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1. INTRODUCTION

Per- and polyfluorinated alkyl substances (PFAS) are a large group of industrial chemicals (Jahnke & Berger, 2009) with oleophobic and hydrophobic properties (Houde et. al, 2005). These substances have an alkyl chain which is partly or fully fluorinated, typically containing between 4 and 18 carbon atoms (Jahnke & Berger, 2009). PFASs are employed in a wide spectrum of industrial and commercial applications due to their unique properties provided by the extreme strength of C-F bonds and their surfactant nature (Gredelj et al 2020). The production of PFASs started since the late 1940s (Jahnke & Berger, 2009) and from then on, a broad range of these substances have been detected in the environment, wildlife, and humans (Buck et al., 2011).

In the environment, PFAS are expected to undergo some transformations to partly form PFSAs (Perfluoroalkane sulfonates) or PFCAs (Perfluoroalkyl carboxylates) (Jahnke & Berger, 2009). PFCAs include Perfluorobutanoate PFBA, Perfluoropentanoate PFPeA, Perfluorohexanoate PFHxA, Perfluoroheptanoate PFHpA, Perfluorooctanoate PFOA, Perfluorononanoate PFNA, Perfluorodecanoate PFDA, Perfluoroundecanoate PFUnA, Perfluorododecanoate PFDoA, Perfluorotridecanoic acid PFTrDA, Perfluorotetradecanoic acid PFTeDA, while PFSAs include Perfluorobutane sulfonate PFBS, Perfluorohexane sulfonate PFHxS, Perfluorooctane sulfonate PFOS, Perfluorodecane sulfonate PFDS, Perfluoroheptane sulfonic acid PFHpS, Perfluoropentane sulfonic acid PFPeS (Jahnke & Berger, 2009; Spaan et al., 2020).

Studies on laboratory animals revealed the persistence and the toxic effect of some PFAS, leading international regulatory agencies to ban the production or the import of some compounds (Houde et. al, 2005). In addition, many compounds result to be bioaccumulative (Jahnke & Berger, 2009). Studies revealed that the bioaccumulation potential of PFASs is strongly correlated with perfluoroalkyl chain length (Spaan et al., 2020). Due to their environmental behaviour, the industry shifted towards shorter chain PFASs, believed to bioaccumulate and biomagnify less, although their persistence in the environment (Lopez-Berenguer et al., 2020).

Dolphins are apex predators of the marine food chain because of their higher trophic level and their ability to deeply influence the ecosystems (Wallach et al., 2015). Top-level predators concentrate contaminants through bioaccumulation (Wells et al., 2004) and are particularly susceptible to exposure to persistent and bioaccumulative substances, such as PFASs (Spaan et al., 2020).

Many studies investigate PFASs in liver, since in this organ they can concentrate (Dassuncao et al., 2017). In fact, since PFASs have a binding affinity to proteins and serum albumin, liver can be the target organ for accumulation and risk assessment of PFASs (Lam et al., 2016).

The Adriatic Sea lies in the northernmost part of the Mediterranean Sea with a unique connection with the Mediterranean Sea trough the Otranto Strait (Lipizer et al., 2014). Some authors believe that the inflow of Atlantic water to the Western Mediterranean basin and river discharges are probably the dominant PFAS inputs to the Mediterranean basin (Brumovský et al., 2016). Nevertheless, local input must be considered: in 2013 for instance, a large-scale contamination of PFAS was discovered in the Veneto region, Northern Italy, as a consequence of the emissions of a fluorochemical plant in the province of Vicenza (Bonato et al. 2020). The contamination plume extends over an area of 190 km^2 reaching the provinces of Vicenza, Verona, and Padua (Canova et al., 2020).

Our results will fill an important gap in the knowledge of PFAS in bottlenose dolphins, *Tursiops truncatus*, in the Northern Adriatic Sea.

2. MATERIAL AND METHODS

2.1 Sample collection

Our sample consisted of 20 bottlenose dolphins all found dead along the Northern Adriatic Sea between 2008 and 2020. Samples were archived at the Mediterranean Marine Mammal Tissue Bank of the University of Padova.

The heterogeneity of the sample allowed to assess the variability in PFAS concentrations related to body length and sex. In fact, samples belonged to 4 females (1 calf, 2 juveniles, 1 adult) and 16 males (3 calves, 9 juveniles, 4 adults). Following Benvegnù et al., (2013) adults were defined by lengths >290 cm for males and >270 cm for females, juveniles by lengths between 200-290 cm for males and 200-270 cm for females, calves by lengths under 200 cm.

