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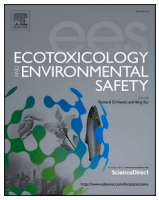

Phthalate esters (PAEs) concentration pattern reflects dietary habitats ($\delta^{13}C$) in blood of Mediterranean loggerhead turtles (*Caretta caretta*)

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ABSTRACT

Phthalic acid esters (PAEs) are classified as endocrine disruptors, but it remains unclear if they can enter the marine food-web and result in severe health effects for organisms. Loggerhead turtles (*Caretta caretta*) can be chronically exposed to PAEs by ingesting plastic debris, but no information is available about PAEs levels in blood, and how these concentrations are related to diet during different life stages. This paper investigated, for the first time, six PAEs in blood of 18 wild-caught Mediterranean loggerhead turtles throughout solid-phase extraction coupled with gas chromatography-ion trap/mass spectrometry. Stable isotope analyses of carbon and nitrogen were also performed to assess the resource use pattern of loggerhead turtles. DEHP (12–63 ng mL^{-1}) and DBP (6–57 ng mL^{-1}) were the most frequently represented PAEs, followed by DiBP, DMP, DEP and DOP. The total PAEs concentration was highest in three turtles (124–260 ng mL^{-1}) whereas three other turtles had concentrations below the detection limit. PAEs were clustered in three groups according to concentration in all samples: DEHP in the first group, DBP, DEP, and DiBP in the second group, and DOP and DMP in the third group. The total phthalates concentration did not differ between large-sized (96.3 ± 86.0 ng mL^{-1}) and small-sized (67.1 ± 34.2 ng mL^{-1}) turtles ($p < 0.001$). However, DMP and DEP were found only in large-sized turtles and DiBP and DBP had higher concentrations in large-sized turtles. On the other hand, DEHP and DOP were found in both small- and large-sized turtles with similar concentrations, i.e. ~ 21.0/32.0 ng mL^{-1} and ~ 7^{-1}/9.9 ng mL^{-1}, respectively. Winsored robust models indicated that $\delta^{13}C$ is a good predictor for DBP and DiBP concentrations (significant Akaike Information criterion weight, AIC_{wt}). Our results indicate that blood is a good matrix to evaluate acute exposure to PAEs in marine turtles. Moreover, this approach is here suggested as a useful tool to explain the internal dose of PAEs in term of dietary habits ($\delta^{13}C$), suggesting that all marine species at high trophic levels may be particularly exposed to PAEs, despite their different dietary habitats and levels of exposure.

1. Introduction

Phthalic Acid Esters (PAEs) are widely used as plasticizers in industrial sectors for enhancing the properties of polymers, such as flexibility, softness, and workability; more common plastic products encompass 20–40% (w/w) of these chemical compounds (Xie et al., 2014). Because PAEs are not covalently bound, but simply mixed with the plastic polymer, they can be easily released in the environment (Sun et al., 2021; Lee et al., 2020; Liu et al., 2020; Arfaeinia et al., 2019), especially when plastics products are degraded to debris (Przybylińska

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and Wyszkowski, 2016; Net et al., 2015) introducing potentially hazard to biota and humans (Lithner et al., 2011). Specifically, PAEs may affect marine organisms acting as endocrine disrupters (EDCs), or by competing with the synthesis of endogenous hormones (Fossi et al., 2012). To protect human health, the European Food Safety Authority (EFSA) established a Total Daily Intake (TDI) for some of these pollutants (Smith et al., 2018). Most of PAEs classified as plasticizers are included on the list of priority compounds of the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention) (Tromp and Koos, 1994). In the European Union (EU) restrictions have been imposed to phthalates, in particular to low-molecular weight phthalates, which are classified as very dangerous substances in Category 1B of REACH Regulation because they are toxic to reproduction (Ventrice et al., 2013).

Knowledge of internal dose of PAEs in the marine food-web is extremely important, especially in the Mediterranean area, where marine debris and microplastics are a widespread source of concern for biota (Fossi et al., 2014; Cole et al., 2011; Tomás et al., 2002; Bjorndal, 1994; McCauley and Bjornal, 1999). The Marine Strategy Framework Directive (MSFD) remarks the importance of monitoring the presence of plastics and microplastics in the sea as indicator to improve the knowledge of the qualitative descriptor n.10 (Marine Litter) (Galgani et al., 2013). Many studies have recorded the ingestion of plastic debris and microplastics in a range of marine taxa in the Mediterranean Sea (Camedda et al., 2021; Fossi et al., 2014; Cole et al., 2011), a process which may facilitate the transfer of chemical additives or hydrophobic pollutants to biota. However, it remains unclear whether exposure to these chemicals can routinely be passed throughout the marine food-web or whether such a contaminant will result in adverse health effects for marine organisms.

Sea turtles have been proposed as sentinel species and environmental health indicators for marine habitats and human health (Aguirre and Lutz, 2004), due to their longevity, vagrant lifestyle, diverse diet and high susceptibility to different anthropogenic pollutants (Aguirre and Luts, 2004; Aguirre and Tabor, 2004; Lutcavage et al., 1997). The loggerhead turtle (*Caretta caretta*) is the most common sea turtle species in the Mediterranean Sea, where it can experience severe anthropogenic impacts (Campani et al., 2013; Tomás et al., 2002; Bjorndal, 1994) and bioaccumulate heavy metals, organic contaminants and marine toxins from food, sediment, and water (Blasi et al., 2020; D'Ilio et al., 2011; Gramentz, 1988). In the Mediterranean Sea, the loggerhead turtle shows significant spatial variation in diet feeding on diverse prey items, according to local distribution and abundance, from planktonic to benthic species (Blasi et al., 2018; Casale et al., 2008), or also opportunistically preying on fishery discards in fishing areas and close to marine debris in polluted areas (Blasi et al., 2018; Blasi and Mattei, 2017; Blasi et al., 2016). Loggerhead turtles may accidentally ingest plastic debris, which are mixed with normal dietary items or misidentify items as prey and actively select them for consumption (Camedda et al., 2021). Additionally, microplastics may also be absorbed indirectly through the consumption of prey items throughout biomagnification (Cole et al., 2011). The increasing concentration of plastic debris recorded in the Mediterranean Sea in the last-half century might have extensively influenced loggerhead turtle diet, leading to bioaccumulation of plastic-related pollutants in the loggerhead food-web (Seney and Musick, 2007). Several studies have been conducted in the Mediterranean Sea on the presence of plastics and microplastics and their potential impact on different marine organisms (Campani et al., 2013; Lazar and Gracan, 2011). The effects of plastic ingestion in marine turtles can be both lethal and sublethal, the latter being more frequent but more difficult to detect (Gall and Thompson, 2015; Hoarau et al., 2014; Schuyler et al., 2014a, 2014b). In the long-term, response to plastic ingestion could have severe consequences, such as under-nutrition through dietary dilution (Plot and Georges, 2010; McCauley and Bjorndal, 1999; Rhind, 2009). Microplastics, due to their hydrophobic properties, are known to attract heavy metals and other toxins from the marine environment (Mattei et al., 2015; D'Ilio et al., 2011) that have been shown to accumulate into tissues and cause developmental and reproductive abnormalities in several taxa (Rhind, 2009). Finally, plasticizers can be also absorbed into tissues, potentially acting as endocrine disrupters (Beltifa et al., 2017; Oehlmann et al., 2009). To date however, the basic knowledge of this issue in marine turtles is limited, especially for acute exposures.

