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Assessing microplastic ingestion and occurrence of bisphenols and phthalates in bivalves, fish and holothurians from a Mediterranean marine protected area

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ABSTRACT

Microplastic (MP) ingestion, along with accumulated plasticizers such as bisphenol A (BPA), bisphenol F (BPF), and bisphenol S (BPS), and phthalates represented by diethyl phthalate (DEP), dibutyl phthalate (DBP) and bis (2-ethylhexyl) phthalate (DEHP), were quantified in bivalves, fish, and holothurians collected from a coastal pristine area at the western Mediterranean Sea. MP ingestion in sediment-feeders holothurians (mean value 12.67 ± 7.31 MPs/individual) was statistically higher than ingestion in bivalves and fish (mean 4.83 ± 5.35 and 3 ± 4.44 MPs/individual, respectively). The main ingested polymers were polyethylene, polypropylene, and polystyrene. The levels of BPS, BPF, and DEHP were highest in bivalves' soft tissue; BPA and DBP had the highest levels in the holothurians' muscle. In addition, the levels of all plasticizers assessed were lowest in fish muscle; only BPA levels in fish were higher than in bivalves, with intermediate values between those of bivalves and holothurians. This study provides data on exposure to MPs and plasticizers of different species inhabiting Cabrera Marine Protected Area (MPA) and highlights the differences in MP ingestion and levels of plasticizers between species with different ecological characteristics and feeding strategies.

1. Introduction

The ubiquity of plastic pollution in the marine environment has been reported worldwide (Peng et al., 2020). Once in the marine environment, and as a result of several processes and factors (water hydrodynamics, temperature, and/or salinity, among others) plastics can become distributed from the sea surface (Compa et al., 2020; Fagiano et al., 2022) along the water column (Rios-Fuster et al., 2022b), and over the seabed (Alomar et al., 2016). In addition, plastics in the marine environment are continuously affected by degradation processes such as photooxidation and fragmentation, which increase the number of microplastics (MPs; < 5 mm) released into the environment. MPs become available to marine organisms with harmful and sub-lethal effects on organisms' physiology (Fossi et al., 2017). Numerous studies report the ingestion of these man-made particles by species with different feeding strategies and from different trophic levels, such as invertebrates and fish (Fossi et al., 2017).

Additional concerns in the scientific community are the ecotoxicological effect of plasticizers (Rios-Fuster et al., 2022a) and the presence of Persistent Organic Pollutants (POPs, which can be absorbed into organisms) over MPs surfaces (Agbo and Abaye, 2016; Rios-Fuster et al., 2021), as both plasticizers and POPs show a different type of interaction with plastics. Bisphenols compounds like bisphenol A (BPA), bisphenol F (BPF), and bisphenol S (BPS); and phthalates like diethyl phthalate (DEP), dibutyl phthalate (DBP) and bis (2-ethylhexyl) phthalate (DEHP), are added to plastics as plasticizers during the manufacturing and processing of plastic to improve their properties for the intended use (Beltifa et al., 2017; Net et al., 2015; Rochester and Bolden, 2015). The presence of these plasticizers in the marine environment can be attributed to their release during the degradation process of plastics in the marine environment (Fikarová et al., 2019). Previous studies have evidenced that both bisphenols and phthalates have ecotoxicological implications for

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marine organisms causing immunotoxicity, neurotoxicity, and oxidative stress disorders, in marine species exposed to these chemicals (Oehlmann et al., 2009; Seoane et al., 2021).

The marine environment is complex; it includes a wide range of species with different trophic levels and different feeding strategies, such as filter-feeders, or selective or non-selective predators. In addition, there is evidence of transference of MPs (Carbery et al., 2018) and other chemical pollutants (Batel et al., 2016) throughout the trophic chain. The species addressed in the present study are bivalves, which have been previously used as bioindicator species in several monitoring programs (Bartolomé et al., 2010; Vidal-Liñán et al., 2010; Viñas et al., 2018), particularly because of their life-span, ubiquity in the marine environment, abundance, and extensive filter-feeding capacity that exposes them directly to pollutants. This latter feature allows for easier detection and quantification of different types of pollutants. There is indeed scientific evidence of MP ingestion (Klasios et al., 2021; Van Cauwenberghe and Janssen, 2014) and of presence of plasticizers (Rios-Fuster et al., 2022a) in bivalves. In fish species, there are multiple feeding strategies (Bernal et al., 2015) leading to MP ingestion (Compa et al., 2018; Rios-Fuster et al., 2019); and the accumulation of phthalates and bisphenols compounds has also been well documented (Barboza et al., 2020; Panio et al., 2020). Previous studies in sedimentivorous species have documented MP ingestion in holothurians (Graham and Thompson, 2009) and the bioaccumulation of several chemicals such as BPA from the industry (Martín et al., 2020).

Marine Protected Areas (MPAs) are management tools designed for conservation purposes with specific levels of protection aiming at the conservation of areas with high ecological value (in terms of habitats and biodiversity) and the increase of biomass of species of interest for fishing. Although MPAs are protected from direct anthropogenic impacts such as boat anchoring, maritime traffic, or extraction and exploitation of marine resources, recent studies have reported that these areas are also affected by the presence of pollutants, mainly plastics, along beaches (Giovacchini et al., 2018), sediments (Alomar et al., 2016), the sea surface (Fagiano et al., 2022), and the water column (Panti et al., 2015); the presence of these plastics evidences the transference of pollutants to MPAs from anthropogenized areas. Moreover, plasticizers have already been detected in sediments from MPAs (Alkan et al., 2021) highlighting the ubiquity of these chemicals even in pristine areas. These studies confirm that marine organisms from MPAs are exposed to MPs and plasticizers.

This study aims to simultaneously evaluate the ingestion of MPs and the accumulation of plasticizers (such as phthalates and bisphenols) in various marine organisms with different biological and functional traits within the scenario of a pristine MPA.