2.2 Extraction and analysis

The 20 liver samples of bottlenose dolphins were frozen and stored at −20 °C until chemical analysis. The samples were then analysed at the laboratory Mérieux NutriSciences Italia, Rag. Soc. Chelab S.r.l.. Seventeen PFASs including PFCAs and PFSAs were quantified: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, LPFBS, LPFHxS, LPFOS, PFUnA, PFDoA, LPFPeS, LPFHpS, LPFDS, PFTrDA, PFTeDA.

2.3 Statistical analysis

Concentrations (mean, standard deviation) of PFASs in the liver samples were statistically analysed by the statistical software package IBM SPSS Statistics 21. The normality in the distribution of concentrations of PFASs was evaluated by the Kolmogorov-Smirnov test. Once it was verified that the data were normally distributed ($p < 0.05$), the parametric T-student for independent samples and the ANOVA tests were used to determine whether concentration of PFASs found in the liver of bottlenose dolphins found dead along the cost of the Adriatic Sea varied in relation to the body length and sex. For the comparative statistical analysis 2 factors were chosen: sex (2 categories: males ($n = 16$) and females ($n = 4$)), body length (3 categories following Benvegnù et al., (2013): male adults defined by lengths >290 cm and >270 cm for females, juveniles by lengths between 200-290 cm for males and 200-270 cm for females, calves by lengths under 200 cm).

3. RESULTS & DISCUSSION

3.1 Characterization of PFASs in the liver of bottlenose dolphins

Of the 17 target PFASs analysed in this work, all of them were quantifiable in more than one sample. PFOS was the most predominant PFASs in the tissues analysed. The greatest PFOS concentration found in the liver of a bottlenose dolphin was 629,73 ng/g, wet weight. **Table 1** shows the descriptive statistics of the 17 PFASs selected for the analyses. Data were normally distributed (Kolmogorov-Smirnov test) and therefore parametric statistical analysis were performed.

Table 1: Hepatic PFAS concentrations (ng g^{-1} ww) in bottlenose dolphins at Northern Adriatic Sea during 2008-2020.

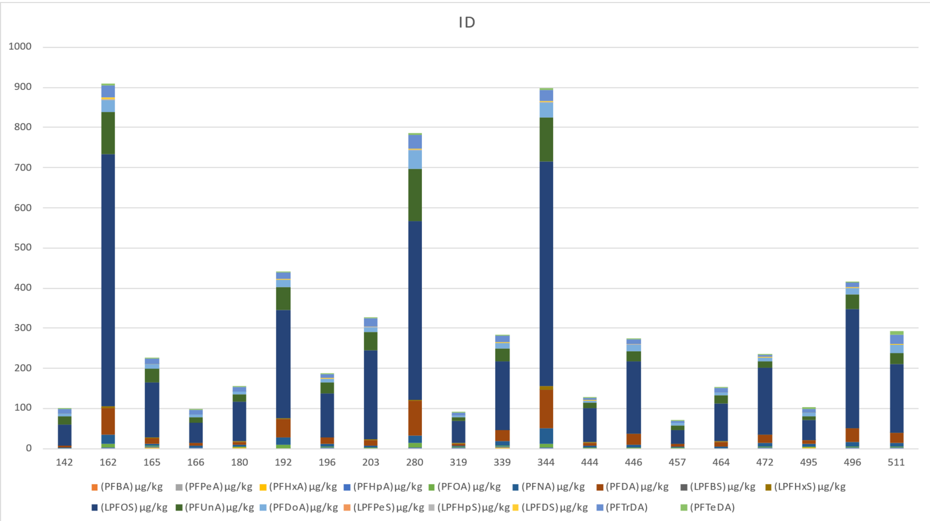
	N	Minimum	Maximum	Mean	Std. Deviation
PFBA	4	0,058	0,082	0,06800	0,010863
PFPeA	20	0,054	0,600	0,27885	0,184063
PFHxA	20	0,138	1,750	0,61150	0,402202
PFHpA	20	0,052	1,940	1,03895	0,629587
PFOA	20	0,470	10,750	3,39000	3,399797
PFNA	20	0,990	38,070	9,96240	8,691937
PFDA	20	4,240	96,430	26,33850	27,271877
LPFBS	6	0,000	0,133	0,08117	0,051890
LPFHxS	20	0,235	7,330	1,44865	1,573246
LPFOS	20	34,250	629,730	194,06100	170,605338

PFUnA	20	10,300	130,050	38,18200	35,578992
PFDoA	20	3,060	47,080	14,21100	11,257832
LPFPeS	3	0,000	0,122	0,06133	0,061003
LPFHpS	20	0,118	3,300	0,74385	0,835088
LPFDS	20	0,297	3,670	1,12875	0,883373
PFTrDA	20	4,980	34,500	14,45400	8,479360
PFTeDA	20	0,810	7,850	2,45850	1,675207

3.2 PFAS profiles

Figure 1 shows PFAS profiles in each specimen. The PFASs profile in liver of the 20 bottlenose dolphins was generally composed of this same five dominant PFASs: PFOS>PFUnA>PFDA≈PFDoA≈PFTrDA.

