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Organophosphate ester plasticizers in edible fish from the Mediterranean Sea: Marine pollution and human exposure☆

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ABSTRACT

Concentrations of organophosphate esters (OPEs) plasticizers were analysed in the present study. Fifty-five fish samples belonging to three highly commercial species, European sardine (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), and European hake (*Merluccius merluccius*), were taken from the Western Mediterranean Sea. OPEs were detected in all individuals, except for two hake samples, with concentrations between 0.38 and 73.4 ng/g wet weight (ww). Sardines presented the highest mean value with 20.5 ± 20.1 ng/g ww, followed by anchovies with 14.1 ± 8.91 ng/g ww and hake with 2.48 ± 1.76 ng/g ww. The lowest OPE concentrations found in hake, which is a partial predator of anchovy and sardine, and the higher $\delta^{15}N$ values (as a proxy of trophic position), may indicate the absence of OPEs biomagnification. Eleven out of thirteen tested OPEs compounds were detected, being diphenyl cresyl phosphate (DCP) one of the most frequently detected in all the species. The highest concentration values were obtained for tris(1,3-dichloro-2-propyl) phosphate (TDClPP), trihexyl phosphate (THP), and tris(2-butoxyethyl) phosphate (TBOEP), for sardines, anchovies, and hakes, respectively. The human health risk associated with the consumption of these fish species showing that their individual consumption would not pose a considerable threat to public health regarding OPE intake.

1. Introduction

Plastic pollution is an increasing threat for marine ecosystems globally (Eriksen et al., 2014; Worm et al., 2017) where the Mediterranean Sea stands out as a remarkably impacted area (García-Rivera et al., 2017; García-Rivera et al., 2018; De Haan et al., 2019; Compa et al., 2019) with estimates of up to 11.5 million items of floating marine macro-litter (Lambert et al., 2020) and a density of 16 items km^{-2} along the North Mediterranean Sea (Garcia-Garin et al., 2020a). Marine organisms are capable of ingesting plastics and microplastics with potential perturbations at different physiological levels (Gabriel et al., 2020). Beyond the ingestion of plastic itself, it is an increasing concern the accumulation of plastics additives and pollutants and the capacity of some of those to bioaccumulate and/or biomagnify (Bekele et al., 2019; Ding et al., 2020). Chemical additives associated with plastics, such as plasticizers and flame retardants like organophosphates ester (OPEs), deserve special attention due to their environmental widespread and their toxicological effects. OPEs are increasingly being used in several products (i.e. plastics, textile, furniture, etc.) and can be released into the environment through different processes (like abrasion and volatilization) resulting in a wide range of concentrations levels detected throughout the environment: indoor/outdoor air, water, soil, and sediment, as well as in animals and humans (Li et al., 2019).

Some OPEs are volatile and predominate in the air phase, while others are water soluble or sorb strongly to particulate matter (Reemtsma et al., 2008). Air deposition, river flux, air-water distribution and macro- and micro-plastics are different sources of OPEs in the marine environment. Due to their ubiquity and increasing environmental

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levels, OPEs have been the focus of recent studies in the marine biota (Sala et al., 2019; Garcia-Garin et al., 2020b; Aznar-Alemany et al., 2021), with increasing interest to investigate the potential derived toxicological effects in biota. For instance, some OPEs were proven to be neurotoxic and carcinogenic (van der Veen and de Boer, 2012) and *in vitro* studies also confirm OPEs to cause developmental neurotoxicity, as well as adverse transcriptomic, reproductive, endocrine, and carcinogenic effects (Frydrych, 2015; Du et al., 2019). Moreover, adverse effects on female reproduction and fetal development in humans have been described (Hu et al., 2017). Nevertheless, the information related to the occurrence of OPEs in marine organisms is still limited and the ultimate implications for human health are mostly unknown, with no reference doses for most OPEs (Bekele et al., 2021).

In the Northwestern Mediterranean Sea, European anchovy (*Engraulis encrasicolus*) and European sardine (*Sardina pilchardus*) are two of the most abundant and commercially important small pelagic fish (SPF) species (Cury et al., 2000). Beyond their economic importance, both species play a key ecological role at the intermediate levels of the food web (Coll et al., 2008; Palomera et al., 2007). Sardine and anchovy are planktivorous feeders, inhabiting the continental shelf and shelf-break, with sardine selecting shallower waters and anchovy occupying larger off-shore areas (Palomera et al., 2007). An important decline in sardine and anchovy populations in the Western Mediterranean Sea has been observed in the last decades, especially evident in the subareas GSA06 and GSA07 (as defined by the General Fisheries Commission for the Mediterranean (GFCM) (Coll et al., 2019; Coll and Bellido, 2019; Palomera et al., 2007; Saraux et al., 2019). This decline has not only implied a reduction of biomass and landings, but also a decrease in individual's total body size and condition (Albo-Puigserver et al., 2019; Van Beveren et al., 2014). Various hypotheses have been considered to explain these trends; for instance, an increase in fishing pressure, environmental changes (*i.e.* variations in temperature), changes in plankton composition, disease (including parasites), the impacts of recovering predators or competitors, or an increase in pollution (Albo-Puigserver et al., 2019; Coll et al., 2019; Coll and Bellido, 2019; Palomera et al., 2007; Pennino et al., 2020; Saraux et al., 2019). Precise knowledge of potential threats affecting SPF dynamics is important, as changes in their population may have consequences in higher trophic levels within marine ecosystems and in regional fisheries. In fact, a recent study expressed concern about the underlying effect for the stock of European hake (*Merluccius merluccius*), an important predator of anchovy and sardine (Sion et al., 2019).