2. Material and methods

2.1. Study area

To assess MP presence and the levels of various plasticizers (bisphenols and phthalates) in key species of Mediterranean coastal

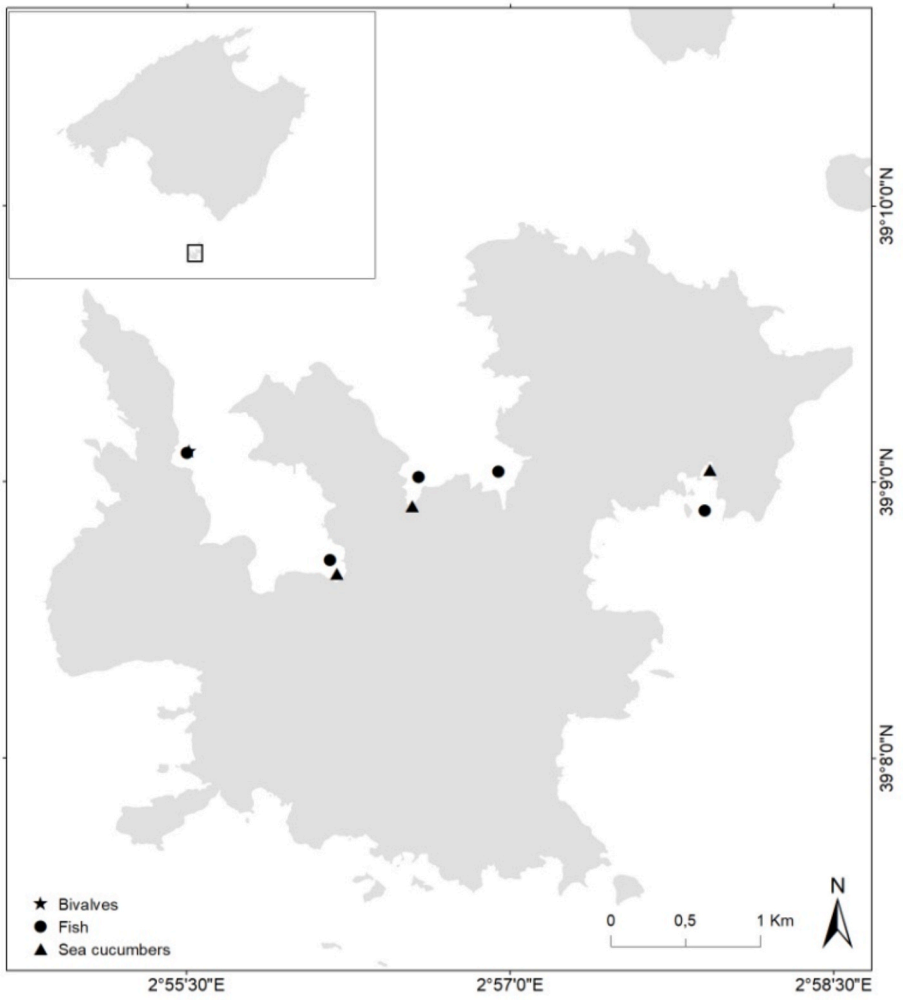

Fig. 1. Locations selected to assess microplastic ingestion, and levels of phthalates and bisphenols in bivalves, fish, and holothurians from Cabrera MPA.

ecosystems, a total of 72 individuals from different trophic level species were sampled: one bivalve species (33 individuals), four fish species (24 individuals), and three holothurians (15 individuals). The organisms were collected in 2019 and 2020 during two scientific surveys carried out at the archipelago of the Marine Protected Area of Cabrera National Park (henceforth, Cabrera MPA) in the Balearic Islands (Fig. 1).

The bivalve *Arca noae* was collected from submerged buoys in the port area of Cabrera MPA. The fish species *Oblada melanura*, *Diplodus vulgaris*, *Serranus cabrilla*, and *Serranus scriba* were sampled from different locations in the park. Finally, the three species of holothurians *Holothuria forskalii, Holothuria poli*, and *Holothuria tubulosa* were collected from scuba diving surveys conducted to quantify marine litter in coastal seafloor areas at different locations (Fig. 1).

2.2. Reagents and materials

Analytical standards of bisphenol A (BPA, purity ≥99.9%), bisphenol F (BPF, purity ≥98%), bisphenol S (BPS, purity ≥98%), diethyl phthalate (DEP, purity ≥99%), dibutyl phthalate (DBP, purity ≥99%), and bis (2-ethylhexyl) phthalate (DEHP, purity ≥99.5%) were supplied by Sigma-Aldrich (Madrid, Spain). The organic solvents acetonitrile (ACN) and methanol (MetOH), acquired from Scharlab (Barcelona, Spain), were graded through high-performance liquid chromatography (HPLC). Formic acid was also purchased from Sigma-Aldrich (Madrid, Spain). All aqueous solutions were prepared with ultrapure water (18 MΩ/cm) using a Milli-Q water system (Millipore Ibérica, Madrid, Spain).

Stock standard solutions of the individual compounds were prepared in methanol and then stored in darkness at 4 °C until use. In these conditions, all solutions remained stable for at least three months. The working standard solutions at the desired concentration of each analyte were prepared daily from stock solutions using the dilution mixture of methanol/water (85:15, v/v).

Florisil (<200 mesh) (Sigma-Aldrich, Madrid, Spain), sodium sulphate anhydrous (Na_2SO_4, purity ≥99.9%) (Panreac, Barcelona, Spain), and washed sea sand (0.25–0.30 mm) (Symta, Madrid, Spain) were used as solid phase materials for matrix-solid phase dispersion (MSPD).