Several studies have reported phthalates in different aquatic organisms, such as protozoans, invertebrates, plankton, macroalgae, mollusks, crustaceans, alligators, different types of fishes (Notardonato et al., 2021; Sala et al., 2021; Baini et al., 2017; Brock et al., 2016; Fourgous et al., 2016; Guerranti et al., 2016; Hu et al., 2016; Net et al., 2015; Natesan, 2012), and high-level consumers in the marine food-web, i.e. marine mammals (Hart et al., 2020; Rian et al., 2020) and sea turtles (Savoca et al., 2021; Savoca et al., 2018). Particularly, in the Mediterranean Sea, PAEs concentrations have been detected in muscle, eggs, gonads and liver of marine turtles (Savoca et al., 2021; Savoca et al., 2018) and in blubber of fin whale (*Balaenoptera physalus*) (Fossi et al., 2012). To the best of our knowledge, there is no indication of PAEs doses in marine organisms' blood, a tissue that could potentially represent a more recent (week to months) exposure to these chemicals when compared to other tissues (Camacho et al., 2012). Since the transport of exogenous substances is realized by means of blood, this matrix could be useful for monitoring chemicals associated at different specimens and, consequently, for estimating recent exposure in relation to turtle diet. Blood is generally used for the evaluation of the health status of organisms by means of analyzing its biochemical parameters, but only recently the normal range of conditions for phthalates analyses in loggerhead turtle blood has been established (Notardonato et al., 2021).

Stable isotopes analysis is a useful tool for studying the ecology of marine consumers, as carbon and nitrogen isotope ratios may reflect individuals' patterns of diet and habitat use. In marine environments, as a result of the effect of biogeochemical processes (Pajuelo et al., 2010), a consumer's stable isotopic ratio of $^{13}C/^{12}C$ ($\delta^{13}C$) reflects foraging habitat (France, 1995a, 1995b; Fry and Sherr, 1984), and the ratio $^{15}N/^{14}N$ ($\delta^{15}N$) indicates the trophic position (Peterson and Fry, 1987). Consequently, an assumed constant trophic fractionation of $^{15}N/^{14}N$ between consumer and prey allows to infer about feeding interactions and trophic level in food web studies (Post, 2002; France, 1995a, 1995b; Fry and Sherr, 1984). $\delta^{13}C$ and $\delta^{15}N$ values at the base of the food web are conserved and amplified by ~1‰ and ~3‰, respectively, through higher trophic levels (Pajuelo et al., 2010; De Niro and Epstein, 1978). These values, however, are not fixed but can vary due to geographic location, seasonal habitat, life stages but also variations in diet (trophic differences) and health of individuals. For example, the long residence time of plastic debris in the gastrointestinal system may increase significantly the $\delta^{15}N$ of Mediterranean loggerhead turtles, probably due to plastic ingestion, which might cause under-nutrition and consequent absorption of chemical contaminants (Blasi et al., 2018).

Stable isotopes and chemical compounds concentrations assessment can be used together in ecotoxicological approaches to trace chemicals assimilation from a trophic habitat (Rodriguez et al., 2020). Regardless of the loggerhead turtle's food web that regulates the internal dose of PAEs, it is probable that the concentrations in blood derive from a plastic-related diet.

In this study, six PAEs, i.e. dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DiBP), dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP) and di-n-octyl-phthalate (DOP), were investigated in the blood of Mediterranean loggerhead turtles and compared to stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes values in the same blood samples. This study focused on evaluating the concentration of those PAEs that are more commonly used as plasticizers in plastic debris, thus potentially more hazardous for marine food-webs. Furthermore, PAEs concentrations were correlated with blood's $\delta^{13}C$ and $\delta^{15}N$ values, to assess if dietary habitats can contribute to exposure to these chemicals (Donaton et al., 2019).

2. Methods

2.1. Study area and samples collection

Blood samples were collected from 18 loggerhead turtle specimens wild-caught in their feeding habitats with detectable diseases or problems (Table 1) in different parts of the Tyrrhenian Sea (Fig. S1-Supplementary material) from April to September 2019. All turtles were hospitalized in Sea Turtles Rescue Centers in Italy, due to the defecation of large numbers of plastic debris items (macro/meso plastic items; data not shown) (Table 1 and Fig. S1). Specifically, 14 turtles were found in the Aeolian Archipelago waters and sampled in the Filicudi Wildlife Conservation (FWC) First Aid Center (1), 5 turtles were found in the Central Tyrrheanian Sea and sampled in the Stazione Zoologica Anton Dohrn (SZN) Campania (2) and IRSA-CNR (CNR) Sardinia (3) Rescue Centers (Table 1 and Fig. S1).

All turtles underwent examination according to standard procedures (Blasi et al., 2016) (Table 1). Causes of rescue were assigned if clear evidence/injuries were observed on the body of turtles (i.e., the presence of longlines or fishing lines entangled around the flippers or neck or evidence of injuries) (Table 1) (Blasi et al., 2016). If multiple causes of rescue were observed, the most severe and recent was assigned as the primary cause, while the less severe as secondary cause. Defecation of plastic debris items was observed in all turtles (100%) during hospitalization (from 7 to 15 days) as primary (11 turtles) or secondary (7 turtles) cause of rescue (Table 1). For each individual the notch-to-tip curved carapace length (CCL, cm), curved carapace width (CCW, cm) and weight (kg) were also measured (Bolten, 1999).

The collection of blood samples was performed as part of routine veterinary examinations at the turtles' arrival at the Rescue Center and before starting veterinary treatments and before the turtles were fed. During clinical investigation blood samples were collected from the cervical sinus to obtain information on the blood chemistry and blood count for diagnostic purposes. An aliquot of 3.5 mL of blood was taken for this study and placed in heparinized microtubes S-Monovette® (7.5 mL LH, code 01.1604.400, orange). 2 mL were immediately transferred in glass containers for PAEs extraction and analyses, while the remaining 1.5 mL were used for stable isotope analyses. All samples were then stored at −20 °C.