In this context, a better understanding of the extent of plastic pollution, direct and/or indirect (such as through the herein analysed OPE plasticizers) in sardine and anchovy can help to identify the true extent of this threat. Previous studies have documented microplastics and fibres in the stomach contents of sardines and anchovies (Compa et al., 2018; Pennino et al., 2020), with values up to 58% and 60% recorded, respectively (Pennino et al., 2020) but their levels of OPEs in this areas had not been assessed before. We also included European hake in our analysis to compare the concentration of OPE plasticizers with those of sardine and anchovy and investigate potential bioaccumulation and/or biomagnification of these pollutants. European hake, with a wide bathymetric range occupying most of the shelf and shelf-break (Demestre et al., 2000; Fisher et al., 1987), is one the most important demersal target species for commercial fisheries in the Mediterranean Sea (Sánchez et al., 2007) and it is known to partially prey on sardine and anchovy with clear ontogenetic changes on their feeding patterns (Bozzano et al., 1997; Cartes et al., 2009, 2004; Ferraton et al., 2007; Lloret-Lloret et al., 2020).

In this study, our main objectives were: (i) to investigate the incidence of OPE plasticizers in three edible fish, two small pelagic fish and a higher trophic predator, along a latitudinal gradient in the Northwestern Mediterranean Sea; (ii) to relate the recorded contaminant values with potential biomagnification process; and (iii) to calculate the human daily intake of these pollutants based on estimates of fish consumption. The information generated here can provide insight towards the Sustainable Development Goals (SDG), covering not only SDG_{14}; the restoration and conservation of marine and coastal areas and ecosystems, but also SDG_3, aiming to improve both human and ecosystem health as well as animal well-being (Borja et al., 2020; United Nations, 2016).

2. Materials and methods

2.1. Sample collection and study area

Fish samples of European anchovy, European sardine, and European hake were collected in the Western Mediterranean area (Fig. 1), in Geographical Sub-Areas 6 (GSA06) during the MEDITS 2019 survey (MEDiterranean International bottom Trawl Survey (Bertrand et al., 2002a; Bertrand et al., 2002b). Samples were obtained through a bottom trawl GO73 with a 20 mm cod-end mesh size net (Bertrand et al., 2002). The GSA06 includes the Spanish continental waters of the Western Mediterranean Sea, from "Cape of Creus" in the north, to "Cape of Palos" in the south. It is an important fishing ground in the Mediterranean Sea (FAO, 2020; Papaconstantinou and Farrugio, 2000) characterized by topographic and hydrographic features with a latitudinal gradient (Estrada, 1996). The continental shelf broadens in the south, and it is widest around the Ebro Delta. It is considered a highly productive area due to the run-off from the Ebro River and the Liguro-Provençal-Catalan current which is the main current in the area, flowing south-westwards along the continental slope (Estrada, 1996).

Fifty-five fish samples were collected during May and June 2019 (Table S1) from the four sampling sites selected: Cape of Creus, Ebro Delta, Gulf of Valencia and Gulf of Alicante (Fig. 1). Samples were wrapped on aluminium foil, to prevent cross-contamination from plastic material, and were frozen onboard for further analysis (ICES-CIEM, 2015). Once in the laboratory, samples were defrozen and total length, sex and maturity stage were noted. Muscle samples of each fish were

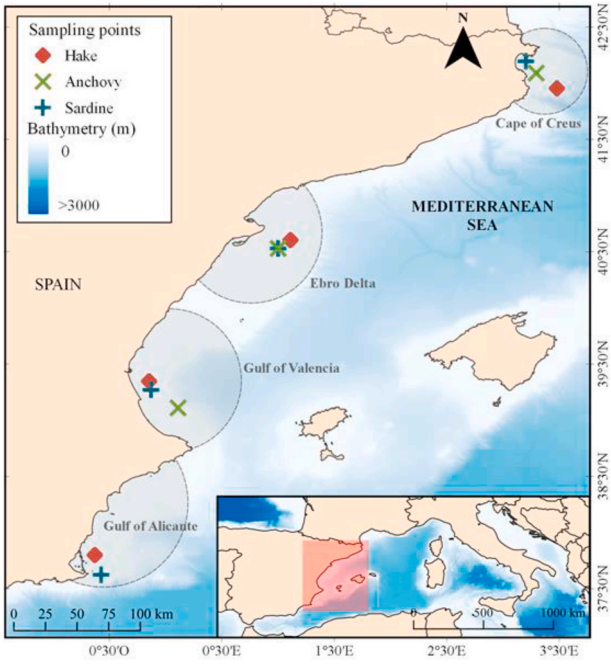

Fig. 1. Map of the Western Mediterranean Sea showing our four study areas (Cape of Creus, Ebro Delta, Gulf of Valencia and Gulf of Alicante) (in shaded grey) and the geographical locations where individuals of European hake (red rhombus), European anchovy (green cross) and European sardine (blue cross) were collected. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

extracted for stable isotope determination and analysis of contaminant concentration. Throughout this process, samples were always handled over a non-plastic surface and only metal instruments were employed. European hake individual length ranged between 24.2 and 41.1 cm, being most of them in active mature stage; European sardine were between 13.5 and 16.3 cm and all of them were in the mature stage; and European anchovy individuals presented the smallest length, which ranged between 11.4 and 15.4 cm and they were mostly at mature stage (Table S1).

2.2. Standards and reagents

Thirteen OPEs were included in this analytical study. Different analytical standards were used. Tris(2-chloroethyl)-phosphate (TCEP), TBOEP, THP, tris(2-ethylhexyl) phosphate (TEHP) and tris (chloroisopropyl)-phosphate (TClPP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2-ethylhexyldiphenyl phosphate (EHDPP) and isodecyldiphenyl phosphate (IDPP) were purchased from AccuStandard (New Haven, CT, USA). Tri-cresyl phosphate (TCP) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Diphenyl cresyl phosphate (DCP), triphenyl phosphate (TPHP), TDClPP, tri-n-butyl phosphate (TNBP), and triphenylphosphine oxide (TPPO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Labelled internal standards (IS) were also used. d_{15}-TPHP was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), whereas d_{27}-TNBP, d_{15}-TDClPP, $^{13}C_2$-TBOEP and d_{12}-TCEP were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada).

Acetone and hexane solvents for organic trace analysis were purchased from J.T. Baker (Center Valley, PA, USA). Methanol and water solvent were obtained from Merck (Darmstadt, Germany).

