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Evaluation of per- and poly-fluorinated alkyl substances (PFAS) in livers of bottlenose dolphins (*Tursiops truncatus)* found stranded along the northern Adriatic Sea.☆

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ABSTRACT

Per-and poly-fluorinated alkyl substances (PFAS) are a group of chemicals used in a wide variety of commercial products and industrial applications. These chemicals are persistent, can accumulate in humans' and animals' tissues and in the environment, representing an increasing concern due to their moderate to highly toxicity. Their global distribution, persistence and toxicity led to an urgent need to investigate bioaccumulation also in marine species. In 2013 PFAS contamination was detected in a vast area in Veneto region, mainly in Adige and Brenta rivers. In order to investigate any relevant presence of these substances in marine vertebrates constantly living in the area, PFAS were measured in hepatic tissue samples of 20 bottlenose dolphins (*Tursiops truncatus*) stranded along the northern Adriatic Sea coastline between 2008 and 2020. Using high performance liquid chromatography-mass spectrometry, 17 target PFAS (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrDA, PFTeDA, PFBS, PFHxS, PFOS, PFDS, PFHpS, PFPeS), were quantified in the samples. PFAS profiles were generally composed of the same five dominant PFAS (PFOS > PFUnA > PFDA ≈ PFDoA ≈ PFTrDA). The greatest PFOS concentration found was 629,73 ng/g wet weight, and PFOS accounted until 71% in the PFAS profiles. No significant differences between sexes were found, while calves showing higher mean values than adults, possibly indicating an increasing ability in the elimination of PFAS with age. Finally, a temporal analysis was carried out considering three different periods of time, but no temporal differences in concentrations were found. The results suggest that long-chain PFAS are widespread in bottlenose dolphins along the North Adriatic Sea. Furthermore, they represent a baseline to investigate the impact of PFAS on marine mammals' conservation and health. Filling an important gap in the knowledge of PFAS accumulation in bottlenose dolphins, this study highlights the relevant role of Environmental and Tissue Banks for retrospective analyses on emergent contaminants.

1. Introduction

Per- and polyfluorinated alkyl substances (PFAS) are a large group of industrial chemicals (Jahnke and Berger, 2009) characterised by a linear or branched carbon chain (Ferrario et al., 2021) with an alkyl chain which is partly or fully fluorinated, typically containing between 4 and 18 carbon atoms (Jahnke and Berger, 2009). PFAS are employed in industrial and commercial applications, such as fire-fighting foams, non-stick coatings and cosmetics, due to their unique properties provided by the extreme strength of C–F bonds and their surfactant nature (Gredelj et al., 2020).

Studies on humans and animals revealed the persistence and the toxic effect of some PFAS, leading international regulatory agencies in the European Union to ban or regulate the production or the import of some compounds, with the declaration of some PFAS as 'candidate persistent organic pollutants (POPs)' (Houde et al., 2005; EFSA, 2018). The Stockholm Convention listed some PFAS such as perfluorooctanoate (PFOA) and its salts under Annex A, with parties committed to take

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measures to eliminate its production and use; perfluorooctane sulfonic acid (PFOS) and its salts under Annex B, resulting in the restriction of its production and use (EFSA, 2018). Other chemicals such as perfluorohexane sulfonate (PFHxS) are under review by the POPs Review Committee, while for the other compounds no restrictions are established (UNEP). Many PFAS result to be bioaccumulative (Jahnke and Berger, 2009) with bioaccumulation increasing with the length of the carbon chain (Conder et al., 2008; Buck et al., 2011; Spaan et al., 2020). Due to this property, the industry shifted towards shorter chain PFAS, which, despite their lower bioaccumulation and biomagnification compared to long chain PFAS, are anyway persistent in the environment (Lopez-Berenguer et al., 2020). Although some PFAS, such as PFOS, are banned in some countries, they are still manufactured in other areas and might continue to be imported and used in consumer goods such as textiles, packaging, coatings and plastics (Fair and Houde, 2018). In addition, shifts in PFAS released in the environment may lead to changes in overall composition of these compounds, but not necessarily to a decrease in concentrations (Dassuncao et al., 2017). Due to their environmental behaviour, PFAS may constitute a threat to wildlife at high trophic levels (Galatius et al., 2013) and are expected to pose a serious threat to humans and the environment in which they live for many years to come (Bonato et al., 2020). However, knowledge about their toxic effects on wildlife is still scarce (Lam et al., 2016).

Some PFAS are absorbed in the gastrointestinal tract of mammals and distribute predominantly to the plasma and liver (EFSA, 2018). Since PFAS have a binding affinity to proteins and serum albumin, liver can be a target organ for accumulation and risk assessment of PFAS (Lam et al., 2016), as in the case of this study. The exposure to some PFAS can lead to an increase in reactive oxygen species and therefore to cellular oxidative stress (Bonato et al., 2020). In addition, in mammals PFAS are suspected carcinogens and obesogens (Bonato et al., 2020). Although many studies refer to marine environment, they may also suggest potential pathway for human exposures, in relation to atmospheric PFAS precursors or exposure to contaminants with marine food consumption (Dassuncao et al., 2017). Some PFAS might accumulate in human body and their exposure can lead to neurobiological, neuroendocrine and neurobehavioral responses (Piekarski et al., 2020). Exposure to PFAS may also have an influence on lipid metabolism during pregnancy (Dalla Zuanna et al., 2021), and serum PFAS concentrations might be associated with an increased risk of cardiovascular diseases (De Toni et al., 2020; Pitter et al., 2020). While some long-chain PFAS have been phased out in many industries, the less-regulated short chain chemicals, due to their smaller size, might cross more readily the blood brain barrier with consequences on brain-related health (Piekarski et al., 2020).