2.2. PAEs extraction from blood samples and analysis

Standards of phthalates were obtained from Sigma-Aldrich (Milan, Italy). Solvents used for the cleaning up and samples extraction such as acetone, *n*-heptane, methanol, methylene chloride, and cyclohexane, were obtained from Merks (Darmstadt, Germany). Phosphoric acid (85%) was used for the analysis. Phenanthrene was used as Internal Standard (IS), which was also obtained from Merks. A solution of the investigated phthalates and the IS was prepared in acetone, with a concentration respectively equal to 500 ng mL^{-1} and 80 ng mL^{-1}. Moreover, a solution of phosphoric acid/saline solution (9 g L^{-1}) (1 + 1, v/v) (Merks), necessary for the analysis, was prepared. In this study, to prevent cross-contamination, it was applied a severe cleaning procedure, described in detail in previous papers (Notardonato et al., 2019; Russo et al., 2012; Kato et al., 2003).

A simple and reproducible analytical method based on solid-phase extraction (SPE) coupled with gas chromatography-ion trap/mass spectrometry (GC-IT/MS) was used for the determination of phthalates from the blood of turtles (Notardonato et al., 2021; Saliu et al., 2020; Dogruer et al., 2018; Andrade-Eiroa et al., 2016; Eckert et al., 2015). A gas chromatograph (Thermo Fischer Scientific, Milan, Italy) model Finnigan TraceGC ULTRA equipped with a mass selective detector model (PolarisQ) and an analysis software (Xcalibur) was used. A fused-silica capillary column with a chemically bonded phase (SE-54.5% phenyl-95% dimethylpolysiloxane) from Teknokroma (Rome, Italy) was used for the analysis. The main characteristics were as follows: 30 m × 250 μm i.d.; d_f film thickness, 0.25 μm; theoretical plate number, N, 120, 000 for *n*-dodecane at 90 °C; capacity factor, K1, 7.3; optimum linear velocity of carrier gas, hydrogen, u_{opt}, 34.5 cm s^{-1}; utilization of theoretical efficiency, 95% was used (Russo et al., 2014a, 2014b). The cartridges used in this study were 100 mg C_{18} phthalate-free (Chromabond). The extraction was obtained by using C_{18} phthalates-free as the stationary phase. In order to individuate the best working conditions for the extraction, the adsorption isotherms were studied and breakthrough curves were plotted for PAEs in the working solution on the used C_{18} cartridges. The adsorbed PAEs were then eluted by letting organic solvent (*n*-heptane) flow through the cartridge at approximately 2–3 mL min^{-1}. Before performing the SPE procedure, 1 mL of blood was spiked with 50 ppb of PAEs and the solution was then diluted to 10 mL with phosphoric acid/saline solution (9 g L^{-1}) (1 + 1, v/v). The PAEs were extracted by means of a liquid-liquid extraction technique proposed by Eckert et al. (2015). This step was essential for confirming the presence of phthalates in the initial solution. An aliquot of 1 mL of blood was diluted to 100 mL with an aqueous phosphoric acid/physiological solution (1 + 1, v/v), containing PAEs and the Internal Standard (IS). This solution was extracted thrice with 10 mL of *n*-heptane. The apolar phase was then collected and placed inside a glass container. Subsequently, the solution was dried under a gentle nitrogen flow and recovered with 250 μL of methanol. Finally, 1 μL of this solution was injected into the separation system. The overall analytical

Table 1
The 18 loggerhead turtles sampled in the three Rescue Centers with their code, date of sampling, cause of rescue (primary and secondary) and morphometric characteristics, i.e. curved carapace length (CCL, cm), curved carapace width (CCW, cm) and weight (kg).

Rescue Center	Rescue date	Turtle Code	Primary cause of rescue	Secondary cause of rescue	CCL (cm)	CCW (cm)	Weight (kg)
FWC Sicily (1)	31/05/2019	BYO	plastic ingestion	*	67.5	61.0	38.8
FWC Sicily (1)	04/06/2019	REX	longline ingestion/entanglement in debris	plastic ingestion	62.5	55.4	26.7
FWC Sicily (1)	19/06/2019	LUC	plastic ingestion	*	54.0	50	20
FWC Sicily (1)	26/06/2019	V07-CIC	longline ingestion/entanglement in debris	plastic ingestion	39.5	35.5	5.7
FWC Sicily (1)	06/07/2019	TRI	plastic ingestion	*	65	60	36.4
FWC Sicily (1)	21/07/2019	NAP	plastic ingestion	*	70	63	38.3
FWC Sicily (1)	25/07/2019	RAM	plastic ingestion	*	69.0	63	42
FWC Sicily (1)	30/07/2019	SPI	plastic ingestion	*	42.5	38.5	8.7
FWC Sicily (1)	12/08/2019	ACH	plastic ingestion	*	56.5	49.0	20.8
FWC Sicily (1)	06/09/2019	LAD	plastic ingestion	*	70.5	64	43
FWC Sicily (1)	14/09/2019	KON	plastic ingestion	*	55	54	20
FWC Sicily (1)	15/09/2019	WAL	good health	*	59	54	30.9
FWC Sicily (1)	15/09/2019	KIR	good health	*	58.8	56.5	31
SZN Campania (2)	24/09/19	V13/ANA/19	water in lung	plastic ingestion	72.5	67.0	45.7
SZN Campania (2)	06/11/19	V14/MIA/19	longline ingestion/entanglement in debris	plastic ingestion	57.5	52.4	20.45
SZN Campania (2)	21/11/19	V15/NER/19	longline ingestion/entanglement in debris	plastic ingestion	57.9	53.5	19.9
CNR Sardinia (3)	15/09/2019	S507	longline ingestion/entanglement in debris	plastic ingestion	68	61.0	46
CNR Sardinia (3)	11/06/2019	S501	longline ingestion/entanglement in debris	plastic ingestion	71	65	49.9

methodology was validated to determine the limit of detection (LOD, DMP 0.6 ng mL^{-1}, DEP 0.3 ng mL^{-1}, DiBP 0.1 ng mL^{-1}, DBP 0.08 ng mL^{-1}, DEHp 0.08 ng mL^{-1}, DnOP 0.1 ng mL^{-1}), limit of quantification (LOQ, DMP 0.8 ng mL^{-1}, DEP 0.8 ng mL^{-1}, DiBP 0.7 ng mL^{-1}, DBP 0.7 ng mL^{-1}, DEHP 0.4 ng mL^{-1}, DnOP 0.4 ng mL^{-1}), and correlation coefficients (DMP 0.9989, DEP 0.9985, DiBP 0.9971, DBP 0.9965, DEHP 0.9933, DnOP 0.9958) (Notardonato et al., 2021). By using this procedure, percentage recoveries (on 5 replicates) ranging from 89% to 103% were achieved (specifically, DMP 91.4 ± 4.9, DEP 94.1 ± 6.0, DiBP 96.8 ± 7.3, DBP 99.7 ± 7.2, DEHP 96.3 ± 7.6, DnOP 93.1 ± 5.4). Finally, precision parameters such as intra-day and inter-day, were studied, and the obtained values were smaller than 12.5%.

