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Phthalate metabolites in loggerhead marine turtles (*Caretta caretta*) from the Mediterranean Sea (East Spain region)

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A B S T R A C T

The exposure of marine reptiles to phthalates has received considerable attention due to the ubiquitous occurrence of these contaminants in the marine environment. The occurrence of phthalate metabolites is established in human populations and marine mammals, but data is scarce for marine reptiles. In this study, concentrations of 18 phthalate metabolites were determined in liver samples from 79 loggerhead marine turtle (*Caretta caretta*) samples collected between 2016 and 2021 in the limits of the Valencian Community (East Spain). For this purpose, the phthalate metabolites were purified from the livers by solid phase extraction and were quantified by UPLC-MS/MS. Fifteen phthalate metabolites were detected in the samples. Monoethyl phthalate (mEP), monomethyl phthalate (mMP), mono-n-butyl phthalate (mBP), phthalic acid (PA) and mono-n-hexyl phthalate (mHxP) were the most abundant metabolites, accounting for detection rates >85%. The highest median concentrations were found for PA (24.2 ng/g dry weight, d.w.) and mHxP (20.3 ng/g d.w.) followed by mMP (12.0 ng/g d.w.), mEP (5.76 ng/g d.w.) and mBP (4.26 ng/g d.w.). The sum of the medians of these five phthalate metabolites (Σ_5PhMet) indicated that concentrations were higher for turtles during year 2020, while a negative association was found between [Σ_5PhMet] and the turtle size. To our knowledge, this is the first study on the biomonitoring of 18 phthalate metabolites in loggerhead marine turtles, and results show that western Mediterranean loggerhead turtles are usually exposed to these contaminants.

1. Introduction

Phthalate esters (phthalates; PAEs) are widespread contaminants that are usually added to a variety of products including (but not limited to) herbicides and personal care products [1–4], such as perfumes [5,6]. They provide plastics with elasticity, softness, and durability properties [1,7]. They are not covalently bound to the polymeric structure of the plastics [7,8], and consequently, they are prone to leaching into the environment. Phthalates have long half-lives (at neutral pH) that can reach up to decades [8]. Nonetheless, their persistence in the environment is dependent on photo-oxidation and photolytic processes, sorption to other materials and biological uptake [8]. They have a relatively high volatilization potential and are easily transported by air and water currents [8]. Due to the continuous littering in coastal areas and the long-lasting nature of plastics in the marine environment, the ubiquitous occurrence of these additives in the oceans is expected.

Phthalates are well established endocrine disruptors, easily incorporated to biota, and consequently, impacting the reproduction and development of species [7,9]. Endocrine disruption effects are demonstrated in both laboratory and epidemiological studies [10,11], although often using unrealistically high concentrations that are not found in the environment [10]. Exposure to phthalates can induce antiandrogenic activity [11–16], promoting the disruption of hormone release [17], but the actual effects on wildlife are understudied. Despite the global decline and vulnerability of reptiles, they are an understudied taxon [18,19]. Experiments with fenced lizards (*Sceloporus occidentalis*) showed that phthalates tend to be retained in adipose and liver tissue [19]. However, potential exposure effects in most of the reptile species must be inferred from data exported from other animal groups, and therefore, conclusions cannot be reliably drawn.

In the Mediterranean, loggerhead marine turtle (*Caretta caretta*) population numbers are directly associated with the health status of the oceans, where declining populations often indicate habitat degradation. Loggerheads play a key role in the marine food web and positively affect the diversity of species and dynamics of the benthic ecosystem [20]. In the Mediterranean, they are categorized as vulnerable by the IUCN (International Union for the Conservation of Nature), while their population survival is still dependent on the conservation actions [21]. Loggerheads ingest high amounts of plastic litter in this sea basin due to their opportunistic feeding behaviour and the highly populated and

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touristic coastal areas that they tend to use for foraging [22–25]. In addition, turtle hatchlings swim towards convergence zones and oceanic fronts where litter tends to accumulate together with food [26,27]. Their sex determination is dependent on the incubation temperature of the nests in sandy beaches [28,29], which in a global warming scenario could lead towards future generations with a higher percentage of females. These expected outcomes can be further worsened by endocrine disruption chemicals such as phthalates that could affect the population; there are reports of pseudohermaphroditism in Mediterranean populations of loggerheads [30], which highlight the endocrine disruption effects of chemicals.