In marine mammals, high levels of PFAS have been reported in many species, with interspecific differences in the metabolic capacity to transform and eliminate PFAS (Fair and Houde, 2018). Habitat use, diet habits, and migration patterns can also contribute to explain the accumulation profile of PFAS in different species (Fair and Houde, 2018). Marine mammal species feeding closer to the shore might have higher burdens of PFAS because of the proximity to contamination sources (Galatius et al., 2013). Other differences may be related to prey preference resulting in a different specific intake of PFAS (Galatius et al., 2013). For instance, the diet of bottlenose dolphins (*Tursiops truncatus*) has been described as opportunistic (Bearzi et al., 2008a) with some degree of specialization in different foraging techniques (Bonizzoni et al., 2020). Studies in the Mediterranean Sea showed that bottlenose dolphins may feed on different preys, both demersal and pelagic (Miokovic et al., 1999; Blanco et al., 2001; Bearzi et al., 2008a; Bearzi et al., 2008b), and it has also been recorded that in some area they can modify their behaviour taking advantage of human activities such as fishing (Bearzi et al., 2019). Movement patterns of bottlenose dolphins in some area of the Mediterranean Sea suggest different levels of site fidelity with a high degree of site fidelity of some individuals (López, 2019). Dolphins, being apex predators (Wallach et al., 2015), can accumulate high concentrations of POPs (Lam et al., 2016), and they are particularly susceptible to exposure to persistent and bioaccumulative substances, such as PFAS (Spaan et al., 2020). Some authors reported that many PFAS can biomagnify in food chain (Martin et al., 2004; Kelly et al., 2009) and in bottlenose dolphin food web (Houde et al., 2006) with potential impacts in some cetaceans (Kelly et al., 2009).

However, to date, only few studies have investigated PFAS in cetaceans inhabiting the Mediterranean Sea. Among them, Kannan et al. (2002), detected widespread occurrence of some PFAS in bottlenose dolphins, common dolphins (*Delphinus delphi*), striped dolphins (*Stenella coeruleoalba*), long-finned pilot whales (*Globicephala melas*) and fin whales (*Balenoptera physalus*) from the Italian coast of the Mediterranean Sea. The Adriatic Sea lies in the northernmost part of the Mediterranean Sea with a unique connection to it through the Otranto Strait (Lipizer et al., 2014). The inflow of Atlantic waters to the Western Mediterranean basin and river discharges are probably the dominant PFAS inputs to the Mediterranean basin (Brumovský et al., 2016), but also local inputs must be considered: for instance, in 2013 a large-scale contamination of perfluoroalkyl acids (PFAAs) was discovered in the Veneto region, facing the Northern Adriatic Sea, as a consequence of the emissions of a fluorochemical plant in the province of Vicenza (Bonato et al., 2020; Canova et al., 2020).

The Veneto Region, with Resolution of the Regional Council n. 1591/2017, has launched the objective "ZERO PFAS" aimed at achieving the 'virtual absence' of PFAS in the drinking water supply chain (ARPAV, 2019). Since some PFAS can spread to groundwater, a survey on these PFAS was carried out in the period 2016–2017 in transitional and marine coastal environments of Veneto: the PFAS values detected were lower than the limit of quantification (LOQ), except for one sample of marine sediment whose contamination was related to ship traffic and not of fluvial origin (ARPAV, 2019). In northern Adriatic Sea, bottlenose dolphins show a relative high degree of site fidelity (Genov et al., 2016) and therefore PFAS values in livers of bottlenose dolphins might be linked to some source of contamination in the area in which they live, similarly to the high PFAS serum levels found in people exposed to contaminated drinking water in Veneto region (Ingelido et al., 2018). Further studies should be done in order to assess the main source of contamination also for bottlenose dolphins.

This study, representing the first along the Italian coastline of the northern Adriatic Sea, aspires to fill an important gap in the knowledge of the presence of PFAS in bottlenose dolphins in this region of the Mediterranean Sea. We hypothesize that levels of PFAS in the study area might be reflected in stranded bottlenose dolphins, confirming their roles as sentinels. These data may be useful for regulatory agencies, and could open a new scenario for research in cetaceans' conservation and PFAS impact mitigation underlining the important role of Environmental and Tissue Banks for retrospective analyses on emergent contaminants.

2. Materials and methods

2.1. Sample collection

A total of 20 bottlenose dolphins were selected for this study. A full list of samples, including information on the ID of the animals, stranding year, sex, total length, stranding site and decomposition condition code (DCC) of the carcass are provided in Table 1. All the animals were found dead along the Italian coastline of the northern Adriatic Sea between 2008 and 2020 (Fig. 1). According to standard protocols (Geraci and Lounsbury, 2005; IJsseldijk et al., 2019), necropsies were performed for all individuals in order to investigate the causes of death and obtain information about morphometric measurement, DCC and sex. The target tissue selected was the liver, and the samples were taken from the Mediterranean Marine Mammal Tissue Bank of the University of Padova, Italy, opting for individuals with DCC between 1 and 4 (DCC 1: just died; 2: fresh carcass; 3: moderate decomposition; 4: advanced decomposition; 5: mummified or skeletal remains, without liver

Table 1
Data from the 20 bottlenose dolphins collected in 2008–2020, including the ID of the animals, stranding year, sex, total length, stranding site and decomposition condition code (DCC) of the carcass.

ID	STRANDING YEAR	SEX	TOTAL LENGHT	STRANDING SITE	DCC
142	2008	F	276	Jesolo (VE)	2/3
162	2009	M	119	Comacchio (FE)	2
165	2009	M	280	Lido Adriano (RA)	2
166	2009	M	260	Pellestrina (VE)	4
180	2010	M	209	Pellestrina (VE)	3/4
192	2010	F	240	Rosolina (RO)	2
196	2011	M	300	Cervia (RA)	2
203	2011	M	284	Rimini (RI)	2
280	2013	F	178	Pellestrina (VE)	2
319	2014	M	310	Goro (FE)	2/3
339	2014	M	280	Porto Garibaldi (FE)	3
344	2015	M	195	Muggia (TS)	2
444	2018	M	274	Pellestrina (VE)	3
446	2018	M	330	Pellestrina (VE)	4
457	2019	M	285	Bibione (VE)	2
464	2019	M	254	Pellestrina (VE)	3
472	2019	M	286	Pellestrina (VE)	3
495	2020	M	303	Grado (GO)	4
496	2020	F	240	Portotolle (RO)	3
511	2020	M	300	Grado (GO)	4

integrity).