2.3. Sample collection and biological parameters

All samples collected from the marine environment were processed on land, at the laboratory of Cabrera MPA. To assess the biological condition of the studied individuals, different condition indexes were calculated according to the recorded biological parameters. In bivalves, total length (cm), total weight (g), soft tissue weight (g), and shell weight (g) were recorded. Because the whole soft tissue was required for both MP and plasticizers analyses, individuals were divided into groups, accordingly; 18 individuals were stored individually for MP analyses and the other 15 individuals were stored in aluminum foil for plasticizers quantification. All individuals were immediately stored at −20 °C. For each individual, the Condition Index (MCI) was calculated (Park et al., 2012) by applying the following equation:

- MCI = soft tissue wet weight (g)/shell weight (g)

According to fish species, biological parameters such as total length (cm), total weight (g), and gastrointestinal tract weight (g) were recorded. For each individual, the whole gastrointestinal tract was extracted and stored at −20 °C for posterior MP ingestion analysis. In addition, a portion of muscle was immediately stored in aluminum foil at −20 °C for posterior plasticizer analysis. For each individual, the Fulton's Condition Index (*K*) was calculated as follows:

- Fulton's condition index (*K*) = total weight (g)/(total length^3 (cm)) × 100

In holothurians, total weight (g) and the gastrointestinal tract weight (g) were recorded for each individual. In addition, the total length (cm), disc width (cm), and disc height (cm) of the body were measured with a caliper. For each individual, the gastrointestinal tract was extracted and stored at −20 °C for MP ingestion analysis, and a portion of muscle was immediately stored in aluminum foil at −20 °C for posterior plasticizer analysis. To estimate the growth function of holothurians, the square root of the length-width product index (SLW) was calculated (Poot-Salazar et al., 2014):

- SLW = square root of length (cm) × width (cm)

All MP ingestion analyses were performed at the laboratory of the Balearic Centre of Oceanography (CN IEO-CSIC); plasticizers analyses were performed at the Department of Analytical Sciences, Faculty of Sciences, National University of Distance Education (UNED).

2.4. Experimental procedures

2.4.1. Microplastic ingestion analysis

For MP ingestion analysis, the gastrointestinal tract from fish and holothurians and the whole soft tissue of bivalves were defrosted at room temperature. All samples were placed in a glass Erlenmeyer flask containing 10% potassium hydroxide (20 mL of KOH per gram of sample). Erlenmeyer flasks were placed in a bath at 50 °C for 48–96 h. After this digestion step, samples were filtered through a glass fiber filter (FILTER-LAB, pore size 2.4 μm, diameter 47 mm). Due to the high presence of undigested vegetal material in holothurians' gastrointestinal tracts, a different procedure for these species was performed: the content of the gastrointestinal tract was placed on a Petri dish and 96° ethanol was added for density separation (Herrera et al., 2018), subsequently, items were visually sorted under a stereomicroscope (Euromex NZ, 1903-S).

During all laboratory work air currents were reduced to minimize contamination, and technicians wore white 100% cotton laboratory coats. Glass and metal materials were used during all dissection, digestion, and density separation processes, and materials were continuously cleaned with filtered distilled water and 70% ethanol during all laboratory procedures. For every three samples, a procedural blank was included in the digestion and density separation processes. On the other hand, an additional blank for each visual sorting step was also added. A sample correction was performed by subtracting the number of items found in blanks with similar shapes and colors, to the items identified during the visual sorting procedure of biological samples.

All identified items were classified according to shape as fibers, fragments, films, and ropes and filaments (Fossi et al., 2019). For polymer characterization, approximately 10% of the items from the different species were randomly selected for analysis by Fourier-transform infrared spectroscopy (FT-IR) using different FT-IR reference databases (Löder et al., 2015). Only results with a quality hit index >700 (max. 1000) were accepted as confirmed polymers and spectra comparison was performed with the Opus 6.5 software.

2.4.2. Phthalates and bisphenols analysis

To assess plasticizers in the selected coastal key species, a total of 15 bivalves, 24 fish, and 15 holothurians were analyzed. In bivalves, plasticizers were determined in the whole soft tissue; in fish and holothurians, plasticizers were quantified in muscle samples. The bisphenols BPA, BPS, and BPF were included in our analysis; the phthalates analyzed DEP, DBP, and DEHP.

A mass sample of 0.2 g of homogenized fresh raw material was accurately weighed and subjected to the MSPD extraction method; florisil, anhydrous sodium sulphate, and sea sand were used as dispersing agents at a 5:5:2 ratio, according to the sample. The MSPD mixture was packed in a glass Solid Phase Extraction (SPE) cartridge. The analytes were eluted using 9 mL of the ACN/MetOH mixture (70/30). The eluate was evaporated, and the sample was reconstituted using 800 μL of an

ACN/H_2O mixture (45/55). An aliquot of the reconstituted sample was analyzed in triplicate by HPLC with both DAD and mass spectrophotometric detection. Due to DAD detection have not enough sensitivity for all the analytes, their determination and quantification was carried out by HPLC coupled to mass spectrometry. To reduce background analyte contamination, the samples were extracted and analyzed in the laboratory avoiding any type of plastic material or consumables that could contaminate the samples. This laboratory is prepared to keep contamination of all plastic materials to a minimum during the experimental procedures, except for micropipette tips. A MSPD blank was prepared to check for background contamination due to the use of plastic lab ware (no sample, only reagents, or solvents). Except for DEHP, no analytes were detected in these procedural blank assays. The DEHP content was taken into account for the final data derived from the analysis of real organism samples. Moreover, the sample treatment method used to analyze target compounds in selected marine organisms has already been validated for similar species in previous work (Cañadas et al., 2021). To ensure the reliability of the results obtained for each of the samples analyzed, a calibration of standard additions (addition of a known analyte concentration to the eluates obtained from the MSPD extraction process) was carried out.

2.5. Chromatographic analyses

HPLC analyses were performed using an HPLC-MS system composed of an HPLC system model 1200 series (Agilent Technologies, Germany) equipped with a diode array spectrophotometric detector (DAD), a quaternary pump, a thermostated column compartment, and an autosampler controlled by HP Chemstation software from Agilent technologies. This apparatus is coupled to a 6110 simple quadrupole mass spectrometer (Agilent) with an electrospray ionization (ESI) interface. The analytical column was an ACE 5 C18-PFP HPLC column (150 × 4.6 mm, 5 μm) from Symta (Madrid, Spain). Chromatographic analyses were carried out by applying an elution gradient using as mobile phase ultrapure water Milli-Q as component A, and acetonitrile as component B. The composition of the eluent varied from 45% to 80% B in 30 min, 80%–100% B from 30 to 31 min, and 100% B for 9 min; the flow rate was kept at 0.8 mL min^{-1}. Subsequently, the column was equilibrated with 45% B isocratic for 10 min at the same flow rate. The column temperature was kept constant at 20 °C and the injection volume was 40 μL. The quantification of the analytes was performed using external calibration and peak area measurements, selecting the optimum wavelength (210 nm) for all of them.