To date, only few studies have addressed phthalates exposures in Mediterranean populations of loggerheads [31,32], and even in studies with other species, sample sizes are generally limited due to challenges surrounding sample collection from wildlife and/or protected species. Moreover, phthalate biomonitoring is challenging due to high background contamination during bioanalytical determination, often leading to overestimations and even false positive results [8,33]. Phthalates are rapidly metabolised by organisms, and therefore, the biomonitoring of their product metabolites, some of those only produced in vivo [3,33,34], can provide more reliable determination and improved exposure assessment [7]. It is noteworthy that the metabolites of phthalates are documented as standalone contaminants in environmental matrices, as they are currently found widespread in river waters [35], marine and freshwater sediments [36,37], and seawaters [37].

With this background, the present study aimed to establish liver concentrations of phthalate metabolites in a large loggerhead turtles' population ($n = 79$) that was sampled from the western Mediterranean Sea along the coastal waters of Spain. The objectives were to: (1) investigate the occurrence of phthalate metabolites; (2) assess the geographical patterns of the major phthalate metabolites in loggerhead turtles along the Valencian Community coast (East Spain); (3) establish associations among the target metabolites; and (4) establish baseline concentrations needed for determining future trends in exposures. Sex differences and the associations between liver concentrations and body size were also investigated. To our knowledge, this is the first study on the occurrence of 18 phthalate metabolites in loggerhead turtles.

2. Materials and methods

2.1. Chemicals and materials

All relevant information was reported by Rian et al. [33]. All target analyte (TAs; Table S1) standards were purchased from Chiron AS (Trondheim, Norway). The isotopically labelled internal standards (Table S1) were purchased from CDN Isotopes Inc. (Pointe-Claire, Canada). Individual stock solutions were prepared in methanol (MeOH) and stored at −20 °C. Polypropylene (PP) tubes (15 mL) and amber glass LC vials (1.5 mL) were purchased from VWR International AS (Oslo, Norway). Ammonium acetate (≥ 98.0% *w/v*), formic acid (98% *v*/v), acetic acid (≥0.99.0% v/v) and *β*-glucuronidase from *Helix pomatia* (type HP-2, aqueous solution, ≥ 100,000 units/mL) were purchased from Sigma Aldrich (Steinheim, Germany). The Milli-Q water was prepared via a water purification system (Qoption, Elga Labwater, Veolia Water Systems LTD, U.K.). The remaining solvents were purchased from VWR Chemicals (Trondheim, Norway).

2.2. Sample collection and area of study

Loggerhead turtle liver samples ($N = 79$) were collected from dead specimens, between year 2016 and 2021, in the limits of the Valencian Community (East Spain; Fig. 1). The Valencian Community is formed by three administrative provinces: Castellón (587,064 inhabitants), València (2,589,312 inhabitants) and Alicante (1,881,762 inhabitants); spanning geographically from north to south of Spain [38]. The samples were collected by the Valencian Community Stranding Network. This

Fig. 1. Number of loggerhead turtles found in each province of the Valencian Community (East Spain).

network involves private and public organizations, including the University of València (València, Spain), l'Oceanogràfic aquarium (València, Spain) and the Environment Office of the Local Government (València, Spain). Most of the turtle liver samples come from turtles that were by-caught in artisanal fisheries, specifically by trammel nets, which are very common in the area (Fig. S1). The remaining samples came from recently deceased turtles stranded at the beach, trapped by trawling nets, or found floating on the sea surface (Fig. S1). Only one from the individuals was captured by longline fisheries (Fig. S1). Gas embolism was the cause of death for all by-caught individuals. The state of the carcasses was classified according to the Geraci and Lounsbury's criteria [39]: 1. denoting “alive”; 2. “freshly dead”; 3. “starting decomposition, but the organs are basically intact”; 4. “advanced decomposition”; and 5. “mummified or skeletal remains only”. Only the individuals belonging to states 2 and 3 were analysed to minimise tissue post-mortem alteration [40] and to realistically reflect contaminant concentrations, which are not affected by organ degradation. The loggerhead turtles were mostly juveniles [mean Curved Carapace Length (CCL, notch to tip) ± (std. dev.) SD: 43.3 ± 12.9 cm; CCL range: 23.5–80.0 cm]. Only the biggest specimen (80.0 cm CCL) could be considered an adult, according to the size range established to estimate age, which considers 80 cm as the size at which sexual maturity is reached [41].