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. (unpublished data), adults were defined by lengths >290 cm for males and >270 cm for females, juveniles by lengths between 200 and 290 cm for males and 200–270 cm for females, calves (in which we included also newborns) 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.. A total of 17 PFAS were quantified: Perfluorobutanoate (PFBA, $C_4HF_7O_2$), Perfluoropentanoate (PFPeA, $C_5HF_9O_2$), Perfluorohexanoate (PFHxA, $C_6HF_{11}O_2$), Perfluoroheptanoate (PFHpA, $C_7HF_{13}O_2$), Perfluorooctanoate (PFOA, $C_8HF_{15}O_2$), Perfluorononanoate (PFNA, $C_9HF_{17}O_2$), Perfluorodecanoate (PFDA, $C_{10}HF_{19}O_2$), Perfluoroundecanoate (PFUnA, $C_{11}HF_{21}O_2$), Perfluorododecanoate (PFDoA, $C_{12}HF_{23}O_2$), Perfluorotridecanoic acid (PFTrDA, $C_{13}HF_{25}O_2$), Perfluorotetradecanoic acid (PFTeDA, $C_{14}HF_{27}O_2$), Perfluorobutane sulfonate (PFBS, $C_4HF_9O_3S$), Perfluorohexane sulfonate (PFHxS, $C_6HF_{13}O_3S$), Perfluorooctane sulfonate (PFOS, $C_8HF_{17}O_3S$), Perfluorodecane sulfonate (PFDS, $C_{10}HF_{21}O_3S$), Perfluoroheptane sulfonic acid (PFHpS, $C_7HF_{15}O_3S$), Perfluoropentane sulfonic acid (PFPeS, $C_5F_{11}O_3S$).

For PFAS analysis, the sample was homogenized and fortified with isotopically labelled surrogates prior to the addition of water. Two grams of sample (wet weight) were weighed into a 50 mL plastic tube (Falcon), acetonitrile was used as solvent to extract PFAS from the samples and a modified QuEChERS extraction technique was performed (Ragan Norli et al., 2011).

The resulting extract was filtered and fortified with internal standard solution and analysed using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Filtration was done through cartridges capable of retaining PFAS and eliminating interferences: the sample was loaded into the cartridge and afterwards the analytes were eluted with methanol and 0.1% ammonium hydroxide.

The PFAS compounds were identified by multiple reaction mode (MRM) transitions and retention time matching with the calibration standards. Ion ratios, when available, were used to confirm the identity. The concentration of each PFAS was determined using the response ratio of the PFAS quantitation transition to that of the relevant labelled

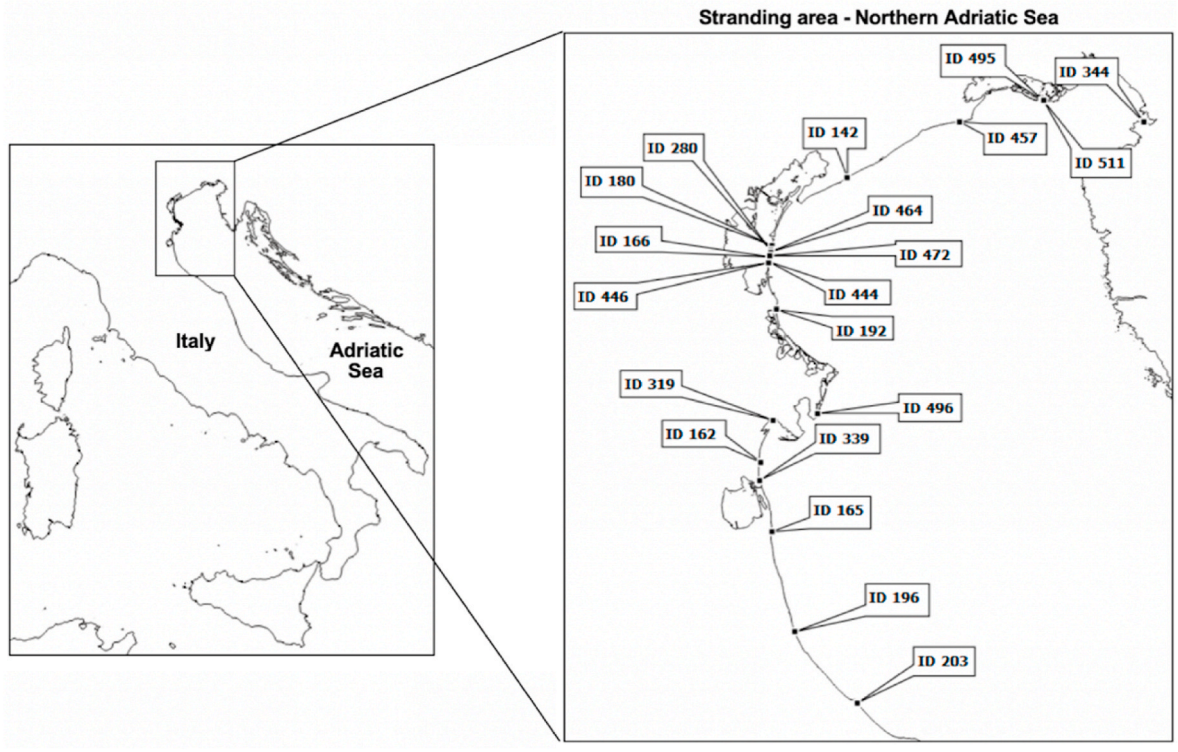

Fig. 1. Sampling area, coastline of Northern Adriatic Sea, Italy. Numbers indicate the ID of the animals collected at the Marine Mammal Tissue Bank of the University of Padova, Italy.

Table 2
Hepatic PFAS concentrations (ng g^{-1} ww) and descriptive statistics of PFAS in bottlenose dolphins found stranded along Northern Adriatic Sea during 2008–2020. N indicates the number of samples in which the target PFAS were detected.