2.3. Stable isotopes analysis

Subsamples of 0.15–0.25 mg were transferred into cylindrical tin capsules (5 mm × 9 mm, Säntis Analitical TM) and oven-dried for at least 24 h at 60 °C at National Institute of Health Labs (Rome, Italy). An aliquot of each lyophilized sample was weighted in tin capsules (1.0–1.5 mg) and then analysed at University of Udine Labs for its carbon (C) and nitrogen (N) isotope content ($\delta^{13}C$ and $\delta^{15}N$, respectively), using a CHNS Elemental Analyser (Vario Microcube, Elementar, Langenselbold, Germany) coupled to a stable isotope ratio mass spectrometer (IRMS; Isoprime 100, Elementar).

$\delta^{13}C$ and $\delta^{15}N$ values were reported per mill (‰) on the relative δ-scale and referred to the following international standards: V-PDB (Vienna Pee Dee Belemnite) for the carbon isotope ratio and atmospheric air for the nitrogen isotope ratio. All the results were calculated according to the following equation:

$$\delta\ (‰) = [(R_{Sample}/R_{Reference}) - 1] \times 1000,$$

where R is the ratio of the heavy to light stable isotope (e.g., $^{15}N/^{14}N$) in the sample (R_{Sample}) and in the standard ($R_{Reference}$). Average analytical reproducibility was about ± 0.15‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$. Samples were analysed in duplicate or triplicate with a standard deviation on average lower than 0.15‰ for $\delta^{13}C$ and $\delta^{15}N$ measurements.

2.4. Statistical analyses for PAEs

The mean (SD) CCL and weight of turtles and PAEs concentrations in blood samples were calculated. Spearman's rank correlation tests were used to correlate CCL and weight and CCL and CCW of individuals. All turtles were grouped into two size classes: CCL ≤ 60 cm and CCL > 60 cm size. The analytical results were preliminarily normalized according to the total concentration of PAEs for each sample. Particularly, the frequency of occurrence (%) of the six phthalates (total concentration) in the different turtles was calculated as (a) ratio of single PAE concentration for each turtle and all samples and (b) ratio of single PAE concentration and total PAEs concentration for each turtle.

PAEs concentrations in blood samples were clustered by using a general agglomerative Cluster Analysis (CA) based on the Paired agglomeration method of squared Euclidean distances (Everitt et al., 2001; Ward, 1963). A dendrogram was obtained where PAEs levels were arranged in a hierarchical fashion with different groups of PAEs clustered together in one axis as with similar distribution and their distance metric on another. The relevance of the dendrogram was assessed by means of the cophonetic correlation coefficient (> 0.80).

A Principal Component Analyses (PCA) was applied using the loggerhead turtles as variables and the PAEs values as statistical units. The component scores, the transformed variable values on the new ordination axes, represent a smaller set of new predictor variables that include the correlation patterns of the original variables (Lebart,1984). The loadings on the leading (> 80% of PAEs variance) principal components (Factors) were used to find the correlation of PAEs concentrations and Factor. The scores on the leading Factors were assessed for significant differences (Mann-Whitney test) between turtles with size higher and lower than 60 cm CCL. All tests were run with the free statistical software R (version 3.6.3, R Foundation).

2.5. Stable isotopes and PAEs correlation

Shapiro-Wilk Normality test was used to test normality assumption and Levene's test to evaluate homogeneity of variance. Outliers and samples with PAEs concentrations below limit of detection were not removed in the statistical analyses as the dataset was limited. Pearson correlation and robust statistical tests were run over nonparametric methods with the free statistical software R (version 3.6.3). The Wincor function ("WRS2" package, version 1.1–1, amount of Winsorization tr = 0.1) was used to obtain reliable correlation coefficients between $\delta^{13}C$ and $\delta^{15}N$ values, CN ratio and PAEs values and significant tests of the resulting correlation matrix was visualized thereafter ("corrplot" package, version 0.84). The linear relationship of $\delta^{13}C$ and $\delta^{15}N$ values with PAEs concentrations among the different turtles was modeled with linear robust models (function "rlm" of MASS package, version 7.3–51.5), whereas model parametrization followed a forward variable selection ("bbmle" package, version 1.0.23). Variable selection followed a forward stepwise selection procedure while using the Akaike Information Criterion (AIC) and AIC weights (Anderson, Burnham & White, 1998). The best robust model was visualized with the "visreg" package (version 2.7.0) together with a standard regression (ordinary least squares) that included and excluded the weighed outliers.

3. Results

The loggerhead turtles had a mean CCL = 60.92 ± 9.45 cm, ranging from 39.5 cm and 72.5 cm. Particularly, 5.5% (N = 1) of turtles had CCL < 40 cm, 44.5% (N = 8) had CCL = 40–60 cm, and 50.0% (N = 9) had CCL > 70 cm (Table 1). The mean weight of turtles was 30.23 (± 13.13) kg. A significant correlation was found between CCL and weight of the individuals (Spearman's rs correlation, R = 0.96, N = 18, p < 0.001). CCL and CCW of turtles were highly correlated (Spearman's rs correlation, R = 0.98, N = 18, p < 0.0001), and consequently only CCL was used as measure of turtle size.

The mean (± SD) PAEs concentration in blood samples is reported in Fig. 1. DEHP, DBP and DiBP were the most commonly found phthalates at higher concentrations. However, DOP, DEP and DMP were also found in some samples.