Sterile and stainless-steel materials were used in all the necropsies and in subsequent steps of the protocol that involved cutting and manipulating tissues. Cross contamination was avoided by changing the scalpel blades between the different organs. Each turtle was necropsied separately, shortly after they died. All samples were wrapped in aluminium foil and stored in the darkness at −20 °C until further analyses. Before sample preparation, portions of the liver tissues were freeze dried at −80 °C and 0.06 mbars to transport to the analytical laboratory. Sex was determined by direct observation of the gonads that resulted in a population of 62 females and 17 males.

2.3. Sample preparation

Extraction of phthalate metabolites was performed according to Asimakopoulos et al. [34] and Rian et al. [33] with minor modifications. Liver was chosen as the target organ as it is a major organ for metabolising the TAs. A portion of 100 mg (±25 mg) homogenized freeze-dried liver was added to a 15 mL PP tube, fortified with 20 ng internal

standard mix (IS) and 600 µL 1.0 M ammonium acetate (aqueous solution) were added, followed by 45 min ultrasonication. Thereafter, 600 µL of 1.0 M ammonium acetate (aqueous solution) that contained 22 units of *β*-glucuronidase (prepared by spiking 25 µL of *β*-glucuronidase into 50 mL of 1.0 M ammonium acetate solution) were added, and the samples were digested for 12 h in an incubator (37 °C, stirred at 120 rpm) to determine total concentrations (free and conjugated species) of the TAs. Thereafter, the samples were centrifuged (10 min, 4000 rpm) and their respective supernatants were transferred to a new 15 mL PP tube and were diluted with 2 mL phosphate buffer (2 g sodium phosphate monobasic dehydrate dissolved in 100 mL milli-Q water with 1 mL orthophosphoric acid 85% *v*/v) prior to the loading SPE step. The ABS Elut-NEXUS solid phase extraction (SPE) cartridges (60 mg/3 mL) were conditioned with 1.5 mL acetonitrile and equilibrated with 1.2 mL phosphate buffer followed by the sample loading step. The cartridges were washed after the loading SPE step with 2 mL aqueous formic acid (1% *v*/v), followed by 1.2 mL milli-Q water. The cartridges were dried under vacuum for 15 min prior to elution with 1.2 mL acetonitrile followed by 1.2 mL ethyl acetate. The eluents were then concentrated to near dryness under a gentle stream of nitrogen. Finally, the eluents were reconstituted to 500 µL with acetonitrile: milli-Q water (1:9 *v*/v) for UPLC-MS/MS analysis.

2.4. UPLC-MS/MS analysis

The chromatographic separation was carried out on an Acquity UPLC I-Class system (Waters, Milford, CT, USA) coupled to a triple quadrupole mass analyser (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, CT, USA). Separation was performed with an Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 µm; Waters, CT, USA) serially connected to a SecurityGuard ULTRA C18 guard column (2.1 mm, sub-2 µm core-shell column; Phenomenex Inc., CA, USA) kept at 30 °C. The mobile phase consisted of solvent (A) water containing 0.1% acetic acid (*v*/v) and (B) acetonitrile containing 0.1% acetic acid (v/v). The flow rate was 350 µL min^{-1} and the injection volume was 2 µL. The gradient elution began with 0% B, held for 0.5 min, increased to 25% B in 0.5 min, held for 2.5 min (3.5 min), increased to 35% B in 0.5 min, held for 1.5 min (5.5 min), increased to 50% B in 0.5 min, held for 1.5 min (7.5 min), further increased to 90% B in 0.3 min, held for 1.7 min (9.5 min) before returning to initial conditions (0% B), and finally held for 1.5 min for a total run time of 11 min. Electrospray ionization was conducted in negative ionization mode (ESI-). Multiple reaction monitoring (MRM) mode was applied, and the optimal source settings of the tandem MS instrument were as follows: source temperature 150 °C, capillary voltage 2.8 kV, desolvation temperature 350 °C, and nebulizer gas pressure 6 bar.