DATA	ID	SEX	LENGTH (cm)	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	LPFBS	LPFHxS	LPFOS	PFUnA	PFDoA	LPFPeS	LPFHpS	LPFDS	PFTrDA	PFTeDA	TOTAL
2008	**142**	F	276		0.05	0.16	0.11	0.86	0.99	5.21		0.24	52.05	21.33	5.74		0.12	0.88	10.25	1.05	99.03
2009	**162**	M	119		0.36	0.82	1.30	10.05	22.7	65.98	0.11	3.23	629.73	104.34	29.47	0.06	2.59	3.67	30.16	3.93	908.50
2009	**165**	M	280	0.06	0.60	1.75	1.56	2.17	6.38	13.76		0.98	137.91	33.18	10.96		0.43	1.58	12.34	1.89	225.55
2009	**166**	M	260		0.10	0.80	0.52	2.03	4.42	6.13		0.58	49.83	13.85	6.07		0.16	0.85	10.23	1.68	97.25
2010	**180**	M	209	0.06	0.52	0.94	1.29	1.68	5.15	6.95		0.81	99.15	19.51	5.41		0.23	0.67	12.02	0.93	155.32
2010	**192**	F	240		0.11	0.14	0.85	7.19	18.6	46.28	0.05	2.05	270.78	56.85	17.03		1.02	1.50	16.31	2.11	440.87
2011	**196**	M	300		0.36	0.68	1.82	2.75	6.7	15.49		0.77	109.79	27.38	8.67		0.41	0.78	8.87	1.5	185.97
2011	**203**	M	284		0.10	0.17	0.36	1.06	6.17	13.25		1.83	222.68	44.27	12.6		1.06	1.26	18.95	1.48	325.24
2013	**280**	F	178		0.36	0.63	1.76	10.61	18.66	87.39	0.13	2.10	444.47	130.05	47.08		1.28	2.93	34.5	5	786.95
2014	**319**	M	310	0.07	0.51	0.67	1.42	1.06	4.28	4.24		0.52	55.17	10.30	3.06		0.17	0.30	7.35	0.81	89.93
2014	**339**	M	280	0.08	0.51	1.12	1.94	3.89	9.98	27.95	0.06	1.02	170.20	32.84	14.03		0.34	0.84	15.53	2.28	282.61
2015	**344**	M	195		0.13	0.30	1.43	10.75	38.07	96.43	0.13	7.33	559.84	111.71	34.51	0.12	3.30	2.16	28.42	3.25	897.88
2018	**444**	M	274		0.09	0.39	0.31	0.71	5.27	7.92		0.76	85.82	13.82	4.67		0.28	0.53	6.28	0.95	127.80
2018	**446**	M	330		0.09	0.28	0.45	1.54	7.85	25.93		1.42	178.66	26.80	16.04		0.79	0.81	11.48	3.31	275.46
2019	**457**	M	285		0.21	0.26	0.37	0.47	2.54	8.06		0.24	34.25	10.35	6.68		0.12	0.32	4.98	1.27	70.11
2019	**464**	M	254		0.06	0.14	0.05	0.48	4.89	10.37		1.36	94.80	19.47	7.70		0.58	0.47	10.55	1.93	152.85
2019	**472**	M	286		0.38	0.79	1.07	3.12	8.32	20.73		0.82	166.51	15.29	9.54		0.58	0.53	5.63	2.57	235.88
2020	**495**	M	303		0.48	0.85	1.93	2.28	6.38	7.94		0.33	50.04	10.47	8.24		0.14	0.40	9.57	3	102.05
2020	**496**	F	240		0.21	0.62	0.85	2.66	12.29	33.2		1.7	297.03	34.46	16.67		0.86	1.02	12.13	2.38	416.07
2020	**511**	M	300		0.36	0.72	1.39	2.44	9.61	23.56		0.89	172.51	27.37	20.05		0.42	1.08	23.53	7.85	291.78
N				4	20	20	20	20	20	20	6	20	20	20	20	3	20	20	20	20	
Minimum				0.06	0.06	0.14	0.05	0.47	0.99	4.24	0	0.24	34.25	10.30	3.06	0	0.12	0.30	4.98	0.81	
Maximum				0.08	0.60	1.75	1.94	10.75	38.07	96.43	0.13	7.33	629.73	130.05	47.08	0.12	3.30	3.67	34.50	7.85	
Mean				0.07	0.28	0.61	1.04	3.39	9.96	26.34	0.08	1.45	194.06	38.18	14.21	0.06	0.74	1.13	14.45	2.46	
Standard deviation				0.01	0.18	0.40	0.63	3.40	8.69	27.27	0.05	1.57	170.61	35.58	11.26	0.06	0.83	0.88	8.48	1.68	