HPLC-ESI-MS analyses were performed in the negative ion mode for bisphenols, and positive ion mode for phthalates. The liquid chromatography (LC) flow rate was 0.8 mL min^{-1}. The operating conditions for the ESI interface were as follows: capillary temperature, 350 °C; capillary voltage, 5000 V; sheath gas (N_2) flow, 11 L min^{-1}. The retention times (R_t, min) and m/z ions for the detection and quantification of each analyte (phthalates and bisphenols) were: DEP (R_t, 10.49 min; m/z: 149, 177), DBP (R_t, 22.68 min; m/z: 149, 205), DEHP (R_t, 34.98 min; m/z: 149, 167, 279), BPS (R_t, 3.38 min; m/z: 92, 108, 156, 249), BPA (R_t, 6.24 min; m/z: 119, 213, 228), BPF (R_t, 4.56 min; m/z: 98, 183, 199, 200).

2.6. Data analysis

To determine differences in MP ingestion and plasticizer levels between groups the following data analysis was performed: a data exploratory test to evaluate the distribution and homogeneity of variances by applying Shapiro-Wilk and Levene's test, respectively. A non-normal distribution was found; therefore, to identify differences in MP ingestion and levels of plasticizers between groups, several non-parametric Kruskal-Wallis (KW) tests were performed. In addition, to evaluate the variability of phthalates and bisphenols between groups a Principal Component Analysis (PCA) was performed.

On the other hand, several Pearson correlations were carried out between the total number of MPs ingested and the condition index; additionally, a correlation analysis was performed between the total number of MPs ingested and the levels of phthalates and bisphenols. Given that Pearson's correlation requires an equal number of samples between variables, we assumed that there is a correlation between MP analysis and plasticizers quantification analysis for bivalves, as all bivalve individuals were collected from the same population.

All data analyses were performed in RStudio version 3.6.4.

3. Results

3.1. Biological parameters

Different condition indexes were calculated for each group of species (Table 1). In bivalves, *Arca noae* had a mean (±SD) Condition Index (MCI) of 1.62 ± 0.44. In fish, the general Fulton Condition Index (*K*) for all species had a mean of 1.3 ± 0.34, ranging from 1.04 ± 0.08 in *Serranus cabrilla* to 1.52 ± 0.32 in *Diplodus vulgaris*. Holothurians presented higher variability in the square root of the length-width product index (SLW) with a general mean of 22.20 ± 6.17 and values ranging from 17.89 ± 4.17 in *Holothuria poli* to 29.20 ± 3.68 in *H. tubulosa* (Table 1).

Table 1
Biological parameters for bivalves, fish, and holothurians. N = sample size. Mean values (±SD) of Mussels Condition Index (MCI) for bivalves, Fulton's condition index (*K*) for fish, and the Square root of the Length-Width product (SLW) for holothurians.

	N	Biological index		
		Mussels Condition Index (MCI)	Fulton Condition Index (*K*)	Square root of the length-width product (SLW)
Bivalves	18	1.62 ± 0.44		
Arca noae	18	1.62 ± 0.44		
Fish	**24**		**1.3 ± 0.34**	
Diplodus vulgaris	6		1.52 ± 0.32	
Oblada melanura	6		1.45 ± 0.21	
Serranus cabrilla	6		1.04 ± 0.08	
Serranus scriba	6		1.20 ± 0.45	
Holothurians	**15**			**22.20 ± 6.17**
Holothuria forskali	5			19.52 ± 2.99
Holothuria poli	5			17.89 ± 4.17
Holothuria tubulosa	5			29.20 ± 3.68

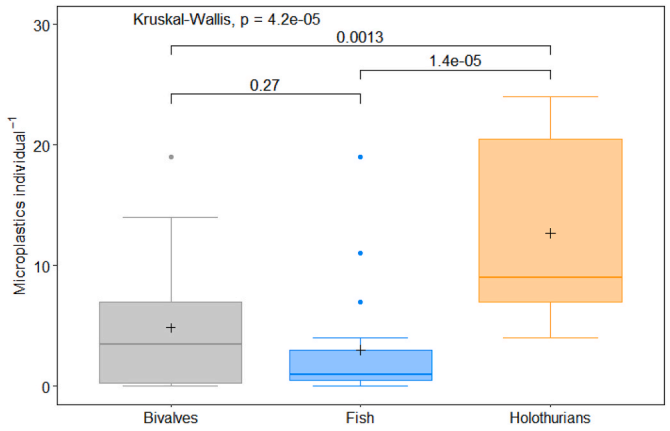

Fig. 2. Boxplot of the mean number of microplastics ingested by bivalves, fish, and holothurians from Cabrera MPA. Differences in microplastic ingestion were assessed with the Kruskal-Wallis test (significance level at $p < 0.05$) according to the group of species.

3.2. Microplastic ingestion

High MP occurrence was detected in the three groups of species. Holothurians had the highest frequency of MP occurrence (15 individuals; 100%), with a mean value (±SD) of 12.7 ± 7.3 MPs per individual. Bivalves were the second group most affected with MP (72.2%; 13 individuals) and a mean value of 4.8 ± 5.4 MPs per individual. Fish showed the lowest frequency of MP occurrence (70.8%; 17 individuals) and a mean of 3 ± 4.4 MPs per individual. The ingestion of MPs was statistically different between groups of species (KW, $p < 0.001$; Fig. 2). Holothurians showed statistically higher ingestion of MPs than bivalves and fish (Fig. 2). However, no differences in MPs ingestion were observed between bivalves and fish ($p > 0.05$). There was no statistical correlation between the abundance of MPs and MCI, *K*, or SLW ($p > 0.05$).

The most common shape of MPs in the three groups of species studied were fibers (238 items), followed by fragments (34 items), films (5 items), and ropes + filaments (18 items). Fig. 3 summarizes the different shapes of MPs identified in the three groups of species, showing a high percentage of fibers identified in bivalves and holothurians. Ropes + filaments were detected only in fish (Fig. 3). Finally, a total of 38 MPs belonging to three polymers were identified by FT-IR analysis (Table 2). The main polymer identified was polyethylene (39% of the items), followed by other minor polymers comprising copolymers and synthetic rubbers among others (26% of the items), propylene (18% of the items) and polystyrene (16% of the items).