Figure 1: Numbers indicate the ID of the animal at the Marine Mammal Tissue Bank of the University of Padova.



As apex predators, cetaceans are particularly susceptible to PFAS (Spaan et al., 2020). In our study, PFOS still represents the major fraction of the PFAS profile, although the production was phased out almost two decades ago (Lopez-Berenguer et al., 2020). PFOS accounted until 71% in the PFAS profile in our sample. In addition, also PFUnA, PFDA, PFDoA and PFTrDA are present as well. PFUnA was the dominant PFCA and the second most abundant PFAS (after PFOS) in liver (mean value 38.182ng/g ww). PFOS, PFUnA, PFDA, PFDoA and PFTrDA are all long chain PFASs and although industries shifted toward shorter chain PFASs (Lopez-Berenguer et al., 2020) and some longer chain PFAS are no longer used in industrial activities, they have bioaccumulative properties and persist in the environment (Bonato et al. 2020). Therefore, this pattern in bottlenose dolphins of Northern Adriatic Sea might be explained by a contamination related to the past. In fact, these findings might be the result of a slow turnover of PFOS and other PFASs in the semi-enclosed Mediterranean Sea basin with concentration of PFASs in cetacean tissues revealing historical releases (Lopez-Berenguer et al., 2020) and longer time scales of removal through sea transport (Dassuncao et al., 2017). The slow response of PFOS to its phase out suggests that the decline in long-chained PFCAs may last decades, also depending on factors such as age and size (Dassuncao et al., 2017).

3.3 Factor analysis

Firstly, it was investigated the effect of the factors sex and body length on the total PFAS concentration and on the 17 PFASs: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, LPFBS, LPFHxS, LPFOS, PFUnA, PFDoA, LPFPeS, LPFHpS, LPFDS, PFTrDA, PFTeDA.

3.3.1 Sex

No significant differences between sexes were found. In fact, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, LPFBS, LPFHxS, LPFOS, PFUnA, PFDoA, LPFPeS, LPFHpS, LPFDS, PFTrDA, PFTeDA and the total PFAS concentration did not differ between the two sexes. This result is not in contrast with previous findings (Lopez-Berenguer et al., 2020).

It is reported that females at reproduction need more proteins for milk production, therefore intensified mobilization of proteins may explain the increase in liver of PFASs, which are compounds bound with high affinity to proteins (Gui et al., 2019). On the other side, lactation may represent an offloading PFAS burden (Gui et al., 2019) possibly resulting in a reduction of PFAS in mature females. The absence of such differences between females and males in our sample might be explained by the fact that only one of the four females of our sample were considered adults according to our classification, therefore they might not have been sexually mature or they might not have given birth to any calves, thus not producing milk.

3.3.2 Body length

Since age was not possible to determine, we divided all our samples into three categories based on their body lengths. PFASs' variation was tested in relation to the body length by ANOVA test followed by a Bonferroni post-hoc. Body length had a significant effect on 11 PFASs (PFOA, PFNA, PFDA, LPFBS, LPFHxS, L-PFOS, PFUnA, PFDoA, LPFHpS, LPFDS, PFTrDA) and on the total PFAS. Significant differences for these PFAS were found between adults and calves and juveniles and calves but not for adults and juveniles. The inclusion of mostly juveniles and adults in the sample set with relative low variation in length could have influenced the absence of link between length and other PFASs concentrations (Lopez-Berenguer et al., 2020).

Differences on PFASs between calves and adults may be explained by the maternal transfer effect and therefore by differences in diet composition (Gui et al., 2019). Calves of bottlenose dolphins mainly rely on mothers' milk for almost 2 years of their lives: age that generally have at weaning (Mazzariol et al., 2015). As they grow, they feed on various preys (Bearzi et al., 2008). As expected, body length had a significant and negative effect with calves showing higher mean values than adults, thus indicating an increasing ability with age of metabolism and elimination of PFASs in dolphins (Gui et al., 2019).

Since the three categories adults (n= 6), juveniles (n=11) and calves (n=3) were not equally distributed, ANOVA test was also performed picking randomly 6 juveniles, in order to have 6 adults, 6 juveniles and 3 calves; again, body length had a significant effect on 11 PFASs (PFOA, PFNA, PFDA, LPFBS, L-PFHxS, LPFOS, PFUnA, PFDoA, LPFHpS, LPFDS, PFTrDA) and on the total PFAS. Significant differences for these PFAS were found between adults and calves and juveniles and calves but not for adults and juveniles.