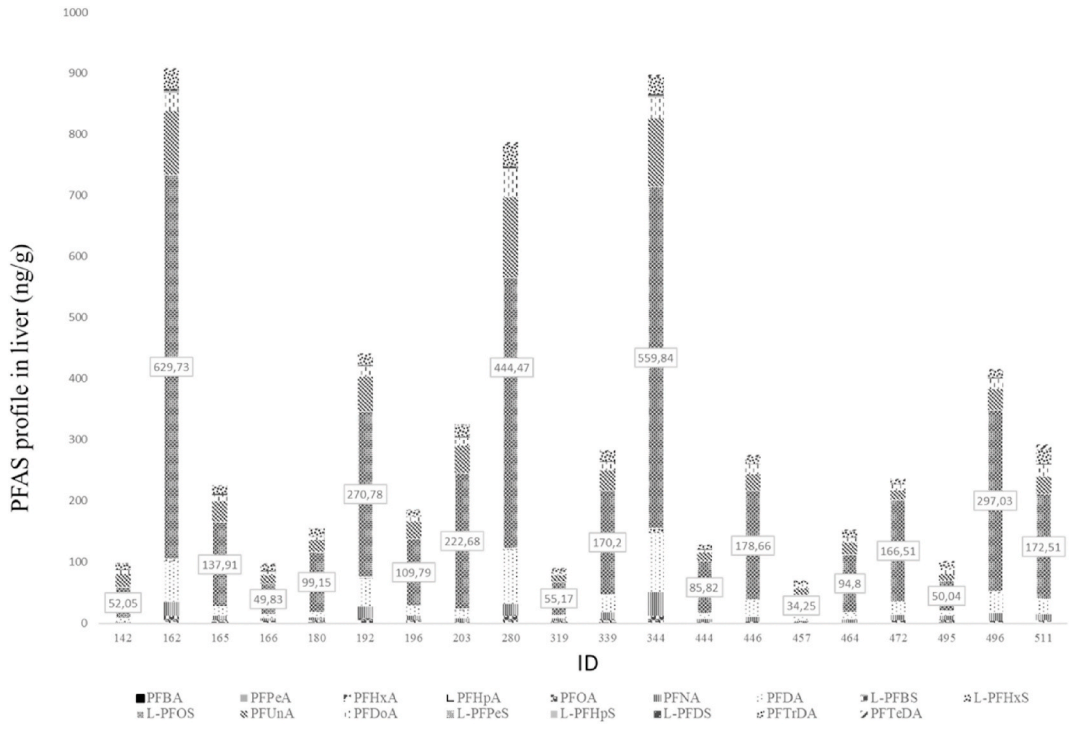

Fig. 2. PFAS profiles in each specimen. Numbers on the axis indicate the ID of the animal at the Marine Mammal Tissue Bank of the University of Padova, Italy; number on the bars indicate the concentration (ng/g) of the most abundant PFAS (PFOS) in the liver of each specimen.

surrogate standard (SS). The concentration was calculated by preparing a calibration curve using response ratios versus concentration ratios for native analytes to that of their labelled-SS.

Quality control samples and method blanks were used during the analysis. Analyte response in method blanks were subtracted from the sample response prior to final quantitation. After determination of the concentration from the curve, the concentration were adjusted for dilution and starting sample mass.

Quality control was made during the different steps of the process: using an initial calibration verification (ICV) of the calibration curve with standard solution from a different supplier (accepted if the values were ±20%); evaluating the recovery rates (90–110) adding to the samples a known quantity of the matrix similar to the one analysed; employing a blank control for checking any contamination in the sample preparation; and performing continuous calibration verification (CCV) to evaluate how the calibration curve reads (accepted if the values were ±20%).

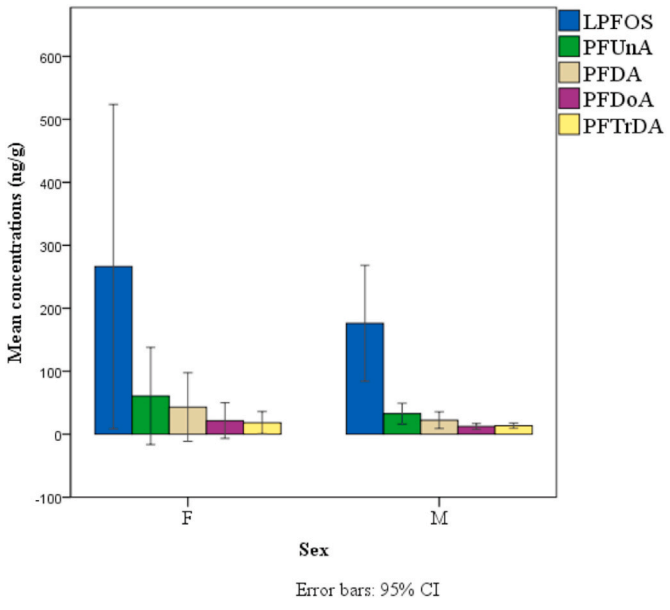

Fig. 3. PFOS, PFUnA, PFDA, PFDoA and PFTrDA concentrations in males and female bottlenose dolphins (error bars represent standard deviation).

2.3. Statistical analysis

Concentrations (mean, standard deviation) of PFAS in the liver samples were statistically analysed by the statistical software package IBM SPSS Statistics 21. The normality in the distribution of concentrations of PFAS was evaluated by the Kolmogorov-Smirnov test and the homoscedasticity by a regression linear test. Once it was verified that the data were normally distributed ($p < 0.05$), the parametric student's T-test for independent samples and the ANOVA tests were used to determine whether mean concentrations of PFAS found varied in relation to the body length and sex. As for the comparative statistical analysis, 2 factors were chosen: sex (2 categories: males (n = 16) and females (n = 4)), body length (3 categories).

Furthermore, ANOVA test was used to carry out a temporal analysis considering three periods of time: 2008–2010 (n = 6), 2011–2015 (n = 6), 2016–2020 (n = 8). 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.

3. Results

3.1. Characterization of PFAS in the liver of bottlenose dolphins

Results of concentrations in liver and the descriptive statistics of the 17 PFAS selected for the analyses are summarized in Table 2. Of the 17 target PFAS analysed in the present work, all of them were quantifiable in more than one sample, and PFOS was the most predominant PFAS in the analysed tissues. The greatest PFOS concentration found in the liver of a bottlenose dolphin was 629,73 ng/g, wet weight.