2.3. Sample preparation

Sample preparation was based on a previously developed method (Giulivo et al., 2016). All samples were lyophilized and homogenized and for every batch of samples, a laboratory blank with hydromatrix was done. To proceed with the ultrasounds extraction, 0.5 g of dry weight (dw) were transferred to a centrifuge tube with 15 mL of hexane:acetone (1:1). After 15 min in an ultrasound bath and 20 min of centrifugation at 22 °C and 4000 r.p.m, the liquid phase was collected. Extraction was carried out twice, and then both extracts were combined. Samples were concentrated to complete dryness under a gentle purified nitrogen source, and then reconstituted with 5 mL of hexane:methanol (1:3). Ten ng of each IS were added to an aliquot of 200 μL, before instrumental analysis (Giulivo et al., 2016). Additionally, another aliquot was used for the lipid content determination gravimetrically. The lipid content referred to wet weight (ww) was between 4.6 and 7.1% for European hake, 7.4 and 47% for European sardine, and 4.9 and 20% for European anchovy.

2.4. Instrumental analysis

Instrumental analysis of OPEs was carried out using a turbulent flow chromatography - high-pressure liquid chromatography - tandem mass spectrometry (TFC-HPLC-MS-MS), using the system Thermo Scientific TurboFlowTM, which allows performing an online sample purification and analysis. This system consisted of two quaternary pumps and three LC columns, two of them for the purification and the last one for separation. The purification columns used were CyclonTM-P (0.5 × 50 mm) and C18-XL (0.5 × 50 mm), and the chromatographic separation was done using the column Purosphere Star RP-18 (125 mm × 0.2 mm), which has a particle size of 5 μm. Detailed conditions used for purification and chromatographic separation were included in Table S2. The tandem MS of this system was a triple quadrupole (QqQ) that uses a heated electrospray ionisation source (H-ESI). Mass spectrometry detection parameters can be found in previous work (Giulivo et al., 2016). Selective reaction monitoring (SRM) mode was used for all compounds with two transitions monitored for each analyte. The most intense transition was used for quantification, while the second provided confirmation. Instrumental working parameters such as retention times, transitions, declustering potential, and collision energies were summarized in Table S3.

2.5. Quality assurance

When working with OPEs, there is an important issue to take into account: the blank contamination. Indoor air is contaminated with OPEs and therefore the contamination during the sample preparation process is an important factor. To solve this problem, the non-volumetric material was heated at 340 °C and wrapped with aluminum foil and lastly rinsed with an appropriate solvent just before use. The volumetric material was always rinsed before use with an appropriate solvent. Even taking these precautions, the blank signal was inevitable and uncontrollable, and was different from day to day. The blank signal can come from different places that cannot be controlled, like the ambient air or the nitrogen from the evaporator. A realistic goal is to minimize as much as possible the blank signal. Thus, for every batch of samples, a blank was included. Blank levels were subtracted from corresponding samples.

Instrumental parameters such as recoveries, limits of detection (LODs) and limits of quantification (LOQs) are summarized in Supporting information (Table S4). Recoveries ranged between 47% and 98%, always being within the range of acceptability (40–120%) (USEPA, 1994) for analytical methods based on quantification by isotopic dilution, with relative standard deviation always below 12%. LODs and LOQs ranged between 0.02 and 0.95 ng/g ww and 0.04–2.62 ng/g ww respectively.

2.6. Stable isotope analysis

In order to directly relate the OPE values detected in the three species with potential biomagnification processes, we used nitrogen stable isotopes analysis (SIA) as a proxy of the trophic position of the studied species (Post, 2002). All muscle samples were freeze-dried, powdered and 0.80–0.85 mg of each sample was packed into tin capsules. Isotopic analyses were performed at the Laboratory of Stable Isotopes of University of La Coruña, Galicia, Spain (*Servicio de Analisis Instrumental* (SAI)) through an elemental analyzer (Carlo Erba CHNSO 1108) coupled to an isotopic ratio mass spectrometer (Finnigan Matt Delta Plus). $\delta^{15}N$ (‰) values are reported relative to atmospheric nitrogen (Coplen, 2011). The accuracy (±SE) of the standards replicates and samples is <0.3%.

2.7. Risk assessment of human exposure

Estimated daily intakes (EDIs), computed for high-exposure scenarios (95th percentile), have been calculated according to the following formula:

$$EDI = \frac{AC^{*}C}{BW}$$

where EDI is estimated daily intake of OPEs (ng/kg body weight (bw)/day), AC is the annual per capita fish consumption in Spain (94.4 g/person/day), C is the concentration detected of each OPE (ng/g ww) and BW is the mean body weight of the human consumers (68.5 kg) (AECOSAN and AESAN, 2006). The mean and 95th percentile concentrations of OPEs were used for the mean and high-exposure scenarios, respectively. It was assumed that all fish consumption corresponded to sardines, anchovies and hakes, and that 100% of the ingested OPEs were absorbed.

2.8. Statistical analysis

The concentration of OPEs in all samples was analysed using one-way analysis of variance (ANOVA) with Tukey's test employed to examine the statistical differences among fish species and sampling areas. The level of significance was set at a $p < 0.05$. All statistical analyses were carried out using R (Version February 1, 5042).

Multivariate statistical analysis on OPE compounds was conducted using principal components analysis (PCA) to investigate potential patterns in OPE data associated with spatial (i.e. Cape of Creus, Ebro Delta, Gulf of Valencia, and Gulf of Alicante) or species-specific differences (i.e. sardine, anchovy and hake) in analysed muscle samples. PCA analysis was performed in *FactorMineR* package (Lê et al., 2008) in R, where data was scaled (i.e. standardized) before PCA. *factoextraExtract* package (Kassambara and Mundt, 2020) in R was used to extract and visualize the results of the PCA.

Biomagnification was assessed using δ^{15}N values as a proxy of trophic position. A lineal model was developed between OPE values (ng/g ww) and δ^{15}N values. The significance level was set at $p = 0.05$.