2.5. Quality assurance and quality control (QA/QC)

The QA/QC procedures were similar to those reported by Rian et al. [33]. Procedural blanks were analysed to monitor and control background contamination arising from laboratory materials and solvents. Quantification of the TAs was accomplished based on the internal standard method and with matrix-matched standard addition calibration standards as described by Rian et al. [33]. A standard solvent calibration curve ranging from 0.02 to 50.0 ng/mL (0.02, 0.05, 0.1, 0.2, 0.50, 1.00, 2.00, 5.00, 10.0, 15.0, 20.0, 25,0 and 50.0 ng/mL) was prepared and satisfactory regression coefficient for most TAs was achieved ($R^2 > 0.998$); while mCPP and PA demonstrated lower regression coefficients at 0.96 and 0.94, respectively. Pre- and post- extraction spiked matrix samples were used as QA/QC samples and were prepared by spiking known concentrations of the TAs and ISs prior to and post sample preparation (extraction and clean-up). To monitor the drift in instrumental sensitivity and carry-over effects during analysis, a calibration check standard and a methanol solvent blank solution were injected, respectively, after the analysis of 25 consecutive samples. More details concerning method details and performance, but also the abbreviations of the parent phthalates are available in the supplementary information (Tables S1-S4).

2.6. Data and statistical analysis

The distribution of the data (concentrations) was non-normal, and consequently, non-parametric tests were used for statistical treatment. Kruskal-Wallis test was used to search for significant differences between the concentration of the TAs and the independent variables of CCL, location, year and the carcass state. Mann-Whitney *U* test was used to perform pairwise comparisons between the concentrations of the TAs and sex. Spearman correlations were used to explore the associations between the phthalate metabolites themselves, and between metabolites' concentration and body size. The probability value of $p < 0.05$ was set for statistical significance in all analyses. All data were recorded in Microsoft Excel (2021) and the analyses and data visualisations were carried out with RStudio [42] and ggplot2 [43]. Chromatographic data was acquired and processed with Intellistart, MassLynx and TargetLynx software packages (Waters, Milford, U.S.). Data analysis did not include non-detects (NDs). Concentrations were reported as ng/g dry weight (d.w.). Wet weight values were estimated dividing by a factor of 3.2, assuming a 68% of water content in the liver from Mediterranean loggerhead turtles [44].

3. Results and discussion

3.1. Liver concentrations of phthalate metabolites

Five TAs (mMP, PA, mEP, mBP, and mHxP) demonstrated detection rates (DRs) > 85% (Table 1). From those the metabolite with the highest median concentration was PA (24.2 ng/g d.w.). The highest detection rates (DRs > 90%) were found for mMP, mEP, and PA that are analogues with shorter carbon chains and lower molecular weights (MW). Regarding their abundance and documented associations with the parent phthalates, lower MW metabolites are considered more reliable biomarkers [7]. The presence of phthalate metabolites in the tissues is indicative of continuous exposure to phthalates since the parent phthalates are rapidly metabolised, although the exact time frame of metabolization depends on factors such as developmental stage and route of exposure [45]. The metabolites mPEP and mIPEP were not detected. The concentrations determined in other aquatic species were presented in Table 2.

The metabolite mEHP is formed by the hydrolysis of di(2-ethyl-1-hexyl) phthalate (DEHP) [46], which can be oxidized and form

Table 1
Occurrence of phthalate metabolites in liver samples of loggerhead turtles ($N = 79$).

Target analytes	Detection rate (%)	Mean (ng/g d.w.)	Median (ng/g d.w.)	Min (ng/g d.w.)	Max (ng/g d.w.)
mMP	100	33.4	12.0	1.56	1463
PA	98.7	74.7	24.2	16.7	657
mEP	96.2	77.6	5.76	0.83	4940
mBP	89.8	5.51	4.26	0.83	17.4
mHxP	87.3	27.7	20.3	0.83	95.9
mNP	84.8	10.6	9.83	0.89	33.1
mEHP	82.2	27.4	22.3	4.19	90.5
mIBP	79.7	4.11	2.21	0.83	91.5
mOP	74.6	5.29	3.93	0.83	24.9
mCPP	73.4	15.6	9.92	3.33	87.8
mBzP	20.2	46.3	5.77	3.45	449
mDeP	27.8	73.7	38.9	0.55	418
mEHHP	5.0	4.64	2.56	1.67	11.3
mCHP	2.5	528	528	60.5	996
mHepP	1.2*	2.08	2.08	2.08	2.08

* one sample.