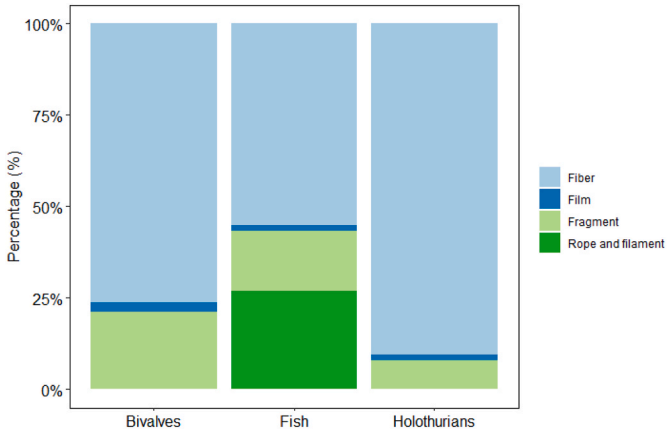

Fig. 3. Stacked barplot for the microplastics identified according to typology (fiber, film, fragment, and rope + filament) in bivalves, fish, and holothurians from Cabrera MPA.

Table 2
Percentage (%) of the polymers identified by Fourier-transform infrared spectroscopy (FT-IR) in the species assessed.

	Polymer			
	Polyethylene	Propylene	Polystyrene	Other
Bivalves	**21.4**	**7.1**	**21.4**	**50**
Arca noae	21.4	7.1	21.4	50
Fish	**33.3**	**–**	**16.7**	**50**
Diplodus vulgaris	–	–	–	100
Oblada melanura	100	–	–	–
Serranus cabrilla	–	–	–	–
Serranus scriba	50	–	50	–
Holothurians	**55.6**	**33.3**	**11.1**	**–**
Holothuria forskali	20	60	20	–
Holothuria poli	50	25	25	–
Holothuria tubulosa	77.8	22.2	–	–
Total general	39.5	18.4	15.8	26.3

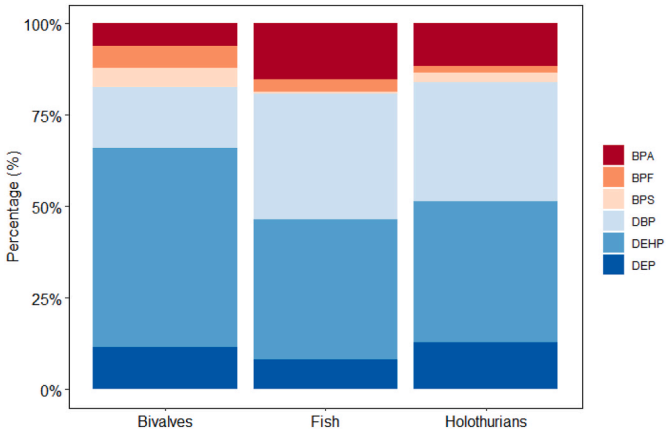

Fig. 4. Stacked barplot representing the percentage of each bisphenol (μg/g ww): bisphenol A (BPA), bisphenol F (BPF), and bisphenol S (BPS); and phthalates (μg/g ww): dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), and diethyl phthalate (DEP) in bivalves, fish, and holothurians from Cabrera MPA.

3.3. Phthalates and bisphenols

The mean levels of phthalates and bisphenols differed between the species assessed. All bisphenols, but not all phthalates, were detected in all individuals. DEHP was detected in 95.83% of the total samples analyzed; it was not present in only two fish individuals of the *O. melanura* species. DEHP was the predominant plasticizer in the three studied groups (Fig. 4). The levels of plasticizers were slightly different between the three groups of organisms: DEHP > DBP > DEP > BPA > BPF > BPS in bivalves, DEHP > DBP > BPA > DEP > BPF > BPS in fish, and DEHP > DBP > DEP > BPA > BPS > BPF in holothurians (Fig. 4).

The concentration levels of all plasticizers were statistically different between groups of species (KW, $p < 0.001$; Fig. 5). In terms of bisphenols, the levels of BPS and BPF (mean 0.25 ± 0.23 μg/g ww and 0.29 ± 0.20 μg/g ww; with a 0.94 and 2.66% of Relative Standard Deviation (RSD), respectively) were statistically higher in bivalves than in fish and holothurians (Fig. 5). Nevertheless, the levels of BPA were statistically higher in holothurians (mean 0.45 ± 0.04 μg/g ww; and RSD 5.56%) than in fish (0.32 ± 0.06 μg/g ww; RSD 3.81%) and bivalves (0.29 ± 0.04 μg/g ww; RSD 3.95%) (Fig. 5). In terms of phthalates, bivalves (mean 0.54 ± 0.26 μg/g ww; RSD 1.96%) and holothurians (mean 0.49 ± 0.26 μg/g ww; RSD 1.56%), showed statistically higher levels of DEP