Our results indicated that all studied PAEs were present in the blood of the examined turtles. Particularly, DBP was found in 13 blood samples (72.2%) with concentrations ranging from 6 to 57 ng mL^{-1}, whereas DEHP was found in 13 samples (72.2%) with concentrations ranging from 12 to 63 ng mL^{-1}. DiBP (found in 12 samples of blood; 66.7%) and

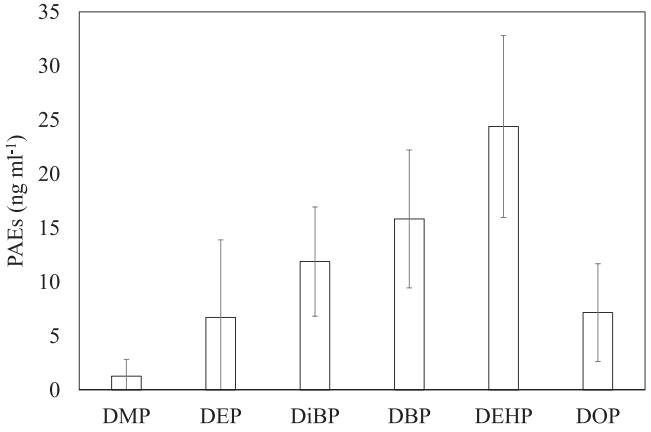

Fig. 1. The mean (± SD) PAEs concentration found in blood samples of loggerhead turtles.

DnOP (found in 7 samples of blood; 38.9%) were found with concentrations ranging from 7 to 41 ng mL^{-1}, and 6–37 ng mL^{-1}, respectively. DMP was found in two samples (1.1%) with concentrations 9 ng mL^{-1} and 14 ng mL^{-1}, respectively. Finally, DEP was found in 5 blood samples (27.8%) with concentrations between 8 and 74 ng mL^{-1}.

The percentages of occurrence per PAE (Fig. 2A) and per sample (Fig. 2B) were calculated showing a different trend for turtles with some PAEs more frequently found at higher concentrations in some turtles than in others.

Three turtles (with CCL > 60 cm) showed the highest total concentration of PAEs (REX = 260 ng mL^{-1}, S507 = 147 ng mL^{-1}, and LUC =124 ng mL^{-1}; Fig. 3A), whereas three turtles were found with concentrations of all PAEs below LODs (KIR, S501 and NAP; LOD < 0.08–0.6 ng mL^{-1}) (Fig. 2A). Furthermore, some turtles were exposed to more types of PAEs than the others (Fig. 2B).

Two principal components Factor 1 (64.79%) and Factor 2 (23.69%) explained 88.5% of variance. On the plot of Factor 1 and Factor 2 scores different groups of turtles were identified (Fig. 3A).

Mean PAEs concentrations were calculated for small-sized (CCL ≤ 60 cm) (67.1 ± 34.2 ng mL^{-1}) and large-sized (CCL > 60 cm) (96.3 ± 86.0 ng mL^{-1}) turtles, excluding samples with concentration below LOD. The total concentration of all PAEs did not differ between large-size and small-sized turtles (Mann-Whitney test, $p > 0.05$). However, the scores on Factor 2 showed a significant difference between small- and large-sized turtles (Mann-Whitney test, $p = 0.01$). Particularly, according to the loadings on Factor 2 (Fig. 3B), DMP, DEP, DiBP and DBP (low-molecular weight PAEs) were higher in large-sized turtles, whereas DEHP and DOP (high-molecular weight PAEs) were higher in small-sized turtles. Indeed, DMP and DEP were found only in large-sized turtles with a mean concentration of 3.3 (± 5.8) ng mL^{-1} and 17.3 (± 25.7) ng mL^{-1}, respectively. DiBP was found in both turtle sizes with higher concentrations in large-sized (18.6 ± 16.2 ng mL^{-1}) than in small-sized (10.5 ± 6.5 ng mL^{-1}) turtles (Mann-Whitney test, $p < 0.001$). DBP was found in both size groups with higher concentrations in large-sized (23.9 ± 20.6 ng mL^{-1}) than in small-sized (14.8 ± 7.5 ng mL^{-1}) turtles (Mann-Whitney test, $p < 0.001$). DEHP was found in both sized turtles with a mean concentration 32.0 ± 21.0 ng mL^{-1} in small-sized turtles and 26.1 ± 17.6 ng mL^{-1} in large-sized turtles (Mann-Whitney test, $p > 0.05$). Finally, DOP was found in both size groups with mean concentration 9.9 ± 14.6 ng mL^{-1} in small-sized turtles and 7.1 ± 8.1 ng mL^{-1} in large-sized turtles (Mann-Whitney test, $p > 0.05$).

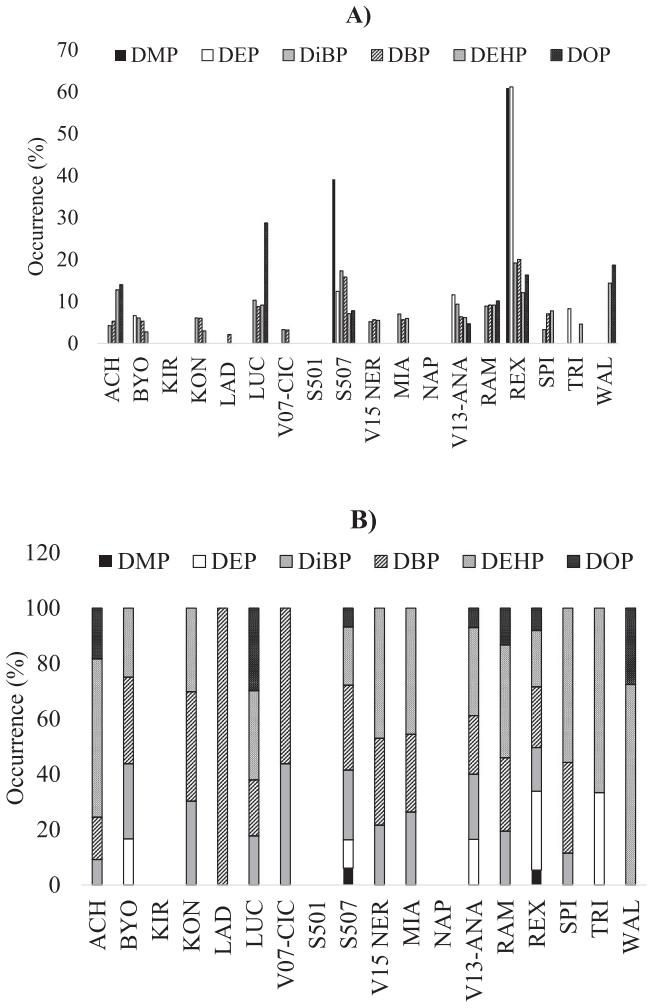

Fig. 2. The frequency of occurrence (%) of the six phthalates in the different turtles calculated as ratio of (A) single PAE concentration for each turtle and total PAE concentration for all turtles, and (B) single PAE concentration and total PAEs concentration for each turtle.

Cluster Analysis using the Paired agglomeration method for the measured values divided the PAEs in three different groups (Clustering coefficient = 0.98) (Fig. 4): DOP and DMP in the first group, DEP, DBP and DiBP in the second group and DEHP in the third group.