3.3.3 Temporal Trends of PFASs

Secondly, a temporal analysis was done and three periods of time were considered: 2008-2010 (n=6), 2011-2015 (n=6), 2016-2020 (n=8). No temporal differences in PFASs concentrations were found for any compounds. Following Lam et al., (2016) only animals with greater body lengths were selected for temporal trend analysis, in order to minimize the possible age-related differences. In addition, male and female adults were pooled together for the analysis since no significant differences between sexes were found. Then, only PFAS in juveniles and adults were tested in the 3 different time periods and no temporal differences in PFASs concentrations were found for any compounds. As previously mentioned, the slow response of some compounds to their phase out suggests that the decline in PFAS concentrations may last decades (Dassuncao et al., 2017).

3.4 Comparison with other areas

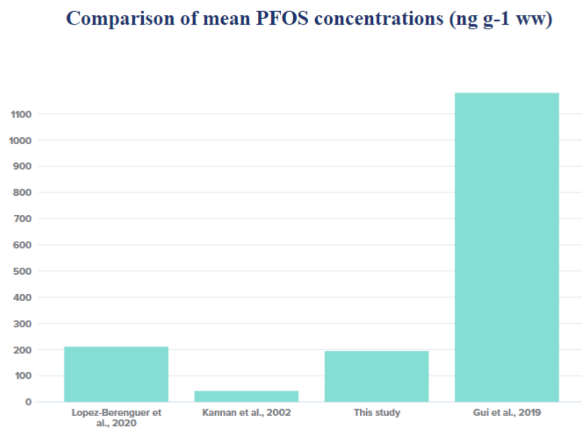
Finally, as shown in **Table 2** and **Figure 2**, our data were compared with data collected in livers of bottlenose dolphins in the Mediterranean Sea (Kannan et al., 2002; Lopez-Berenguer et al., 2020) and with another dolphin species, the Indo-Pacific humpback dolphin, *Sousa chinensis*, from another geographical area, the Pearl River Estuary, China (Gui et al., 2019).

Considering the mean values of PFOS, the most abundant compound in our dataset (n=20), our data resulted to be similar to the mean value of PFOS collected in livers of bottlenose dolphins stranded in the south-eastern coast of Spain (n=5) during 2009-2018 (Lopez-Berenguer et al., 2020). On the other side, our data were greater compared to the value of PFOS in liver reported in a single specimen (n=1) collected in North Adriatic Sea (Croatia) in 1992 (Kannan et al., 2002). These results, although not exhaustive because of the limited sample, might indicate that PFOS is widespread both in western Mediterranean Sea and Adriatic Sea. In addition, differences between our data and the bottlenose dolphin sampled in 1992 (Kannan et al., 2002) may indicate a raise in PFOS contamination in the last 30 years, confirming the persistence of PFOS in the environment.

Gui et al., 2019, reported mean PFOS concentrations in the Indo-Pacific humpback dolphin (n=52) from the Pearl River Estuary and Zhanjiang waters, China, of 1180 ng g^{-1} ww, extremely greater than those collected for bottlenose dolphins in our study. This data might offer an insight on the possible levels of PFAS contamination that can be detected worldwide.

Table 2: Comparison of mean PFOS concentrations (ng g^{-1} ww) in livers of bottlenose dolphins from North Adriatic Sea, south-eastern coast of Spain, Croatia and in livers of Indo-Pacific humpback dolphins from the Pearl River Estuary and Zhanjiang waters, China.

Locations	Species	n	Mean PFOS	Reference
North Adriatic Sea	Bottlenose dolphin	20	194,06	This study
Western Mediterranean Sea	Bottlenose dolphin	5	211.0	Lopez-Berenguer et al., 2020
North Adriatic Sea, Croatia	Bottlenose dolphin	1	42.5	Kannan et al., 2002
Pearl River Estuary and Zhanjiang waters, China	Indo-Pacific humpback dolphins	52	1180	Gui et al., 2019



CONCLUSION

The results of this study suggest that PFAS, especially long-chain, are widely distributed in bottlenose dolphins along the northern Adriatic part of the Mediterranean Sea. In addition, PFOS concentrations in liver of bottlenose dolphins of Northern Adriatic Sea resulted to be similar to those registered in specimens collected in dolphins in western Mediterranean Sea. Our findings can be useful for the understanding of PFASs exposure risk and their emission in the study area.

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