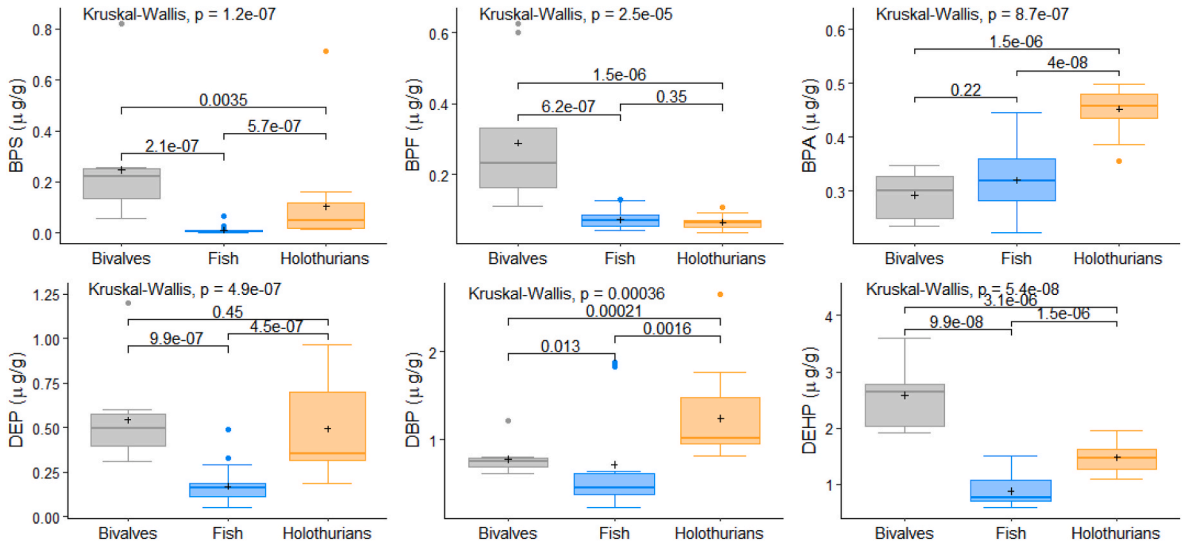

Fig. 5. Boxplot of the levels of the bisphenols (µg/g ww): bisphenol S (BPS), bisphenol F (BPF), and bisphenol A (BPA); and phthalates (µg/g ww): diethyl phthalate (DEP), dibutyl phthalate (DBP), and bis (2-ethylhexyl) phthalate (DEHP) assessed in bivalves, fish, and holothurians from Cabrera MPA. Differences in plasticizer levels values were assessed with the Kruskal-Wallis test (significance level at $p < 0.05$) according to the group of species.

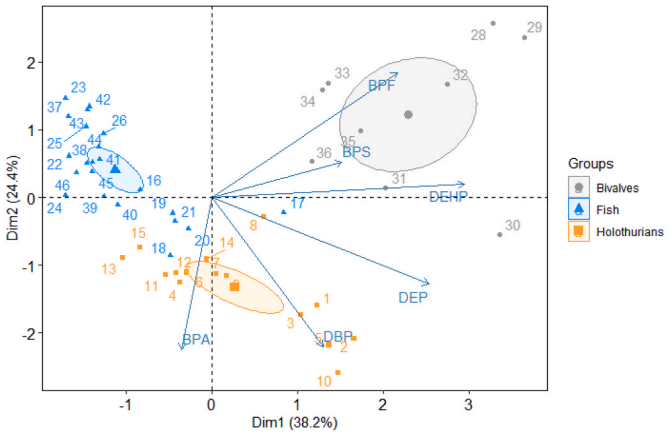

Fig. 6. Plot of the Principal Component Analysis (PCA) of levels of plasticizers assessed in bivalves, fish, and holothurians from Cabrera MPA. Grey dots, bivalves; blue triangles, fish; brown squares, holothurians. Confidence intervals of the groups are represented with ellipses. Biplot has been overlapped to PCA. Small angles between arrows indicate positive high correlations between biomarkers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

than fish (0.17 ± 0.01 µg/g ww; RSD 3.64%) (Fig. 5). Finally, DBP and DEHP levels differed within the three groups. DBP levels were statistically higher in holothurians (mean 1.24 ± 0.49 µg/g ww; RSD 2.16%), than in bivalves (0.78 ± 0.18 µg/g ww; RSD 2.21%) and fish (0.72 ± 0.60 µg/g ww; RSD 3.4%) (Fig. 5). In terms of DEHP, bivalves showed the highest values (mean 2.58 ± 0.55 µg/g ww; RSD 2.93%), followed by holothurians (1.48 ± 0.28 µg/g ww; RSD 3.52%), and fish (0.88 ± 0.26 µg/g ww; RSD 4.36%) (Fig. 5). The levels of plasticizers in bivalves were not statistically correlated with MCI ($p > 0.05$). Fish' K index showed a negative correlation with DBP (rho = −0.45, $p < 0.05$). Finally, in holothurians, SLW showed no correlation with none of the plasticizers assessed ($p > 0.05$).

We used PCA analysis to assess the variability of phthalates and bisphenols levels in bivalves, fish, and holothurians; the two principal components of the PCA explained 62.5% of the total variability (Fig. 6). The main plasticizers in the first dimension were DEHP, DEP, and BPF with a 36.3%, 26.8%, and 19.7% contribution to variability, respectively. BPA, DBP, and BPF were the main plasticizers in the second dimension with 33.0%, 32.0%, and 22.3% of the total contribution, respectively. Bivalves were highly correlated to BPF, BPS, and DEHP, while holothurians had a high correlation with BPA and DBP. On the other hand, fish had no correlation with all the phthalates and bisphenols assessed. In general, the PCA analyses did represent clear differences between species according to the plasticizers assessed (Fig. 6).

3.4. Correlation between MP ingestion and plasticizers

The presence of MPs in the soft tissue of bivalves was statistically correlated with levels of BPF (rho = 0.75; $p < 0.05$); however, no correlation was detected between the presence of MPs and BPA, BPS, or the three phthalates assessed. In fish, no correlation was observed between MPs ingestion and none of the plasticizers assessed. Finally, holothurians showed a negative and statistically significant correlation between MP ingestion and BPA (rho = −0.61; $p < 0.05$), and a positive correlation between MP ingestion and DEP and DEHP phthalates (rho = 0.53, rho = 0.55, respectively; $p < 0.05$).

4. Discussion

To our knowledge, there are no studies simultaneously assessing the ingestion of MPs and the presence of bisphenols and phthalates in marine species with different biological and ecological traits, particularly from marine protected areas (supposedly free of plasticizers pollution). Our results provide evidence of high MP ingestion in bivalves, fish, and holothurians and the presence of phthalates and bisphenols plasticizers in marine organisms from Cabrera MPA. Our findings indicate exposure to and bioaccumulation of these pollutants in marine organisms of a natural reserve.

4.1. Microplastic ingestion

The mean MPs ingestion was different in the three groups of species; holothurians were the group with the highest number of MP items ingested, followed by bivalves, and finally by fish. The mean MP ingestion in holothurians reported here (12.67 ± 7.31 MPs per individual) was within the range of previous results performed under laboratory conditions (4.0 ± 7.2 to 47 ± 72 MPs per individual) (Graham and Thompson, 2009), and from natural ecosystems in the Yellow Sea (1.56 ± 0.96 to 24.2 ± 5.90 MPs per individual) (Mohsen et al., 2019).