mEOHP and mEHHP in humans [34,46]; mEHHP and mEOHP are secondary metabolites and are considered reliable markers of the presence of DEHP in animal tissues because they are produced in vivo [3,33,34]. On the contrary, mEHP can be formed by abiotic processes, and therefore, it is not as reliable biomarker of exposure as mEHHP and mEOHP. However, in the liver of the turtles herein, mEOHP was not detected, while mEHHP had a low DR of 5%. The mEHP concentrations found herein were higher by one order of magnitude (median: 22.3 ng/g d.w.) than those reported in bottlenose dolphin urine (median: 4.57 ng/mL, Table 2) [47] and in Mediterranean tuna muscle [1.73 ng/g (wet weight; w.w.), [48]], while approximately 3000 ng/g w.w. of the parent compound, DEHP, were reported in a *C. caretta* liver [31] and in *C. caretta* eggshells/yolk (medians of 12.5 ng/g and 7 ng/g w.w., respectively, Table 2) [32].

PA is a generic hydrolysis end product of phthalates' metabolism; and therefore, it is a common biomarker of total phthalate exposure [33,49]. Laboratory animal experiments documented that PA could induce oxidative stress [34,50,51]. PA was also found in harbour porpoise livers from Norway [33] at the same order of magnitude [median concentrations in porpoises: 7.75 ng/g w.w; and in turtles: 24.2 ng/g d.w. (estimated: 7.56 ng/g w. w.), Table 2].

mEP is a metabolite of DEP and presented a median concentration of 5.75 ng/g d.w. (estimated: 1.80 ng/g w.w.). mEP was found on the same order of magnitude as the concentrations found in the livers of harbour porpoises from Norway (*Phocoena phocoena*) [33] and in the urine of bottlenose dolphins from Florida, USA (*Tursiops truncatus*) [1,47]. However, a few samples with significant higher concentrations render this metabolite more variable in our study, where the concentrations ranged from 0.83 up to 4940 ng/g d.w. (estimated concentrations: 0.26–1543 ng/g w.w.) vs. the concentrations ranging from 2.62 to 17.4 ng/g w.w. in Rian et al. [33]. Nonetheless, other studies focus on quantifying the parent compound, DEP. For instance, Vorkamp et al. [52] analysed DEP in livers from polar bear (*Ursus maritimus*), minke whales (*Balaenoptera acutorostrata*), pilot whales (*Globicephala melas*) and ringed seals (*Phoca hispida*). By assuming that 100% of the parent DEP is metabolised to mEP by the organism then the DEP concentrations

Table 2
Phthalate concentrations in tissues from different animal species.

Location	Species	Matrix	Concentration	Units and reported statistic	Study
West Atlantic Ocean	Bottlenose dolphin	Urine	mEHP: 4.57 mEP: 4.51	ng/mL (mean)	[47]
North Atlantic Ocean	Polar bear Minke whale Pilot whale Ringed seal Northern fulmar	Liver	DEP: 13.2–76.9 DMP: < 1.5–56.5 DBP: 2.5–28-7 DnHP:2.5–107.4 BBP: 6.3–68.6 DEHA[#]: n.d.* - 144.3 DEHP: n.d. - 151.2 DNOP: < 1.5–45	ng/g w.w. (range)	[52]
North Atlantic Ocean	Fin whale	Skin	DEP: 303 DBP: 303	ng/g d.w. (median)	[53]
Norwegian Sea	Harbour porpoise	Liver	mEP: 5.67 mBP: 25.2 mIBP: 30 mMP: 0.34–8.72 mHxP: 0.63 mOP: 0.63 PA: 7.75	ng/g w.w. (median)	[33]
Central Mediterranean Sea	Loggerhead turtle	Gonads, liver and muscle	DEHP: 3000 DEP: n.d. DBP: 2600–19,000	ng/g w.w. (not specified/range)	[31]
Central Mediterranean Sea	Loggerhead turtle	Eggshell and yolk	DEHP: n.d. - 12.5 DEP: n.d. DBP: 13–40.5	ng/g w.w. (range)	[32]
Central Mediterranean Sea	Bluefin tuna	Muscle	DEHP: 9.43 mEHP: 1.73	ng/g w.w. (median)	[48]
Mediterranean Sea	Fin whale Risso's dolphin Striped dolphin Bottlenose dolphin	Skin	BBzP: 259.9–1629 DEHP: 1130–26,068 DIOP: 306.7–3031 mEHP: 463.7–1770 mBzP: 5.98–93.7 mBP: 780.1–984	ng/g d.w (range)	[56]
North Mediterranean	European eel	Muscle	mEP: 33 mIBP: 206 mBzP: 174 mEHP: 82 mEHHP: 282 mEOHP: 94 mMP: 5.7 mOP: 2	ng/g d.w. (mean)	[55]
Western Mediterranean	Loggerhead turtle	Liver	mEHP: 22.3 mEP: 1.80 mBP: 1.33 mIBP: 0.70 mHxP: 6.33 mMP: 3.76 mOP: 1.22 PA: 7.56	ng/g dw. (median)	This study

*n.d.: Not detected; DEHA: [#]Di(2-ethylhexyl) adipate.