3. Results and discussion

3.1. OPE levels in mediterranean fish

OPEs were detected in 96% of analysed samples with total OPE concentrations ranging between nd (not detected) and 73.4 ng/g ww. The obtained results are summarized in Table 1, indicating the minimum and maximum concentration levels, as well as mean and median values (for individual sample results see Supporting information, Table S5). Sardine presented the highest mean value with 20.5 ± 20.1 ng/g ww, followed by anchovy with 14.1 ± 8.91 ng/g ww and finally by hake with 2.48 ± 1.76 ng/g ww. Significant differences were observed between species (ANOVA, $F_{(2, 52)} = 9.76$, $p = 0.0002$): hake and sardine ($p < 0.001$) and hake and anchovy ($p = 0.05$). However, no significant differences were found between anchovy and sardine ($p > 0.05$).

The three species have different diet composition in the Western Mediterranean Sea. Sardine and anchovy are small pelagic fish and share diet similarities. Both are planktivorous feeders showing an opportunistic ingestion mainly of copepods, cladocerans, euphasiids, decapods and diatoms (Bachiller et al., 2020). In addition, both show particulate feeding capacity but sardines are greater filter-feeders. In the case of European hake, it is an ambush demersal predator with ontogenetic variation in its diet. This species mainly feeds on small crustaceans as well as in small fish, including small pelagic fish like sardine and anchovy (Mellon-duval et al., 2017; Lloret-Lloret et al., 2020). These variations in preying abilities and prey preferences may have resulted in some differences in OPEs ingestion. Nevertheless, OPEs absorption and accumulation does not only result from ingestion but also from other routes like water, sediments and organic matter through the gill and epithelial tissues (Wang et al., 2019).

If we compare concentration levels in the different species by sampling location, significant differences were found in Cape of Creus (ANOVA, $F_{(2, 12)} = 16.9$, $p = 0.0003$) between anchovy and hake ($p < 0.001$) and between anchovy and sardine ($p < 0.01$). In the Ebro Delta (ANOVA, $F_{(2, 12)} = 5.02$, $p = 0.026$), only significant differences between hakes and sardines ($p < 0.05$) were detected. Finally, significant differences were also observed between hakes and sardines in both Gulf of Valencia ($p < 0.05$) and Gulf of Alicante ($p < 0.05$).

The results showed that in all study areas hake was the species with the lowest $\sum$OPEs concentration and the highest δ^{15}N values (Figure S1). A significant negative relationship was found between δ^{15}N and total OPEs ($F_{(1,53)}$, $R^2 = 0.11$, p-value = 0.01, Figure S2), suggesting an absence of biomagnification. Similar results were presented by Brandsma et al. (2015) and Zhao et al. (2018) where they studied trophic transfer through entire marine food webs and found that most OPEs exhibit trophic dilution rather than biomagnification. This contrasts with results from Kim et al. (2011), which found demersal species accumulated more OPEs than pelagic species in Manila Bay (Philippines). They also found a positive correlation of OPEs with δ^{15}N only for one of the congeners (TPHP) but not for the rest. Both, Kim et al. (2011) results and our current results may indicate that the analysed OPEs are not biomagnified through the food web, but they are bioconcentrated by uptake from water and contact with sediments and organic matter through the gill and epithelial tissue.

Nevertheless, if we focus on congeners, TCP was the only one found in hake but absent in anchovy and sardine. This could indicate an uptake from another pathway, as previous research found that OPE accumulation could vary depending on whether congeners are acquired from the benthic or pelagic food web (Kim et al., 2011). In addition, hake feeds on several different prey sources (apart from sardine and anchovy) and with ontogenetic variation, so the TCP could have been acquired from other prey or from the environment itself. On the other hand, our TNBP concentration levels, expressed in lipid weight (lw) (Table S6), were higher in hake (mean value = 79.7 ± 123 ng/g lw) than in sardine and anchovy (22.5 ± 30.3 ng/g lw and 34.5 ± 35.8 ng/g lw respectively), indicating a possible biomagnification of TNBP. Bekele et al. (2019) also reported a significant positive relationship between TNBP concentrations (expressed in lw) and trophic levels. In addition, Garcia-Garin et al. (2020b) also reported results for fin whales and krill from North Atlantic, where TNBP was the most abundant OPE in both organisms,

Table 1
OPE levels (expressed in ng/g ww) in muscles of European sardine (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*) and European hake (*Merluccius merluccius*) from the Western Mediterranean Sea.

	Sardine					Anchovy					Hake				
	Min.	Max.	Mean[a]	Median[a]	DF (%)	Min.	Max.	Mean	Median	DF (%)	Min.	Max.	Mean	Median	DF (%)
TCEP	nd	8.36	2.38	1.87	80	nd	1.64	0.16	0.03	21	nd	nd	nd	nd	0
TPPO	nd	3.64	1.14	0.65	65	nd	3.26	1.21	0.33	36	nd	4.49	0.63	0.33	10
TClPP	nd	6.64	0.54	0.04	10	nd	2.92	0.40	0.03	29	nd	nd	nd	nd	0
TDClPP	nd	62.9	10.1	1.72	65	nd	3.06	0.66	0.03	43	nd	nd	nd	nd	0
TPHP	nd	9.39	2.42	1.30	75	nd	2.28	0.66	0.46	50	nd	nd	nd	nd	0
TNBP	nd	0.70	0.26	0.14	60	nd	0.49	0.17	0.11	64	nd	0.98	0.20	0.01	35
DCP	nd	4.53	2.09	2.14	80	nd	6.05	3.84	3.95	100	nd	1.45	0.31	0.03	35
TBOEP	nd	19.5	1.45	0.01	10	nd	nd	nd	nd	0	nd	3.84	0.98	0.01	35
TCP	nd	nd	nd	nd	0	nd	nd	nd	nd	0	nd	2.34	0.15	0.04	10
EHDPP	nd	nd	nd	nd	0	nd	nq	nd	nd	7	nd	nd	nd	nd	0
IDPP	nd	nd	nd	nd	0	nd	nd	nd	nd	0	nd	nd	nd	nd	0
THP	nd	nd	nd	nd	0	nd	25.1	6.91	4.82	50	nd	nd	nd	nd	0
TEHP	nd	nd	nd	nd	0	nd	nd	nd	nd	0	nd	nd	nd	nd	0
ΣOPEs	1.39	73.4	20.5	14.9	100	3.82	34.7	14.1	12.4	100	nd	6.97	2.48	1.73	90

nd = not detected, below LOD; nq = not quantifiable, below LOQ.
[a] Mean and median values were calculated assigning nd values to ½ LOD, and nq values to LOD.