The assumption of normality was rejected for both $\delta^{13}C$ and $\delta^{15}N$ values and most of the descriptive variables (DiBP, DBP, DEHP and DOP) (Shapiro-Wilk, $p > 0.05$) whereas homogeneity of variance was not rejected (Levene's test, $p < 0.05$). No significant correlation was found between $\delta^{15}N$ values and PAEs concentrations among turtles(Pearson correlation, $p > 0.05$). Differently, a significant correlation between $\delta^{13}C$ and four low-molecular weight PAEs values (DMP, DEP, DiBP and DBP) was found (−0.82, −0.81, −0.72, −0.67) (Pearson correlation, $p < 0.0001$) (Fig. 5).

The correlation between each PAE indicated high correlation between DMP and DiBP, whereas DEP was not correlated to DiBP and DEP was not correlated to DBP (Fig. 5). DEHP and DOP were correlated to each other but not with the other PAEs (Fig. 5). Finally, a significant correlation of δN^{15} with weight was found (Fig. 6). The final robust model included either DBP or DiBP as the best predictors ($AIC_{wt} = 0.143$ and 0.136) (Fig. 6).

The difference between the two models was low, because the correlation between the two variables was very high (0.95). Primarily, δC^{13} values decreased with increasing DiBP (−0.011) and DBP (- 0.009).

The regression with atypical data was rejected for both DiBP and DBP ($p = 0.080$, $p = 0.079$). A robust regression that considered atypical data gave similar intercepts as regressions that excluded atypical data (DiBP: $p = 0.0018$; DBP: $p = 0.0026$), however the intercepts were slightly steeper (DiBP: robust: −0.012; no outlier −0.011; DBP: robust: −0.0087; no outlier: - 0.0082).

4. Discussion

This study represents the first quantitative analysis of phthalates in the blood of a marine organism, the Mediterranean loggerhead turtle, using an effective and simple analytical approach (Notardonato et al., 2021). The multidisciplinary approach GC-IT/MS coupled to stable isotopes analysis also resulted to be a useful tool to explain the internal dose of PAEs exhibit by loggerhead turtles in terms of $\delta^{13}C$.

Several studies reported the presence of phthalates, mainly DEHP and DBP, or their metabolites, in different tissues and with different concentrations, within and across marine species (Jebara et al., 2021; Rian et al., 2020; Brock et al., 2016; Fourgous et al., 2016; Guerranti et al., 2016; Hu et al., 2016), suggesting that all marine species at a high-trophic level may be largely exposed to phthalates, despite their different diet, feeding habitat and levels of exposure to plastic debris (Serrano et al., 2014). Particularly, in the Mediterranean Sea, detectable concentrations of phthalates, mainly DEHP, were found in several tissues with higher concentrations in some species (i.e. bottlenose dolphin, *Tursiops truncatus*: 89% of the total PAEs detected) (Baini et al., 2017).

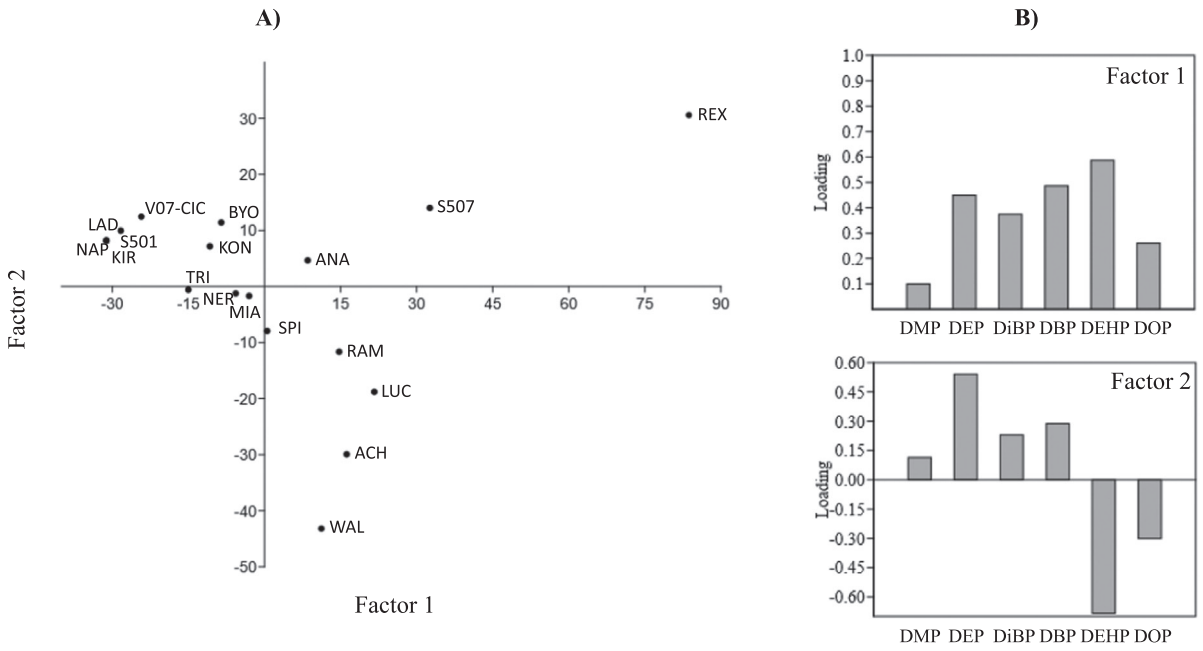

Fig. 3. PCA of PAEs values among samples. (A) Different groups of turtles, each with its code, were identified on the component scores. (B) The loadings (correlation coefficients) of PAEs on the leading principal components, Factor 1 and Factor 2. For turtle code, please see Table 1.

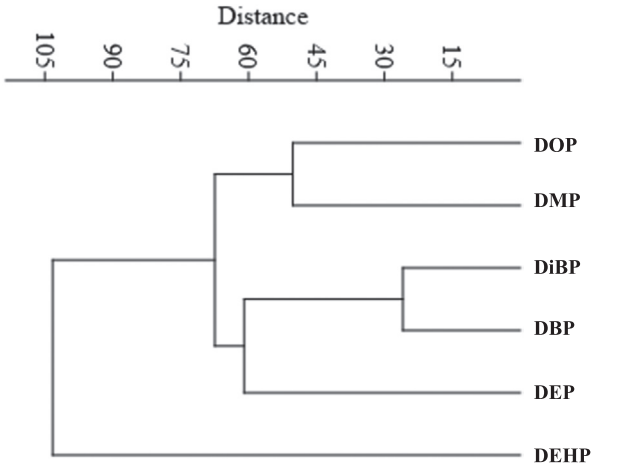

Fig. 4. Cluster analysis using the Paired Agglomeration Method of squared Euclidean distances for the analysed PAEs.