(15.1 to 31.6 ng/g w.w.) in that study differs from the mEP median concentration found herein (5.75 ng/g d.w.; estimated: 1.80 ng/g w.w.). In another case, DEP was found in Icelandic fin whale skin samples, but in much higher concentration (median : 303 ng/g d.w.) [53]. DEP was also analysed in *C. caretta* tissues from Central Mediterranean in Sicily, in which it was not detected [31], and in *C. caretta* eggs from Linosa Island, in which DEP was detected in some samples (max: 79 ng/g, Table 2) [32].

The isomers mBP (median: 4.26 ng/g d.w., estimated 1.33 ng/g w.w.) and mIBP (median: 2.21 ng/g d.w., estimated 0.70 ng/g w.w.) were found herein in 89.8 and 79.7% of the samples, respectively. They are both products of the metabolic breakdown of DBP. The mBP and mIBP concentrations were found lower herein than those in harbour porpoises from Norway, where medians of 25.2 and 30 ng/g w.w. were reported, respectively [33]. Savoca et al. [31] determined a high concentration of DBP (2600–19,000 ng/g w.w.) in liver and muscle of *C. caretta* from the Mediterranean Sea, while García-Garín et al. [53] determined DBP median concentration of 303 ng/g d.w. in Atlantic fin whales. In another study, DBP was determined in *C. caretta*'s eggshells, yolk, and albumen, with median concentrations of 26, 40.5 and 13 ng/g, respectively (Table 2) [32].

mMP, the major metabolite of DMP, was found in all liver samples (median: 12.0 ng/g d.w., estimated 3.76 ng/g w.w.) and in the same order of magnitude as in harbour porpoises (0.34–8.72 ng/g w.w.) [33], and as in the liver and fat of other marine mammal species (2.5–8.4 ng/g w.w.) [52]. Neither mMP nor DMP were documented in marine turtle tissues before. mHxP, the metabolite of DHxP, was found in 87.3% of the samples in this study and demonstrated a median concentration of 20.2 ng/g d.w. (estimated 6.33 ng/g w.w.), which was higher than that determined in the liver of harbour porpoises (detection rate of 45%, median of 0.63 ng/g w.w. [33]. The parent compound, DHxP, was also reported in marine mammal livers [52], and in human urine with detection rate of 59% [54].

Lastly, median concentrations of mOP in turtle livers (3.93 ng/g d.w., estimated 1.22 ng/g w.w.) were in the same order of magnitude with those determined in harbour porpoises' liver (0.63 ng/g w.w.) [33], but lower than those reported in European eel muscle (*Anguilla anguilla*, mean: 82 ng/g d.w.) [55]. Higher concentrations of mOP in eels could be explained by their habitat. Eels live in more enclosed bodies of water, while porpoises and marine turtles live in the open sea, where contaminant concentrations can be more diluted. The mOP precursor, DIOP, was quantified in marine mammals' skin from the Mediterranean, including *Balenoptera physalus*, *Grampus griseus*, *Stenella coeruleoalba* and *Tursiops truncatus* [56]. Although its detection rate was lower (47.4% in contrast to 74.6% in this study), concentrations in marine mammals were found to be higher than herein (306.7–3030.8 ng/g d.w. in contrast to a median of 3.93 ng/g d.w., Table 2).