A previous study assessing the routes of MP uptake in the holothurian *H. cinerascens* highlighted two potential routes of MPs intake: the feeding tentacles and the respiratory trees (Iwalaye et al., 2020). Additionally, it has been hypothesized that under controlled conditions holothurians can ingest MPs along with sediment and that the relative amount of MPs ingested would be consistent with the ratio of MPs in the sediment (Graham and Thompson, 2009). This hypothesis was confirmed in holothurians collected from eight farms along the Bohai Sea and the Yellow Sea in China, where the number of MPs from the intestines increased proportionately with MP abundance in the sediment (Mohsen et al., 2019). The high amounts of MP ingested by holothurians in our study agree with the high amounts of MPs observed in sediments samples from Cabrera MPA by Alomar et al. (2016), who reported values of MPs ranging from 100.78 ± 55.49 to 897.35 ± 103.31 MP kg^{-1} of dry sediment. Our results and the high amounts of MPs in both holothurians and sediments from Cabrera MPA give further evidence that this echinoderm can capture MP particles directly from the sediment, which is reinforced by the fact that holothurians are sedimentivorous non-selective feeding species.

In our study MP ingestion was high in bivalves (mean 4.83 ± 5.35 MPs per individual). Previous studies reported a high variability of MP ingestion in bivalves with mean values ranging from 1.70 ± 0.20 to 2.00 ± 0.20 MPs per individual (in Mediterranean mussels, *Mytilus galloprovincialis*, from the Northern Ionian Sea) (Digka et al., 2018), to values ranging from 0.06 to 2.47 MPs per individual (in Mediterranean mussels collected along the Turkish coasts) (Gedik and Eryaşar, 2020). A strong correlation between MP abundance in water and MP abundance in bivalves has previously been described (Qu et al., 2018). Therefore, the presence of MPs in bivalves is representative of MP abundance in the water column of Cabrera MPA. Samples from the sea surface, collected with a manta trawl in Cabrera MPA, showed mean values ranging from 0.19 ± 0.17 to 19.32 ± 14.45 MP m^{-3} which are higher than those reported for the majority of study areas of the western Mediterranean Sea (Fagiano et al., 2022). Alomar et al. (2016) and Fagiano et al. (2022) use different units to evaluate the abundance of MPs in the sediments and the sea surface, respectively, thus it is not feasible to make a direct comparison of values between these studies. Nevertheless, the high abundance of MPs in sediments provides additional evidence that sediments act as a sink of MPs, and support the hypothesis that the distinct abundance of MP in different groups of species could be related to their feeding strategies and their habitat.

Fish showed the lowest level of MP ingestion out of the three groups of species. Taking into account that fish have high trophic levels on the food web, and the confirmed transfer of MPs throughout trophic levels (Santana et al., 2017), it was expected that fish presented higher MP ingestion values than invertebrates. The non-selective feeding behavior of bivalves and holothurians could explain their higher levels of ingested MPs. Because of their habitat and feeding characteristics, bivalves and holothurians have optimal ecological traits for a bioindicator species for monitoring MPs in specific coastal areas (Fossi et al., 2017).

The most common shape of MPs was fibers, followed by fragments, films, and ropes + filaments. There is an increasing concern in the scientific community regarding the presence of fibers in the marine environment (Chan et al., 2021; Erdle et al., 2021; Rios-Fuster et al., 2022b). Recent studies analyzing the presence of MPs in marine ecosystems have reported a high presence of fibers in both the sea surface and the water column (Rios-Fuster et al., 2022b; Suaria et al., 2020), and in marine organisms (Compa et al., 2018; Rios-Fuster et al., 2019), revealing MP ubiquity in the environment. Previous studies reported domestic textile laundering and wastewater treatment plants as the most common pathway for fibers release into the environment (Browne et al., 2011; Gavigan et al., 2020). Oceanographic processes and marine currents patterns might be involved in the transference of pollution to Cabrera MPA from elsewhere, as Cabrera has no wastewater treatment plants, and therefore no fiber loads are released here directly. Instead, an additional potential source of fibers is the waste released from merchant ships and from the ships that visit the area of Cabrera MPA, especially during the summer months. These results should encourage the development of new technologies designed to reduce the release of textile fibers into the environment (Erdle et al., 2021; Gavigan et al., 2020; Rios-Fuster et al., 2022b), and reinforce the need to implement mitigation measures focusing on reducing marine litter at a global scale.

Slight differences in polymer types were observed between groups; there was a similar number of polyethylene and polystyrene items in bivalves and fish; holothurians showed a high number of polyethylene items, followed by polypropylene items. In general, the predominance of the polymers that we describe here is in keeping with a previous report: 40% of the MPs identified in the water column from the western Mediterranean Sea were low-density polyethylene and polypropylene (20% each), and 14% were polystyrene (Rios-Fuster et al., 2022b). A high percentage of polyethylene items has been previously reported in several field studies from the Mediterranean Sea (Avio et al., 2017; Digka et al., 2018; Suaria et al., 2016). Polyethylene and polypropylene microfibers have been previously associated with laundry and textile washing activities (Naji et al., 2021).

In Cabrera MPA, here we show that marine organisms with non-selective feeding behavior, sessile species, and low mobility species have higher ingestion of MPs than fish (which have a selective feeding behavior and have higher mobility). We conclude that MPs are obtained from the water column and sediments, due to the feeding behavior; this is evidence that both the water column and the sediments in Cabrera MPA have considerable loads of MPs.

4.2. Phthalates and bisphenols

The three groups of species show a different predominance of plasticizers. In general, the levels of two bisphenols (BPS and BPF) and one phthalate (DEHP) are higher in bivalves' soft tissue than in the muscle of fish and holothurians. The levels of BPA and DBP are higher in the muscle of holothurians than in bivalves and fish. Additionally, fish show the lowest levels of plasticizers and only levels of BPA are intermediate between the BPA levels of bivalves and holothurians. It must be noted that plasticizers were quantified in muscle in fish and holothurians and the whole soft tissue of bivalves so a direct comparison is not possible.