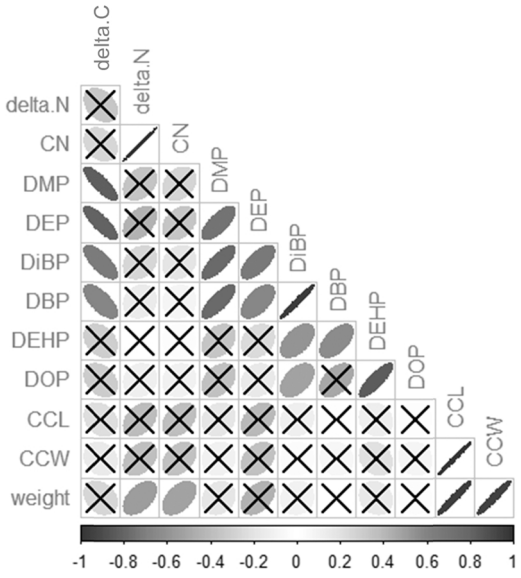

Fig. 5. Correlation plot among PAEs concentrations, stable isotopes $\delta^{13}C$ (delta C) and $\delta^{15}N$ (delta N) and curved carapace length (CCL), curved carapace width (CCW) and weight of turtles. CN is the ratio $\delta^{13}C/\delta^{15}N$.

However, here, appreciable levels of all analysed phthalates were detected in blood samples with concentration levels that varied among specimens and were higher than those found in other tissues of Mediterranean sea turtles (Di Renzo et al., 2021; Sala et al., 2021; Savoca et al., 2018) or in the blubber of cetacean species (Fossi et al., 2012; Baini et al., 2017). In liver and gonads of leatherback turtles (*Dermochelis coriacea*), for example, DEP, DBP, BBP, and DEHP were found in variable concentrations (Savoca et al., 2018), whereas in muscle tissues DBP was found at much lower concentrations than the others. Additionally, from the same study (Savoca et al., 2018), a prevalence of DBP (2,600–19,000 ng g^{-1}) was found in liver and gonads of loggerhead turtles, with high concentrations of BBP (700–9,100 ng g^{-1}) registered only in liver tissues, whereas DEP was absent in all samples. In fat tissues of loggerhead specimens, a major prevalence of DEHP (up to 6000 ng g^{-1}) and DOTP (up to 14,000 ng g^{-1}) was also detected (Savoca et al., 2018). Regular ingestion of plastic debris and contamination from their prey in loggerhead turtles may explain the higher levels of phthalates found in this study compared to values reported previously for other marine vertebrates (Sala et al., 2021).

Several factors could be related to absorption of phthalates in loggerhead turtle blood, such as plastic production trend, polymer properties and metabolic pathways, environmental conditions, and degradation times. Most of PAEs analysed in this study are used as additives in a wide variety of consumer products, mainly single-use plastics, which are main sources of floating debris and can be ingested by loggerhead turtles as leach out in the marine environment (Camedda et al., 2021; Domènech et al., 2019). The capacity of DEHP to partially solubilize in biological fluids, such as blood and plasma, has been already demonstrated (Jaeger et al., 1973). DiBP have been detected in several areas, and its own presence was particularly significant in the marine environment (Zhang et al., 2018), probably due to its

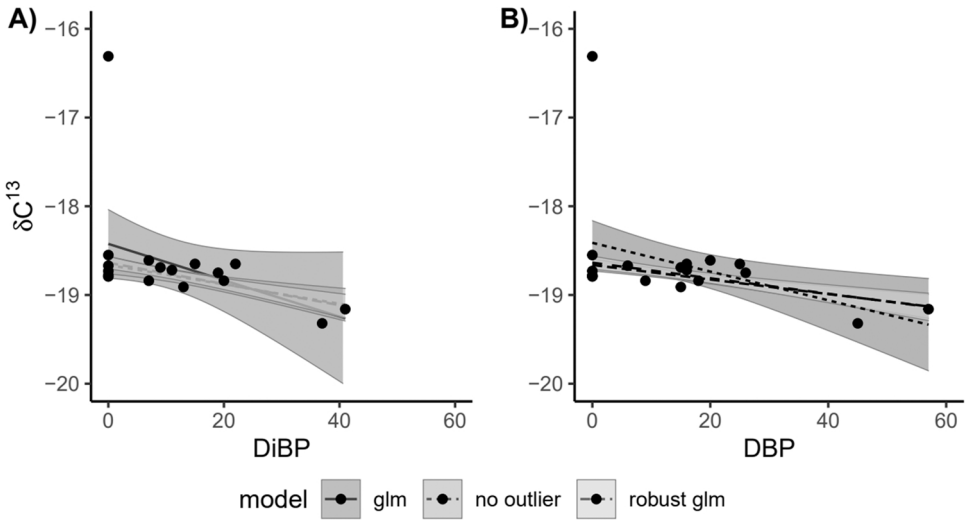

Fig. 6. Final robust model including both DBP and DiBP with the lowest AIC and highest AICwt.

widespread usage in the production of plastic materials. The high concentration of DEHP and DBP in the blood of turtles may be related to their crucial leaching from plastic litter present in the marine environment. Particularly, DEHP is leached by PVC, which is among all polymers the most commonly present in the sea, because it is added to soften PVC during production (Jaeger and Rubin, 1973). DBP, on the other hand, is used primarily as plasticizer to add flexibility to several plastic products found in the form of debris in the marine environment (Manikkam et al., 2013).

Generally, several polymers can release PAEs during degradation and ageing in the marine environment (Paluselli et al., 2019). Thermo-oxidative degradation of plastic debris can lead to the release of chemical products that could have an impact on animals once ingested (Paluselli et al., 2019) and some polymers appear to be more susceptible of others to photodegradation (Fotopoulou and Karapanagioti, 2017). Water salinity, UV radiation and atmospheric deposition may potentially affect the release of plasticizers, specifically for each polymer or type of plastic material, and may be potential sources of phthalates (Suhrhoff and Scholz-Böttcher, 2016). Furthermore, areas with high levels of turbulence, such as coastal areas, might regulate the release of phthalates from different types of plastic debris, as well (Lee et al., 2020). For all these reasons, it cannot be excluded that the detected PAEs concentrations may derived from exposure to phthalates released in the environment during plastics degradation. Among all plastic compounds, polyethylene (PE) and polypropylene (PP) are the most required polymers in term of plastics demand and production worldwide (Plastic Europe, 2019). They are commonly found in the environment (Fotopoulou et al., 2012), and in debris items ingested by loggerhead turtles from the Mediterranean Sea (Camedda et al., 2021; Domènech et al., 2019).