although OPE bioaccumulation in fin whales and biomagnification from krill to whales was not observed. Otherwise, Hallanger et al. (2015) found flimsy biomagnification of OPEs from fish to their predators from Svalbard (Norway) and TNBP was non detected in any sample. Significant negative correlations with lipid contents were found for OPEs concentration in ng/g ww ($r = -0.5$, $p < 0.05$) and with lipid-normalized concentrations of OPEs ($r = -0.61$, $p < 0.01$) in our study. Such results suggest that the bioaccumulation of these compounds is not related to lipid content. Numerous studies have reported that total OPEs were not basically associated with lipids (Sundkvist et al., 2010; Kim et al., 2011; Chen et al., 2012; Brandsma et al., 2015; Malarvannan et al., 2015; Hou et al., 2017). However, Liu et al. (2019b) suggest that chemical affinity of OPEs to lipids still plays a significant role in the deposition of OPEs in tissues. The same study found a relationship between the log K_{ow} values of OPEs and the correlation of OPEs concentration and lipid content. Compounds with a low log K_{ow} had a weak or no correlation, whereas compounds with a log K_{ow} >4 had a positive correlation. Nevertheless, this correlation is not reflected in our study.

In vivo toxicological laboratory tests have shown that TNBP was lethal for certain animal species, mainly rats, chick, rabbits, zebrafish and mice, with a median lethal dose (LD_{50}) of over $1.40*10^6$ ng/g (van der Veen and de Boer, 2012). But, their concentrations in our muscle samples of hake, sardine and anchovy were five orders of magnitude lower (between nd and 0.98 ng/g ww).

Comparing the different sampling sites, significant differences were detected in OPE levels for anchovy (ANOVA, $F_{(2, 12)} = 7.19$, $p = 0.009$) (Fig. 2a), being the levels in the Ebro Delta significantly lower (mean value of 6.96 ± 1.47 ng/g ww) than those in Cape of Creus (mean value of 21.6 ± 9.27 ng/g ww) ($p < 0.01$). Regarding data on sardine (Fig. 2b), no significant differences were observed between the 4 sampling areas (ANOVA, $F_{(3, 16)} = 2.40$, $p = 0.105$). Finally, the concentrations obtained for hake samples presented significant differences between sampling areas (ANOVA, $F_{(3, 16)} = 6.23$, $p = 0.005$) (Fig. 2c), with lower levels in Cape of Creus ($p < 0.05$) and Gulf of Alicante ($p < 0.05$) when compared to those in the Ebro Delta.

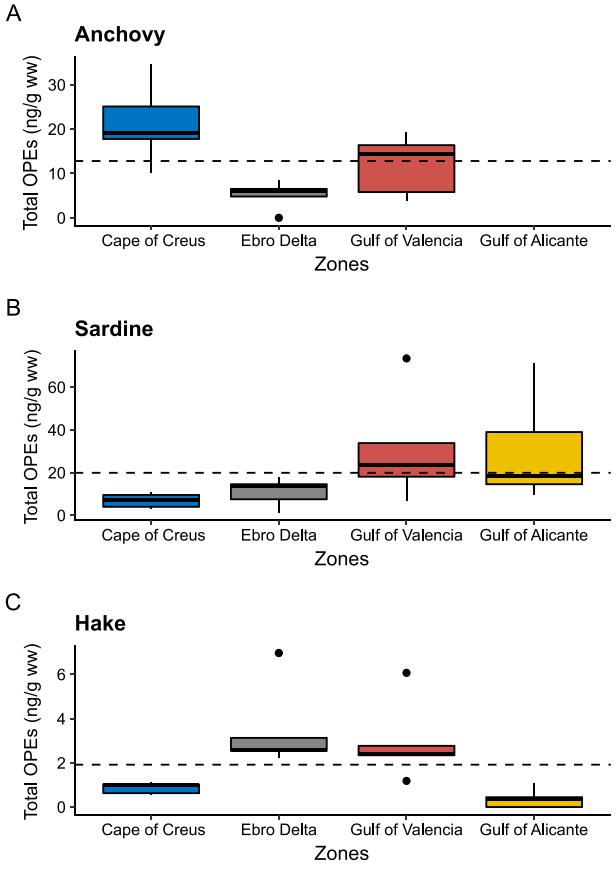

Fig. 2. Boxplot of the total OPEs detected in each of the zones for each species. A: European anchovy, B: European sardine, C: European hake (dotted lines correspond to mean values).

In general, the four studied areas presented OPE contamination levels within a similar range of concentration and no significant differences have been observed with each other (ANOVA, $F_{(3, 51)} = 1.07$, $p = 0.369$). In a recent study, Garcia-Garin et al. (2020a) reported results of the floating marine macro-litter (FMML) observed with drone surveys along the Northwestern Mediterranean Sea. The authors reported that FMML densities were significantly different throughout the surveyed area, where higher densities were found close to the coastline in the Ebro Delta, while Cape of Creus showed the lowest values. It should be noted that the drone surveys in Cape of Creus were conducted one year before those conducted in the other two areas, and during a different season, thus a proper comparison is not feasible (Garcia-Garin et al., 2020a).