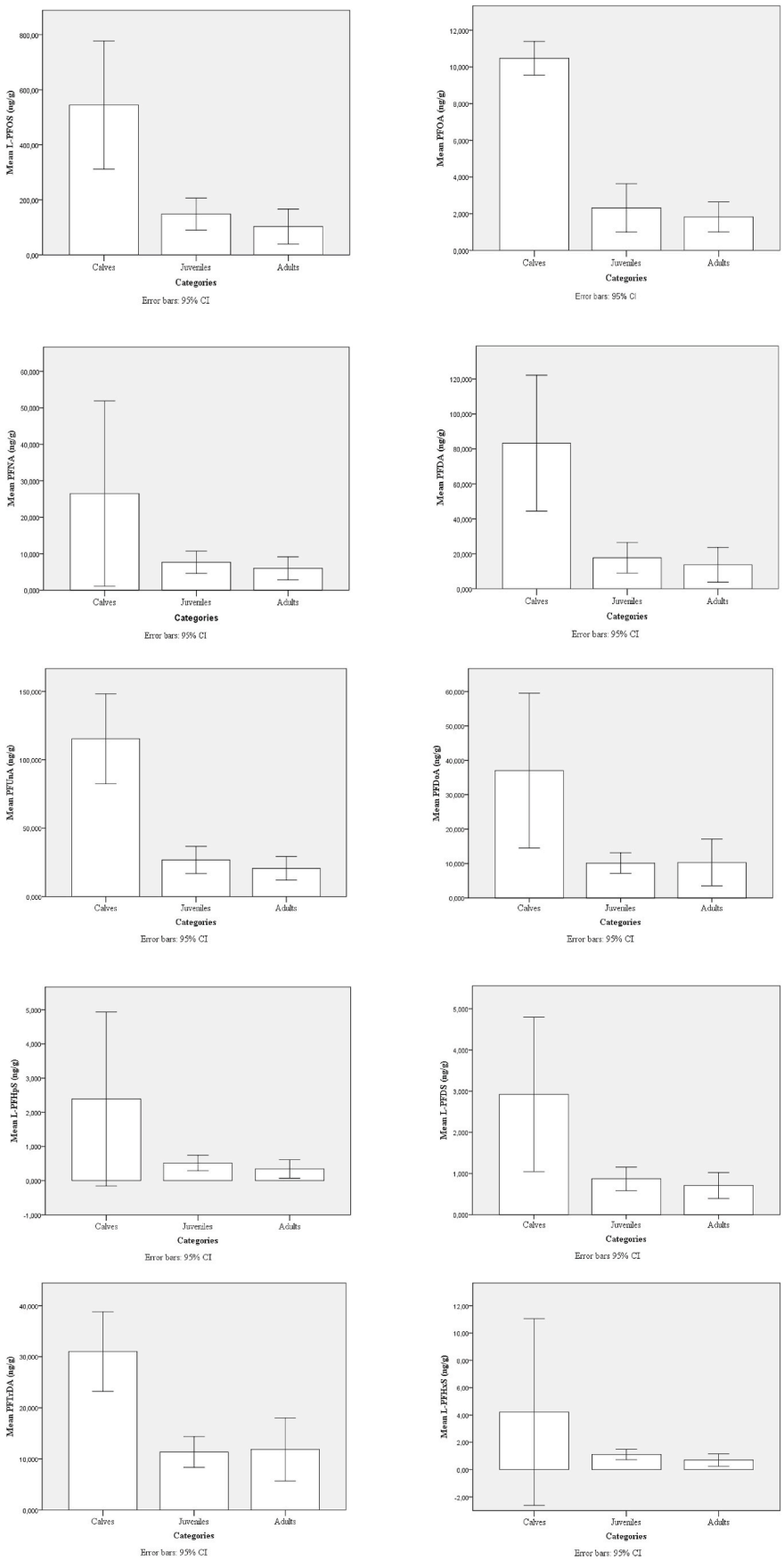

Fig. 4. PFOA, PFNA, PFDA, LPFBS, LPFHxS, LPFOS, PFUnA, PFDoA, LPFHpS, LPFDS, PFTrDA concentrations in calves, juveniles and adult's bottlenose dolphins (error bars represent standard deviation).

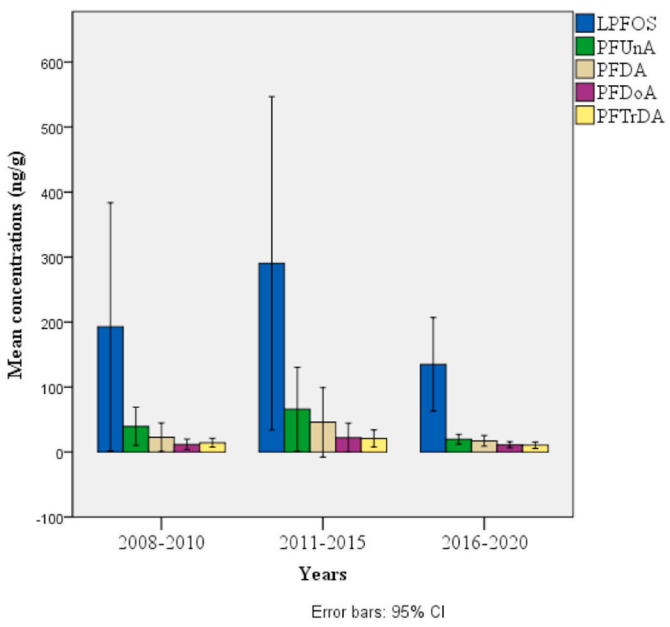

Fig. 5. PFOS, PFUnA, PFDA, PFDoA and PFTrDA concentrations in the three time periods (error bars represent standard deviation).

Table 3
Comparison of mean PFOS concentrations (ng g^{-1} ww) in liver tissues from bottlenose dolphins living in North Adriatic Sea, Mediterranean Sea, Croatia; from Indo-Pacific humpback dolphins in the Pearl River Estuary and Zhanjiang waters, China; from Marine tucuxi dolphin in Guanaraba Bay, Brazil; and from long-beaked common dolphin in Korean Coastal Sea.

Locations	Species	n	Mean PFOS	Reference
North Adriatic Sea, Italy	Bottlenose dolphin	20	194.06	Present study
Western Mediterranean Sea	Bottlenose dolphin	5	211	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 dolphin	52	1180	Gui et al. (2019)
Guanabara Bay, Brazil	Marine tucuxi dolphin	23	268	Dorneles et al. (2008)
Mediterranean Sea	Bottlenose dolphin	5	270	Giesy & Kannan (2001)
Korean Coastal Sea	Long-beaked common dolphin	47	47	Moon et al. (2010)

Data were normally distributed (Kolmogorov-Smirnov test) and therefore parametric statistical analysis were performed. Of the 17 PFAS, 10 (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, LPFHxS, LPFOS, PFUnA, PFDoA, LPFHpS, LPFDS, PFTrDA, PFTeDA) were found in all the livers of the animals, LPFBS in only 6 samples, PFBA in 4, LPFPeS in 3.

3.2. PFAS profiles

PFAS profiles in each specimen are shown in Fig. 2. The PFAS profile in livers of the 20 bottlenose dolphins was generally composed of five dominant PFAS: PFOS > PFUnA > PFDA ≈ PFDoA ≈ PFTrDA.