To sum up, the median concentrations of the respective metabolites [mBP, mIBP, mBzP, mEHP; range: 2.21–22.3 ng/g d.w.] reported herein are lower than the concentrations of the parent phthalates (if assumed that the parent phthalates convert 100% to their respective metabolites) found in *C. caretta* tissues from the coasts of Sicily (DBP: 1250–19,000 ng/g; DEHP: 2000–5900 ng/g w.w. approximately) [31]. Concerning other species from the Mediterranean Sea, phthalate concentrations found herein are generally lower to those reported in cetacean blubber by Baini et al. [56] (mBzP = 32.1 ng/g d.w. in one individual; mBP: 780.1–983.7; mEHP: 463.7–1770; BBzP: 259.9–1629.1; DEHP: 1130–26,068; and DIOIP: 306.7–3030.8 ng/g d.w); but higher than those concentrations found in tuna muscle samples (mEHP: 1.5–6.3 ng/g w.w.) analysed by Guerranti et al. [48].

It is noteworthy that the turtles that demonstrated the highest concentrations of PA (end product of phthalates metabolism), did not present high concentrations of the other metabolites, potentially due to their metabolic elimination to PA. Metabolic profiles vary depending on the species, and therefore, further studies concerning phthalates' metabolism in reptiles are deemed necessary.

The correlation between mBP and mHxP ($r = 0.68$) indicated a concomitant source of exposure. This could be explained by the fact that parent compounds of mBP and mHxP are co-occurring in many materials and consumer goods [33,57,58]. The sum of the medians of the five phthalate metabolites with higher DRs (Σ_5PhMet; including, in descending order, mMP, PA, mEP, mBP, and mHxP) demonstrated strong correlation with mIBP ($r = 0.96$), and a weaker positive correlation was found between Σ_5PhMet and mEHP ($r = 0.22$).

3.2. Concentrations of phthalate metabolites and their relationship with biometric and spatial-temporal variables

The location and year of sampling, and the origin, size and sex of the turtles, did not significantly influence the profile or concentrations of the metabolites in liver. This finding agrees with previous studies on marine mammals [47,53] and European eels [55]. However, a slight negative correlation was found between Σ_5PhMet (mMP, PA, mEP, mBP and mHxP) concentration and size (CCL, $r = -0.296$). This correlation indicated that the larger the size of the turtle, the lower the concentration of those metabolites. However, a wider size range would allow to better assess size effect on metabolites' concentration, as turtles in this study belong to a juvenile stage, except for one specimen. A negative correlation between the harbour porpoises' size (*Phocoena phocoena*) and PA was previously observed [33]. Previous studies indicated that contaminants, including phthalates and their metabolites, tend to be more concentrated in smaller individuals [33,59]; although other studies show no relationship between contaminant concentrations and size in marine turtles and other species, including cetaceans and fish [31,33,47,48,55,60,61]. Similarly, no correlations were found between the concentration of phthalate metabolites and the sex of the analysed turtles. However, more research is needed concerning these variables, as this is the first study to explore a possible relationship between phthalates metabolites and sex in marine turtles. The sex of this sample set was biased towards the females (females = 62, males = 17), which is an additional reason to further explore the relationship between phthalate content and sex. Furthermore, it is noteworthy that sex determination in marine turtles depends on the incubation temperature of the nest. Increasing temperatures in a climate change scenario could bias the population towards more females; effect that could add up to the potential antiandrogenic effects triggered by phthalates and, eventually, hindering long-term population stability.

Phthalate metabolites concentrations statistically differed depending on the year ($p < 0.005$). The Σ_5PhMet concentration was increasing steadily since 2016 and a steeper increase in 2021 (Fig. 2, median: 229 ng/g d.w.) than in previous years (Fig. 2, median in 2016: 63.2 ng/g d.w.; 2017: 67.6 ng/g d.w.; 2018: 78.4 ng/g d.w.; 2019: 56.3 ng/g d.w.; and in 2020: 88.4 ng/g d.w.) was observed.

This increase (Fig. 2) could be explained by an abnormally high

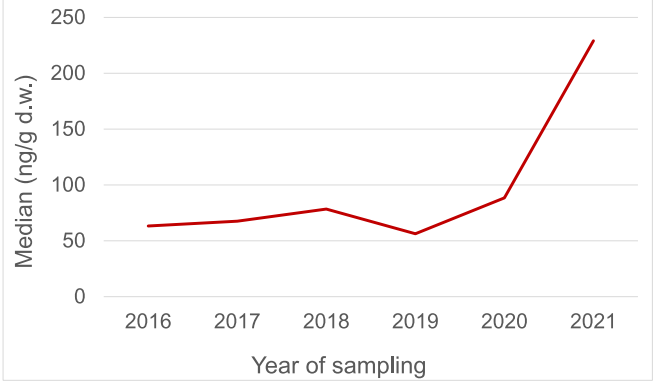

Fig. 2. Evolution of Σ_5PhMet concentrations in the years of sampling (2016–2021). Σ_5PhMet includes mMP, PA, mEP, mBP, and mHxP.