Recent research in the same area reported higher concentrations of microplastic ingestion in the Gulf of Alicante for sardines and in the Ebro Delta for anchovies (Pennino et al., 2020). Authors showed higher microplastic ingestion was found in individuals with lower body condition; a similar correlation could occur in the case of contaminant concentrations and should be further investigated. Despite spatial variation was not always significant for OPE concentrations, sardine also showed the highest OPE levels in the Gulf of Alicante; however, anchovy showed an opposite pattern with lower OPE levels in the Ebro Delta. In fact, no relationship was detected between OPE levels and microplastic ingestion for another species (bogue, *Boops boops*) in the same area (Garcia-Garin et al., 2020c). Further analysis should focus on the direct relationship, if any, between ingestion of microplastics and concentration of OPE from the same individual samples. In another recently published study of OPEs in sea turtles (*Caretta caretta*) from the Balearic coast, 10 compounds (TEP, TClPP, TDClPP, TPHP, TNBP, DCP, 2IPPDPP, 4IPPDPP, TCP and EHDPP) were detected in plastic debris, turtle muscle and prey, demonstrating that turtles could acquire them from plastic waste, diet or both (Sala et al., 2021).

Some studies are available on the occurrence of OPEs in marine fish and there is evidence of its occurrence for different marine and freshwater species worldwide, such as in Pearl River Delta, China (Liu et al., 2018; Zhang et al., 2018; Liu et al., 2019a, 2019b), Swedish lakes and coastal areas (Sundkvist et al., 2010) and also in the Mediterranean Sea (Castro et al., 2020; Garcia-Garin et al., 2020c). In fact, recent research proved the presence of OPEs in the muscle of bogue from Barcelona and Cape of Creus, and reported levels of 10.6 ± 4.9 and 7.9 ± 6.5 ng/g ww, respectively (Garcia-Garin et al., 2020c). These results were similar to our OPE levels in hake (1.45 ± 0.24 ng/g ww) and sardine (7.65 ± 3.29 ng/g ww) from Cape of Creus, whereas anchovy presented levels of one order of magnitude higher (21.6 ± 9.27 ng/g ww). Kim et al. (2011) detected mean concentrations of TBOEP, TNBP and TCP in fishes collected from Manila Bay (Philippines) similar to concentrations found in our study. Notwithstanding, OPEs accumulation and elimination may be compound specific and may vary between fish species, and even the different observed OPE patterns may also reflect the different OPEs usage per region.

3.2. OPE patterns in mediterranean fish

Eleven out of thirteen tested OPEs were detected. Only IDPP and TEHP were not detected in any sample. For sardine, compounds with the highest detection frequencies were DCP and TCEP with 80% of positive samples, followed by TPHP (75%) and TPPO and TDClPP (65%). For anchovy, DCP was detected in all samples, followed by TNBP (64%) and TPHP and THP (50%). Finally, the most frequent OPEs in hake samples were TNBP, DCP and TBOEP (35%). As regards concentration levels, the highest values in sardine were obtained for TDClPP (10.1 ± 17.8 ng/g

ww); for anchovy, THP was the predominant compound (6.91 ± 7.97 ng/g ww), and for hake, TBOEP (0.98 ± 1.39 ng/g ww). The OPE profiles in all species by sampling area are presented in Fig. 3. As can be seen, OPE profiles exhibited several differences between species. However, within each species, OPE distribution profile was similar among different sampling areas, except for Ebro Delta for anchovy and Cape of Creus for hake.

Samples were displayed in a biplot using PC1 (23.8%) vs. PC2 (16.7%) and PC2 vs. PC3 (11.6%) scores to investigate potential patterns in OPE data associated with spatial or species-specific differences. The original variables (i.e. OPE compounds) were also displayed (Fig. 4). OPE compounds with high (positive or negative) values along a PC axes in a biplot have high importance for that PC axis. PCA is showing that the separation is bigger between species (Fig. 4a-d) rather than between areas where the overlap is higher (Fig. 4b-e). These results would indicate a similar use of the different OPEs throughout the sampled area. And the differences in the OPE profiles may be related to different capacities of acquisition, bioaccumulation, and metabolism, for each species. Differences in feeding behavior and metabolic efficiency among different individuals or species could greatly influence the accumulation potential of OPEs. Due to the lack of research on metabolites of OPEs, the understanding of the complete phenomenon of OPEs in the aquatic organisms tested is still insufficient, considering that the biomagnification potential of some OPEs could be affected due to their high metabolization potential. Liu et al. (2019b) calculated that the ratios of the concentrations of OPEs in other tissues to the liver (OLR) of wild aquatic species from a closed e-waste contaminated pond in Qingyuan, southern China, were larger than 0.5, indicating the effect of metabolism on the deposition of OPEs in the liver. In two of the studied species (i.e. snakehead (*Ophiocephalus argus*) and mud carp (*Cirrhinus molitorella*)), the OLRs of kidney were significantly lower than those in other tissues ($p < 0.05$), suggesting that the kidney may also be involved in the metabolism of OPEs. Snakehead showed higher OLR values than mud carp, which could be due to the higher metabolism potential of the former, considering that snakehead occupied a higher trophic level (Liu et al., 2019b). It is necessary to take in to account the different physiological strategies of each species. Sardines and anchovies have opposite reproductive strategies (capital-breeder *vs* income-breeder, respectively), presenting different reproductive periods in this area (Palomera et al., 2007; Albo-Puigserver et al., 2020). Sardine accumulates mesenteric fat during the spring-summer season prior to the reproduction period (capital breeder behaviour), while anchovy present a lower seasonal variability on fat reserves, as expected for an income breeder. Hake is considered an income breeder (Domínguez-Petit et al., 2010), i.e. the acquired energy is immediately used for reproductive investment, meaning there is no need for related, extensive storage (Rijnsdorp, 1990). The liver is an important organ for energy storage and is usually the first site for lipid (energy) storage in a number of benthic and demersal species such as gadoids (e.g., Kjesbu et al., 1991; Lambert and Dutil, 1997; Lloret et al., 2008). Lipids normally constitute nearly 70% of the dry liver of hake (Lloret et al., 2008), confirming the important role of the liver in energy storage in these species. In contrast, lipids constituted on average only 3% of the dry muscle of hake (Lloret et al., 2008).