In this study, the total concentration of PAEs did not differ between large-size turtles (CCL $>$ 60 cm) (96.3 $\pm$ 86.0 ng mL^{-1}) and small-size turtles (CCL $\leq$ 60 cm) (67.1 $\pm$ 34.2 ng mL^{-1}). Additionally, DEHP and DOP were found in both small and large sizes turtles with similar concentrations, suggesting that, given their widespread use, all loggerhead specimens during different life stages may regularly encounter opportunities for exposure to the most commonly used polymers (Camedda et al., 2021). Accordingly, some organophosphate esters (OPEs) were detected in loggerhead turtle muscle in the western Mediterranean (Sala et al., 2021) but not in their prey, thus suggesting that direct ingestion of plastic debris may be the main source of exposure to these chemicals. A recent study (Baini et al., 2017) reported a significant correlation between the phthalate's levels and the microplastic size, suggesting that phthalates concentrations may be related to the microplastic size rather than to other microplastic characteristics (colour and shape), or the zooplankton taxa abundance. Finally, it is expected that vinyl plasticizers with more carbon (C_8-C_{13}), such as DEHP, are more liposoluble and consequently might be absorbed more quickly in the different tissues, including blood. However, low-molecular weight PAEs (DMP, DEP, DiBP and DBP), generally used as scent stabilizers in commercial products (Ventrice et al., 2013), were found at higher concentrations in large-size turtles. As already documented in other marine organisms (Savoca et al., 2018), the different distribution of phthalates with turtle size (CCL) may be associated with accumulation of these chemicals during turtles' lives. Considering that Mediterranean loggerhead turtles may reach sexual maturity at about 23–29 years when their size spread 70 cm CCL (Casale et al., 2011), it is expected that the concentration of these phthalates in blood might increase with time, thus showing their higher values in adult individuals. The relationship of phthalates with turtle size was already observed in muscular tissues of Mediterranean loggerhead turtles, where DBP (1250–3200 ng g^{-1}) was found only in samples taken from medium size specimens (56–64 cm CCL) (Savoca et al., 2018). It was also shown that phthalates and other chemical pollutants may be transferred from the mother to the lipid contents of egg yolk in marine turtles (Cerón et al., 2000).

In this study, the internal dose of PAEs exhibit by loggerhead turtles was assessed in terms of dietary habits. In particular, δ^{13}C values appeared to be good predictors for the concentration of DBP and DiBP in loggerhead blood (AIC_{wt} = 0.143 and 0.136), suggesting that turtle diet and foraging habitats might have contribute to exposure among turtles reflecting further metabolic transformation at higher trophic levels.

Stable isotope analyses on scute of Mediterranean loggerhead turtles have shown that pelagic prey at low trophic levels were preferred to those at higher trophic positions with some variations in terms of prey consumption, such as benthic prey and fishery discards, depending on turtle size, health status and trophic differences among individuals (Blasi et al., 2018). Mean carbon isotope ratios estimated here for blood (δ^{13}C: −18.68 $\pm$ 0.56‰) are comparable to those reported for scute by Blasi et al. (2018) (δ^{13}C: −18.1 $\pm$ 0.4‰). However, scute, considered to represent older stable isotope inputs, resulted to be always enriched in ^{13}C compared to blood, which conversely integrates a more recent isotope contribution (data not shown). The wide range of phthalate concentrations found in some specimens might be explained by spatial variation in diet according to local distribution of prey (Revelles et al., 2007; Seney and Musick, 2007) and specific environmental conditions (Casale et al., 2008; Revelles et al. 2007; Hatase et al., 2002). The significant influence of δ^{13}C on DBP and DiBP concentrations might reflect feeding habitats and diet differences among turtles (Blasi et al., 2018;

Snover et al., 2010; Hatase et al., 2002; Godley et al., 1998). It is well known that prey at a higher trophic level also have higher isotopic values (Goodman Hall et al., 2015; Pajuelo et al., 2010), reflecting also high levels of absorption of these chemicals in some tissues (Godley et al., 1999). Heavy metals and organic contaminants have been largely reported at high concentrations in several tissues of loggerhead turtles, including blood, and it is not excluded that part of these xenobiotics might derive from plastic ingestion (D'Ilio et al., 2011). A different distribution of heavy metals along scute of Mediterranean loggerheads have been also documented (Mattei et al., 2015), suggesting accumulation of these contaminants during life stages characterised by different dietary habitats (Musick and Limpus, 1997). Therefore, the inter-individual variability in terms of feeding habits may have influenced also the concentration of PAEs found in this study. It is not excluded that faster growth (Casale et al., 2009) might increase levels of absorption of these chemicals in blood, too, as already demonstrated for other chemicals (Godley et al., 1999). Finally, higher concentrations of PAEs may be partially attributed to the health status of the individual that, given its inability to dive, is forced to feed on prey related to floating-plastic (Blasi et al., 2018).

5. Conclusions

The monitoring of exposure to phthalates is crucial for animal health in the marine environment since several studies have demonstrated their toxicological action as endocrine disruptors, even at low exposures. However, the ecological risks deriving from these chemicals may vary among marine species and tissue, and more studies are necessary to establish the effects of many other compounds. Therefore, we recommend a continuous monitoring of phthalates concentrations in different tissues of those organisms that act as sentinels of the marine environment, as turtles and cetaceans. It can be useful to couple phthalates and stable isotopes analyses in order to link dietary habitats to levels of exposure.

CRediT authorship contribution statement

Monica Francesca Blasi: Conceptualization, Data curation, Methodology, Statistical Analysis, Software, Investigation, Writing – original draft. **Pasquale Avino:** Methodology, Formal analysis, Writing – review & editing. **Ivan Notardonato:** Methodology, Formal analysis, Writing – review & editing. **Cristina Di Fiore:** Methodology, Formal analysis. **Daniela Mattei:** Supervision, Writing – review & editing. **Marco Friedrich Walter Gauger:** Statistical analysis, Software, Methodology, Writing – review & editing. **Michelle Gelippi:** Writing – review & editing. **Davide Cicala:** Methodology, Formal analysis, Writing – review & editing. **Sandra Hochscheid:** Data curation, Writing – review & editing. **Andrea Camedda:** Data curation, Writing – review & editing. **Giuseppe Andrea de Lucia:** Data curation, Writing – review & editing. **Gabriele Favero:** Supervision, Writing – review & editing.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113619.

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