The results showed that PFOS represents the major fraction of the PFAS profile. In fact, PFOS accounted until 71% in the PFAS profile in our sample. In addition, also PFUnA, PFDA, PFDoA and PFTrDA have a strong presence, with PFUnA beingthe second most abundant PFAS (after PFOS) in liver (mean value 38.18 ng/g ww).

3.3. Sex and body length

The parametric student's T-test, showed no significant differences between sexes for all the investigated compounds and the total PFAS concentration. Concentrations of the five dominant PFAS in female and male bottlenose dolphins are shown in Fig. 3 and in Supplementary Table 1; PFBA and LPFPeS were excluded from the analysis since they were found only in males. Due to the lack of data about the age of the animals, the sample was divided into three categories based on animals' body lengths. PFAS' variation was tested in relation to the body length by ANOVA test followed by a Bonferroni post-hoc (PFPeS was excluded since it was found only in calves). Body length had a significant effect on 11 PFAS (PFOA, PFNA, PFDA, LPFBS, LPFHxS, LPFOS, PFUnA, PFDoA, LPFHpS, LPFDS, PFTrDA) as shown in Fig. 4 and in Supplementary Table 2, and on the total PFAS concentration. Significant differences were found between adults and calves (except for PFBS, that was not found in adults) as well as juveniles and calves, but not between adults and juveniles. 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 PFAS (PFOA, PFNA, PFDA, LPFBS, LPFHxS, LPFOS, PFUnA, PFDoA, LPFHpS, LPFDS, PFTrDA, Supplementary Table 3) and on the total PFAS concentration. Significant differences for these PFAS were found between adults and calves and juveniles and calves, but not between adults and juveniles. In addition, since body length is a continuous variable, a correlation analysis using Pearson was carried on with total PFAS concentration, resulting in a significant and negative correlation between the two variables (r = −0.8).

3.4. Temporal trends of PFAS

No temporal differences in PFAS concentrations were found for any compounds (concentrations of the five dominant PFAS in the three periods are shown in Fig. 5 and Supplementary Table 4; PFPeS was excluded for the scarcity of data). Since no significant differences were found between sexes, male and female adults were pooled together for the analysis. Furthermore, PFAS were tested only in juveniles and adults in the 3 different time periods and no temporal differences in concentrations were found for any compounds (Supplementary Table 5).

3.5. Comparison with other areas

Finally, as shown in Table 3, the results of this study were compared with data reported in cetacean livers of bottlenose dolphins in the Mediterranean Sea (Giesy & Kannan, 2001; Kannan et al., 2002; Lopez-Berenguer et al., 2020) and with other dolphin species, the Indo-Pacific humpback dolphin (*Sousa chinensis*), from the Pearl River Estuary, China (Gui et al., 2019), the marine tucuxi dolphin (*Sotalia guianensis*) from the Guanabara Bay, Brazil (Dorneles et al., 2008) and the long-beaked common dolphin (*Delphinus capensis*) from Korean Coastal Sea (Moon et al., 2010). Taking into account only the mean values of PFOS, the most abundant compound in our dataset (n = 20), our data resulted to be similar to those reported in livers of bottlenose dolphins stranded in the south-eastern coast of Spain (n = 5) (Lopez-Berenguer et al., 2020) and in Mediterranean Sea (Giesy & Kannan, 2001), and of marine tucuxi dolphin in Brazil (Dorneles et al., 2008). On the other side, mean hepatic PFOS concentration found in our data resulted to be more than 4.5 times higher than that reported in a single specimen (n = 1) collected in North Adriatic Sea (Croatia) in 1992 (Kannan et al., 2002), and more than 4 times higher than those reported for long-beaked common dolphin in Korean Coastal Sea (Moon et al., 2010). Differently, Gui et al. (2019) reported 6 times higher mean hepatic PFOS concentrations in the Indo-Pacific humpback dolphin (n = 52) from the Pearl River Estuary and Zhanjiang waters, China.

4. Discussion

Dolphins are apex predators of the marine food chain because of their higher trophic level and their ability to deeply influence the ecosystems, limiting the density of preys and smaller predators (Wallach et al., 2015). Due to their longevity and long-term residency, dolphins can serve as important sentinels of the health of coastal marine ecosystems (Wells et al., 2004). In addition, considering the high level of site fidelity for some bottlenose dolphins (López, 2019) and the potential biomagnification of PFAS for this species (Houde et al., 2006), they may also represent sentinels for the impact of PFAS in the environment and for the potential effects that bans of these substances could have on it. In the Mediterranean Sea, bottlenose dolphins are listed as "Vulnerable" according to the IUCN and are protected under the Habitats Directive (Directive 92/43/EEC), the Bern Convention, the SPA/BIO protocol (Barcelona Convention) and the Bonn Convention. The Mediterranean Sea is a deep oligotrophic ocean (Brumovský et al., 2016) and its semi-enclosed nature may explain the slow turnover of PFAS (Lopez--Berenguer et al., 2020). Since cetaceans are apex predators particularly susceptible to PFAS (Spaan et al., 2020), this study could help with the conservation of bottlenose dolphins in the Mediterranean Sea.

PFAS are primarily investigated in protein-rich tissues such as liver, but few studies have explored their occurrence in lipid-rich tissues such as blubber (Fair and Houde, 2018; Schultes et al., 2020). Blubber investigations might be useful to carry on analyses on living animals with non-invasive methods: skin biopsies (blubber tissue) may be collected using a non-destructive method such as remote dart sampling (Baini et al., 2017), offering a more complete overview of PFAS compound in bottlenose dolphin populations.

In this study, PFOS still represents the major fraction of the PFAS profile, although its production was phased out almost two decades ago and the Stockholm Convention in 2009 listed it under Annex B, resulting in the restriction of its production and use (Wang et al., 2013). The five dominant PFAS found in this study (PFOS, PFUnA, PFDA, PFDoA and PFTrDA) are all long chain PFAS and although industries shifted towards shorter chain PFAS (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). The reported results might be explained by a contamination related to past events or to sources of secondary contaminations: a slow turnover of long chain PFAS, like PFOS, in the semi-enclosed Mediterranean Sea basin could lead to a high concentration of these chemicals 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 PFAS may last decades (Dassuncao et al., 2017). Knowledge about the toxic effects of PFAS in cetaceans is still scarce but the presence of these substances in the Adriatic Sea highlights the importance of evaluating possible threats to the species.

