115367. doi:10.1016/j.envpol.2020.115367

[10] Marsili, L., A. D'Agostino, D. Bucalossi, T. Malatesta, M.C. Fossi (2004) - *Theoretical models to evaluate hazard due to organochlorine compounds (OCs) in Mediterranean striped dolphin* (Stenella coeruleoalba), Chemosphere 56, 791-801. doi:10.1016/j.chemosphere.2004.03.014

[11] Mazzetti M., Agostini A., Altemura P. (2022) - *Notiziario dei Metodi Analitici and IRSA news*. ISSN 2465-017X, 2 aprile 2022, pag 17

[12] Muir D.C.G, L.T. Miaz (2021) - *Spatial and Temporal Trends of Perfluoroalkyl Substances in Global Ocean and Coastal Waters*, Environ. Sci. Technol. 55, 9527 – 9537.

[13] Sciancalepore, G., G. Pietroluongo, C. Centelleghe, M. Milan, M. Bonato, G. Corazzola, S. Mazzariol (2021) - *Evaluation of per- and poly-fluorinated alkyl substances (PFAS) in livers of bottlenose dolphins* (Tursiops truncatus) *found stranded along the northern Adriatic Sea*, Environ Pollut 291, 118186. doi:10.1016/j.envpol.2021.118186

[14] http://mammiferimarini.unipv.it/