3.3. Risk assessment of human exposure

Fish species analysed in the present study are commonly consumed by humans. Therefore, obtained OPE levels can provide reliable information for human exposure assessment to these contaminants through fish ingestion. Table 2 showed the different EDI values obtained for individual OPEs as well as for ∑OPEs. The primary contributors to the total OPE exposure through fish consumption (calculated mean and 95th percentile) were TDClPP (4.94 and 12.6 ng/kg bw/day respectively), followed by THP (3.20 and 8.57 ng/kg bw/day respectively) and by DCP (2.87 and 5.05 ng/kg bw/day respectively), and an EDI of 16.9 ng/kg bw/day for mean concentration and 37.1 ng/kg bw/day for 95th percentile was obtained based on the ∑OPEs. In addition, the EDI values of each congener of the OPEs analysed in this study were compared with the EDI values calculated from existing studies (Table S8). Our EDI values were well below the oral reference dose (RfD) values proposed by USEPA (EPA, 2018), which ranged between 600 ng/kg bw/day for EHDPP to 100,000 ng/kg bw/day for TEHP.

Hazard quotients (HQ) were calculated by dividing the obtained EDI values by the corresponding RfDs (Table 2). It was reported that if the HQ was higher than 1, then a potential risk to humans might occur (Ding et al., 2018). The obtained HQ were between 5.00 * 10^{-6} and 7.33 * 10^{-4} (Table 2), being much lower than the threshold value of 1. Thus, if fish consumption was only coming from those species, the consumption of fish from the Northwestern Mediterranean Sea would not pose a

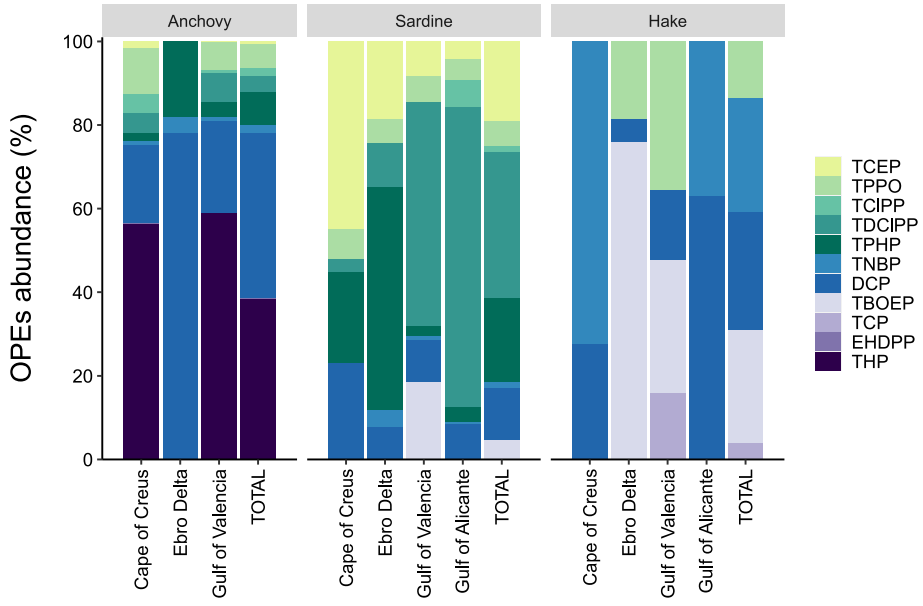

Fig. 3. Percentage contribution of detected OPEs to the total concentration levels in European anchovy, European sardine and European hake from Cape of Creus, Ebro Delta, Gulf of Valencia and Gulf of Alicante.

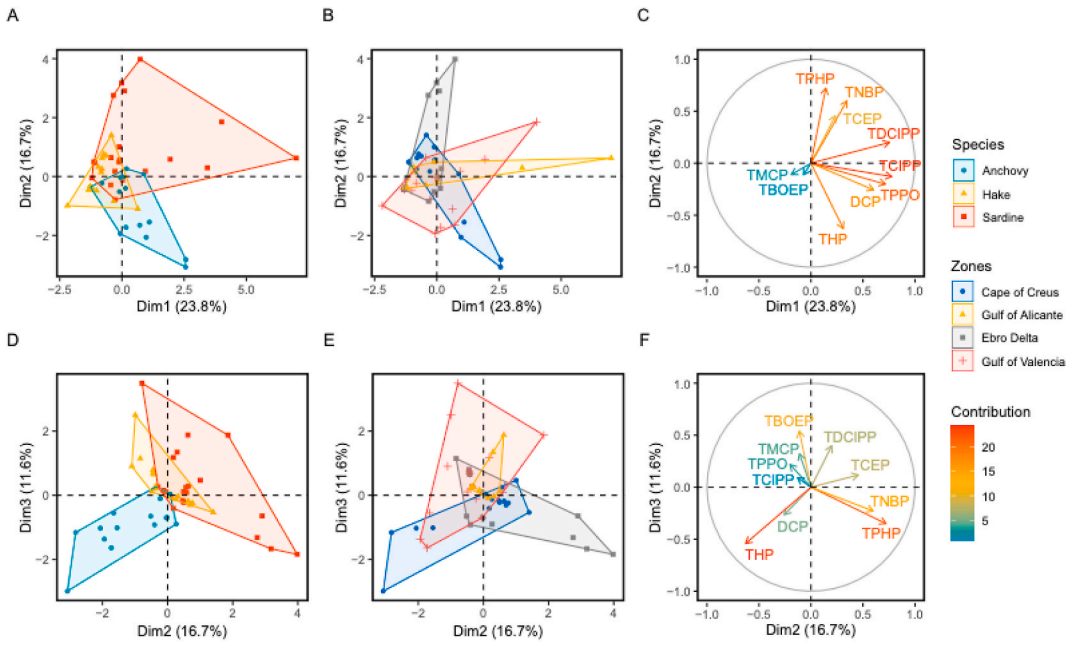

Fig. 4. Principal component analysis (PCA) of OPEs congeners. PC1: A,B,C; PC2: D,E,F. Convex hulls for species (A and D) and zones (B and E) are delimitated in different colours. Loadings are shown in subplot C for PC1-PC2 and in subplot F for PC2-PC3. The further away from the origin an OPE congener is located in the plot, horizontally or vertically, the more important it is for the direction of the first, second or third component of the PCA (*i.e.* more contribution). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Estimated daily intakes (EDIs) of OPEs via fish ingestion (ng/kg bw/day) and associated hazard quotients (HQ).