Data obtained in this study confirm the results obtained by Lopez-Berenguer and collegues (2020) and no differences were found between sexes for any compounds. Different studies reported the possibility of maternal transfer of PFAS between mother and offspring in different marine mammal species (Houde et al., 2005; Fair and Houde, 2018) with lactation possibly representing an offloading PFAS burden in mature females (Gui et al., 2019). Nevertheless, females may have higher values of PFAS since reproduction implies a greater need of proteins for milk production, therefore the consequent intensified mobilization of proteins may explain the increase of PFAS in liver, due to their high affinity to proteins (Gui et al., 2019). In the present study, the absence of a relationship between sex and higher concentration of target pollutants may be related to the small number of samples: 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.

On the contrary, the differences found on PFAS 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, that is at the time of weaning (Geraci and Lounsbury, 2005). As expected, body length had a statistically significant and negative effect: calves show higher mean values than adults, thus confirming an increasing age-related ability of metabolism and elimination of PFAS in dolphins (Gui et al., 2019) or a growth dilution effect. No significant differences were found between adults and juveniles: this might be explained by the fact that most of the juveniles and adults included in the sample set had relatively low variation in length, which could have influenced the absence of a link between body length and other PFAS concentrations (Lopez-Berenguer et al., 2020).

In 2013 PFAS contamination was detected in a vast area in Veneto region, mainly on the Adige and Brenta rivers draining basins (Bonato et al., 2020; Canova et al., 2020), which are two of the major rivers debouching in the North Adriatic. No differences in hepatic PFAS concentrations of bottlenose dolphins were detected before and after this year and no temporal changes in concentrations were found for any PFAS during the three periods of time: 2008–2010, 2011–2015, 2016–2020. 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).

The comparison with other studies (Giesy & Kannan, 2001; Kannan et al., 2002; Dorneles et al., 2008; Moon et al., 2010; Gui et al., 2019; Lopez-Berenguer et al., 2020) suggests that although the limited quantity of the considered samples, PFOS might be widespread also in western Mediterranean Sea and the Adriatic Sea. In addition, higher PFOS concentrations found in our data compared with those of Kannan et al. (2002), may indicate a raise in PFOS contamination in the last 30 years, confirming the persistence of PFOS in the environment. Although the ban of some PFAS in European Union, these substances might be imported from other countries, therefore, the stop in production in one country does not directly imply their complete absence. In addition, as bottlenose dolphins' prey preferences vary depending on the different geographical area (Miokovic et al., 1999; Blanco et al., 2001; Bearzi et al., 2008a; Bearzi et al., 2008b; Bearzi et al., 2019; Bonizzoni et al., 2020), the results obtained in northern Adriatic Sea might also be explained by differences in feeding habits. As far as the authors' knowledge, studies on PFAS evaluation in bottlenose dolphins' preys in northern Adriatic Sea have not been done. Future studies might focus on these species in order to compare PFAS in other environmental compartments, trying to calculate bioaccumulation factors.

The limited sample size might affect the outcomes of the study, however, the obtained results allow to get a first insight of these compounds in the study area. Another point to take in consideration is that depuration half-lives of PFAS varies between different animals and biological persistence of PFAS in different species may be partly related to the body size, with slower rates of depuration for larger animals (Martin et al., 2003). Therefore, considering that DCC is strongly influenced by the time of recovery of the carcasses, data collected from very decomposed animals (DCC 4) may represent an underestimation of real PFAS concentrations. In addition, in larger organisms, PFOS can also be formed by environmental degradation and by metabolism (UNEP, 2006), therefore different PFAS might be potential precursors of PFOS, possibly explaining the greater presence of this compound in our results.

5. Conclusion

The results of this study suggest that PFAS, especially the long-chain ones, are widely distributed in bottlenose dolphins along the northern Adriatic Sea. According to the descriptor 8 of the Marine Strategy Framework Directive (MSFD), the concentrations of contaminants must be controlled in order to avoid pollution effects (EC, 2008), therefore this study could be useful to assess PFAS exposure risk and their

emission in the investigated area, highlighting once again the role of marine mammals as a tool of passive monitoring of marine environment. Since some PFAS might be linked to adverse outcomes for human health (Bonato et al., 2020; De Toni et al., 2020; Piekarski et al., 2020; Pitter et al., 2020; Dalla Zuanna et al., 2021), further studies should investigate the effect of these contaminants on other mammals' health, such as dolphins. New approaches and technologies could be used: the application of -omics technique applied to cell cultures could help to understand the effects of chemicals on different species (Augustyniak et al., 2019). These new perspectives could allow to obtain a more complete view of the conservation status, also considering other stressors impairing marine mammals' conservation.

As stated above, Veneto Region and its hydrographic basins were involved in a severe emergency related to PFAS exposure in terrestrial mammals, including humans. The main goal of this study was to use samples of bottlenose dolphins stranded along the north eastern Italian coastline between 2008 and 2020, in order to assess the presence of PFAS and the possible impact of this incident to marine megafauna living in the northern Adriatic Sea. The possibility to use frozen samples of tissues stored in the Mediterranean Marine Mammals Tissue Bank, highlights the important role of Environmental Banks. Carrying these types of retrospective analyses, gives the possibility to evaluate past exposure of new and emergent chemicals, and their impacts on wildlife health, thus assessing the outcome of policies adopted to reduce pollutants' effect in order to strengthen marine conservation efforts.

Credit author statement

Giuseppe Sciancalepore: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Guido Pietroluongo:** Visualization, Writing – review & editing. **Cinzia Centelleghe:** Conceptualization, Writing – review & editing. **Massimo Milan:** Visualization, Writing – review & editing **Marco Bonato:** Methodology, Writing – review & editing. **Giorgia Corazzola:** Writing – review & editing. **Sandro Mazzariol:** Conceptualization, Supervision, Writing – review & editing.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118186.

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