discharge of waters coming from the surrounding agricultural fields and wastewater treatment plants (WWTPs) during 2020 and 2021. Several beaches in the area were repeatedly closed to the public due to unknown polluted water discharges [62,63]. Nevertheless, it is known that phthalates do appear frequently in this type of water discharges in Spain [64] and that the Valencian Community is the second region in Spain with the highest volume of urban wastewater discharges [65]. Another possible explanation could be the constant increase in the amount of waste reaching the ocean, which is expected to continue [66,67], but it may also be the result of the increase of single-use plastic formulations and the mismanagement of plastic disposal following the Sars-CoV-2 pandemic [68]. It is noteworthy that the Σ_5PhMet concentrations were higher in the more industrialised and populated province (Fig. 3), e.g., Valencia, although differences were not significant. The occurrence profile of the metabolites was similar in all the three provinces, which indicated a similar metabolic profile regardless of the origin of the turtles. Concentrations of phthalate metabolites and other contaminants are not necessarily associated; and therefore, the concentration of a contaminant in one environmental or biological compartment does not necessary allow to assess the risks in biota [69]. However, it is necessary to continue monitoring these chemicals in a variety of matrices and study their mobility through the ecosystems to better understand risks and consequences of exposure.

4. Conclusions

This is the first study to document phthalate metabolites concentrations in loggerhead turtles, suggesting a novel approach in assessing phthalate exposures in marine reptiles by measuring their metabolites, and not their respective precursors. This method can overcome some common problems of parent phthalates biomonitoring, such as overestimation and high background contamination. As a result, we demonstrated that phthalate metabolites are commonly found in western Mediterranean loggerhead turtles, suggesting continuous exposure of turtles to artificial materials that leach these types of chemicals, including plastics. A significant increase in phthalate metabolites' concentration was documented during 2020, possibly due to an increase in single-use plastics during the Sars-CoV-2 pandemic or in uncontrolled wastewater discharges. Finally, a negative association was found between phthalate metabolites' concentration and turtle size, suggesting a dilution effect.

5. Animal ethics permit

Ethical approval was not sought for the present study because no handling or sampling were performed on live animals. All samples were opportunistically sourced from either dead stranded animals or from dead animals that were found as part of by-catch in commercial trawling or artisanal fisheries in the area. The responsible network of this sourcing is the Valencian Community Stranding Network, operated by the University of Valencia, l'Oceanogràfic Aquarium and the Environment Office of the Local Government (all located at València, Spain).

CRediT authorship contribution statement

Olga Novillo Sanjuan: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Shannen Thora Lea Sait:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. **Susana V. Gonzalez:** Resources, Methodology, Writing – review & editing. **Jesús Tomás:** Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Juan A. Raga:** Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Alexandros G. Asimakopoulos:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

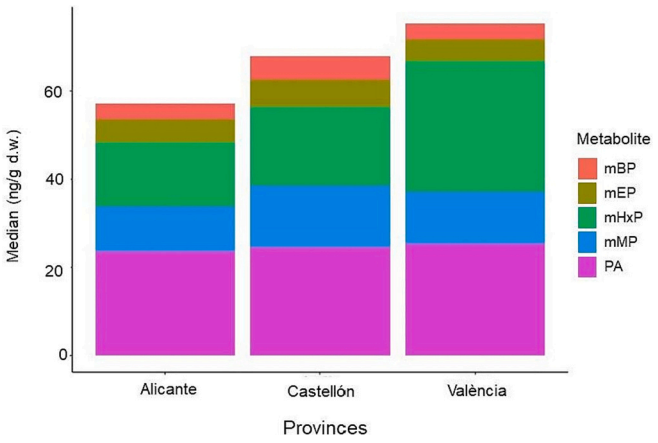

Fig. 3. Sum concentrations (Σ_5PhMet) of the five most abundant phthalate metabolites in liver samples of fresh carcasses of loggerhead turtles stranded or bycaught along the Valencian Community coast (E Spain; 15 individuals from Alicante, 41 individuals from Castellón and 23 individuals from València).

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enceco.2023.08.003.

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