	RfD*	C** (Mean)	EDI (Mean)	HQ (Mean)	C** (95th percentile)	EDI (95th percentile)	HQ (95th percentile)
TCEP	7000	0.86	1.18	$1.68 * 10^{-4}$	2.16	2.98	$4.25 * 10^{-4}$
TPPO	20,000	0.99	1.37	$6.85 * 10^{-5}$	1.20	1.65	$8.25 * 10^{-5}$
TClPP	10,000	0.33	0.45	$4.50 * 10^{-5}$	0.52	0.72	$7.20 * 10^{-5}$
TDClPP	20,000	3.58	4.94	$2.47 * 10^{-4}$	9.13	12.6	$6.30 * 10^{-4}$
TPHP	7000	1.04	1.43	$2.04 * 10^{-4}$	2.25	3.10	$4.43 * 10^{-4}$
TNBP	10,000	0.21	0.29	$2.90 * 10^{-5}$	0.26	0.35	$3.50 * 10^{-5}$
DCP	–	2.08	2.87	–	3.67	5.05	–
TBOEP	1500	0.81	1.1	$7.33 * 10^{-4}$	1.40	1.93	$1.29 * 10^{-3}$
TCP	20,000	0.07	0.10	$5.00 * 10^{-6}$	0.14	0.20	$9.82 * 10^{-6}$
EHDPP	600	nq	–	–	nq	–	–
IDPP	–	nd	–	–	nd	–	–
THP	–	2.32	3.20	–	6.22	8.57	–
TEHP	100,000	nd	–		nd	–	–
∑OPEs	–	12.3	16.9	–	27.0	37.1	–

*Oral reference dose (RfD) expressed in ng/kg bw/day, and recommended by the USEPA (EPA, 2018).
**OPEs concentration, expressed in ng/g ww.
nd = not detected, below LOD; nq = not quantifiable, below LOQ.

considerable threat to public health regarding OPE intake. Similar results, with a low level of risk of OPE associated with fish consumption, were found by Bekele et al. (2021) for ten fish species from coastal area of Laizhou Bay, North China (EDI ranged between 3.1-22.1 and 1.7–12 ng/kg bw/day for urban and rural residents, respectively).

However, it is important to note that OPEs can be ingested through the migration of plasticizers in packaging plastics to food (Ma et al., 2019), as well as through other food items. Some studies reported EDI values via ingestion of different food groups, with values of 85 ng/kg bw/day for the sum of five OPEs in Sweden (Poma et al., 2017) or of 103 ng/kg bw/day for the sum of seven OPEs in Belgium (Poma et al., 2018). Other similar studies, such as those of Zhang et al. (2016), Xu et al. (2017), Ding et al. (2018) and Wang and Kannan (2018) presented EDI values for the sum of OPEs between 9 and 52 times higher than those computed for this study (Table S8). In all studies TCEP and TPHP were detected and presented the lowest EDI mean value in Spain (1.18 and 1.43 ng/kg/bw/day respectively) and highest in China (182 and 77 ng/kg/bw/day respectively).

Moreover, human OPE exposure also occurs by other routes, such as indoor/outdoor inhalation (Wong et al., 2018), dermal absorption (He et al., 2018) and dust ingestion (Kim et al., 2019). The sum of all these exposures can bring the EDI values closer to the established safety limits.

4. Conclusions

OPEs were detected in almost all the fish samples with concentration levels ranging from not detected to 73.4 ng/g ww. Small pelagic fish had higher amounts of OPE compounds in their muscle tissue than their predator, hake. Even if there is no evidence of OPE biomagnification, certain congeners such as TCP and TNBP showed higher concentration levels in hake than in their prey. It must be considered that there are metabolic differences between hake and small pelagic fish, which affect the bioaccumulation and metabolization capacity of OPEs. These compounds can also bioconcentrate through different sources, through

water and/or contact with sediments and organic matter.

Sardine was the most contaminated fish with a mean OPE level of 20.5 ± 20.1 ng/g ww, and concentrations differing significantly by location and with the highest value in the Gulf of Valencia. Further research is needed to explore correlations between human activities around studied areas and OPE concentrations.

Estimated daily intake of OPEs through fish consumption was assessed, showing no considerable threat to public health. However, OPEs can arise human body through diverse pathways such as food ingestion, indoor/outdoor inhalation, dermal absorption and dust ingestion. The sum of all these exposure sources can pose certain risks to human health. Therefore, it is recommended to minimize human exposure to OPEs, reducing pollution in marine ecosystems, and preventing an increase in OPE levels in marine fish.

Author statement

Berta Sala: formal analysis; data treatment; methodology; validation; writing of the original manuscript draft and revision of the manuscript. Joan Giménez: sampling; formal analysis; data treatment; writing of the original manuscript draft and revision of the manuscript. Julio Fernández-Arribas: formal analysis; data treatment; methodology. Carlota Bravo: formal analysis; data treatment; methodology. Elena Lloret-Lloret: sampling; formal analysis; data treatment; writing of the original manuscript draft and revision of the manuscript. Antonio Esteban: sampling; revision of the manuscript. José María Bellido: sampling; revision of the manuscript. Marta Coll: conceptualization and coordination of the study; supervision; writing of the original manuscript draft and revision of the manuscript; resources; funding acquisition. Ethel Eljarrat: conceptualization and coordination of the study; supervision; methodology; validation; writing of the original manuscript draft and revision of the manuscript; resources; funding acquisition.

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.envpol.2021.118377.

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