Bivalves were collected from submerged buoys in a port area with medium/high maritime traffic. Therefore, ship traffic or recreational activities could explain the higher presence of this type of pollution in bivalves. A previous study in the northwestern Mediterranean Sea reported a higher abundance of phthalates and organophosphate esters in sediments close to large ports (Alkan et al., 2021). Although the maritime activity in the Port of Cabrera MPA and its surroundings is significantly lower than the maritime activity in large Mediterranean ports, the presence of these chemicals in ports suggests that ships may be a potential source of these plasticizers. In general, in *A. noae* from Cabrera MPA the levels of DEP (0.54 ± 0.26 μg/g ww) were higher, and the levels of BPA (0.29 ± 0.04 μg/g ww) were lower in comparison with the Mediterranean mussel (*M. galloprovincialis*) from Port d'Andratx, located at the south-western of Mallorca in an anthropogenically impacted area with high maritime traffic and a highly populated coast (mean values 0.27 ± 0.63 and 5.56 ± 2.33 μg/g ww, respectively) (Rios-Fuster et al., 2022a). Because the biological half-life of BPA in the Mediterranean mussel is 26 days, and because its depuration rate is lower than its uptake rate (Gatidou et al., 2010), the low levels of BPA in mussels from Cabrera MPA suggest that these individuals have low exposure to BPA.

In our study, fish had lower levels of MP ingestion and lower levels of plasticizers in comparison to bivalves and holothurians. The levels of BPA in fish muscle (0.32 ± 0.06 μg/g ww) were higher than values reported in red mullets (*Mullus barbatus*) collected from the Northern Coast of Sicily, which ranged from 46.7 ± 7.6 to 58.9 ± 14.7 ng/g ww (Errico et al., 2017). In fresh fillets of European seabass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*), and yellowfin tuna (*Thunnus albacares*) blast-chilled fillets purchased in a supermarket,

DEHP had the highest relative values of all phthalates, followed by DBP and DEP (Panio et al., 2020). Finally, the lower values of plasticizers in the muscle of fish, relative to invertebrates, can be associated with lower MP ingestion. In this study, BPA was the only plasticizer with a higher abundance in fish than in bivalves. Differences between the levels of plasticizers in different species could be due to metabolic differences (Sala et al., 2022).

Some organophosphorus flame retardants in a lake food web exhibit trophic dilution rather than bioaccumulation (Zhao et al., 2018). Additionally, the plasticizers assessed in our study were previously detected in gilthead seabream (*Sparus aurata*) from aquaculture facilities, and a decrease in the concentration of BPA throughout a four months experiment was reported (Capó et al., 2022). Taken together, all these results demonstrate the complexity of the bioaccumulation processes of chemical pollutants within trophic levels, and suggest that further investigations should be performed, taking into consideration several factors, such as the molecular structure of the chemicals and the metabolic characteristics of the species under study.

The high levels of BPA and DBP quantified in holothurians suggest a high bioaccumulation capacity of these chemicals in the muscle of this species. A previous study reported that the levels of some emerging contaminants, such as benzophenone 3 and the biocide triclosan, were ≥50-fold higher in holothurians than in the water and the sediment (Martín et al., 2020). Moreover, several emerging contaminants derived from personal care products had a higher affinity for sediments than for water, under controlled conditions (Martín et al., 2020). It is thus possible that sedimentivorous species are more exposed to emergent contaminants than pelagic species and that holothurians have a high capacity to bioaccumulate these types of chemical contaminants. The respiration system of holothurians may be the second pathway of MPs intake, as during the breathing process holothurians pump water into the organism without a prior filtering process (Iwalaye et al., 2020), explaining the high abundance of these pollutants in holothurians in our study. In addition, higher concentrations of the phthalates DBP and DEHP were reported only in the bottom water of the northwestern Mediterranean Sea, suggesting that these chemicals have high degradation rates in the upper layers of the water column (Paluselli et al., 2018). Phthalates have also been detected in sediments from different locations in the northwestern Mediterranean Sea (Schmidt et al., 2021), demonstrating that sediments act as deposits for these chemicals. The correlation between the MPs ingested and the concentrations of the phthalates DEP and DBP indeed suggest that sediments might act as a sink for these contaminants, which are consequently available for sedimentivorous organisms.

4.3. Correlation between microplastic ingestion and plasticizers

The high negative correlation between the number of MPs and BPA levels that we observed in holothurians highlights a complex interaction between these pollutants. Similar results were reported in mussels collected from several locations in the western Mediterranean (Rios-Fuster et al., 2022a). The correlation between polymers and plasticizers exhibits a complex pattern, and environmental factors such as salinity or pH can influence the sorption/desorption rates of these chemicals from polymers to sea water (Liu et al., 2019), in turn affecting their bioaccumulation rates in marine organisms. Polyethylene and polypropylene polymers exhibited a high and reversible sorption capability (Liu et al., 2019). Further investigations should be performed to correlate the total number of MPs with BPF concentrations in the soft tissue of bivalves. We did not find a statistically significant correlation between ingested MPs and the levels of plasticizers in fish species; this observation differs from the significant positive correlation between the total concentration of bisphenols in muscle and the number of MPs ingested in several fish species (Barboza et al., 2020). These differences suggest a tissue and/or location effect affecting the interaction between these pollutants. The exploration of these interactions requires further investigation. Joint reduction and mitigation actions and policies should be conducted on a global scale to preserve biodiversity from plastic pollution and the associated contaminants. Our results should encourage the European Member States to implement stronger measures to reduce marine pollution (especially plastic litter), reduce the transference of plastics and MPs from urban to protected areas, and reduce the presence of plasticizers in the marine environment, which we evidenced here.

5. Conclusions

Our simultaneous assessment of the ingestion of MPs and environmental plasticizers (phthalates and bisphenols) in bivalves, fish, and holothurians from a MPA suggests that feeding traits and habitat use of individuals can directly affect the ingestion of MPs and the accumulation of plasticizers. Bivalves had intermediate values of MPs ingestion and the highest concentration of some plasticizers (BPS, BPF, and DEHP), suggesting that a filter-feeder feeding strategy exposes them to MP pollution. Conversely, fish had low values of both MPs and plasticizers, suggesting that selective feeders could reduce the risk of plastic ingestion and associated plasticizers. Holothurians seem to be a suitable bioindicator for sediment contamination of MPs and plasticizers. Our results evidenced that, throughout the water column, MPAs are not free from MPs and plasticizers.

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.

Data availability

Data will be made available on request.

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