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Phthalate levels in common sea anemone *Actinia equina* and *Anemonia viridis*: A proxy of short-term microplastic interaction?

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ABSTRACT

Phthalates are widely employed plasticizers blended to plastic polymers that, during plastic aging and weathering are prone to leach in the surrounding environment. Thus, phthalates were proposed to indirectly evaluate MPs contamination in marine environments, with still uncertain and scarce data, particularly for wildlife. This study investigates simultaneously microplastics (MPs) and phthalates (PAEs) occurrence in wild *Actinia equina* and *Anemonia viridis*, two common and edible sea anemone species. Both species had a 100 % frequency of MPs occurrence, with similar average concentrations. PAEs were detected in 70 % of samples, with concentrations up to 150 ng/g in *A. equina* and 144.3 ng/g for *A. viridis*. MPs and PAEs present in sea anemone tissues appear to reflect seawater plastic contamination conditions in the study area. Given the rapid biodegradation of PAEs, occurrence and concentrations of both these additives and their metabolites could be useful tracers of short-term plastic debris-biota interactions.

1. Introduction

Due to its increasing production, indiscriminate discards and durable nature, plastic has become a prevalent element of marine litter worldwide (Andrady, 2011). It is particularly important to monitor and assess microplastics (MPs), plastic particles smaller than 5 mm in size (Arthur et al., 2009) which are recognised as one of the most urgent global marine environmental issues (Fries et al., 2013; Galgani et al., 2015). MPs mainly consist of polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), or polyethylene terephthalate (PET) (Andrady, 2011; de Haan et al., 2019), with fibres and fragments as the shapes more commonly found in aquatic samples (Dusaucy et al., 2021; Sharma et al., 2021). Beyond mechanical impacts, MPs can lead to toxic effects related to biota exposition to plastic associated contaminants, either adsorbed from the environment or added during the manufacturing process (Luís et al., 2021). Indeed, plastics are rarely pure polymers, as during their manufacture a mix of additives such as plasticizers, flame retardants, fillers and stabilisers, pigments and reinforcements are usually added to them (Fries et al., 2013; Hermabessiere et al., 2017). Among them, phthalate esters (PAEs) are the family of chemicals most widely used as plasticizers to render plastic more flexible, durable, and transparent (Luís et al., 2021). Short chain phthalates like diethyl phthalate (DEP), di-n-butyl phthalate (DBP) and butyl benzyl phthalate (BBP) are common in non-PVC applications such as plastic bags and personal care products (Luís et al., 2021). The use of long-chain phthalates, like di-(2-ethylhexyl) phthalate (DEHP), is common in plastic polymers and construction materials (Net et al., 2015; Luís et al., 2021). Such chemicals are added to plastic polymers in high

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relative mass amount (Hermabessiere et al., 2017; Paluselli et al., 2019; Luís et al., 2021) and in different combination (Zhang et al., 2019).

Since PAEs are not covalently bound to the polymeric matrix, they slowly leach into the environment during plastic aging and weathering (Paluselli et al., 2019). Upon entering the aquatic ecosystem, PAEs interact with a variety of organisms, from fishes to marine invertebrates, causing concern due to their toxic nature and endocrine disruptor activity (Baloyi et al., 2021) even at low concentrations (e.g. in the low ng/l to μg/l range, Oehlmann et al., 2009; Zhang et al., 2021). With PAEs being widely used in plastic manufacturing (Paluselli et al., 2019; Zhang et al., 2021), their presence in the seas is linked to the widespread use of plastic products and constant releases of plastic into the sea (Paluselli et al., 2018; Zhang et al., 2018; Luís et al., 2021). Indeed, according to Hermabessiere et al. (2017), PAEs' chemical fingerprint and behaviour in the water column can be determined by MPs accumulation and degradation in the marine environment. The interaction between biota and MPs (e.g., ingestion) has been documented in a wide variety of animal species (Martin et al., 2019; Palazzo et al., 2021; Savage et al., 2022) and may constitute a transfer pathway for PAEs into marine organisms (Zhang et al., 2018). Thus, a possible link between MPs exposure and the presence of PAEs in organism tissues has been proposed in different marine species (Baini et al., 2017; Fossi et al., 2012; Vered et al., 2019). As phthalates have long half-lives under abiotic environmental conditions (Net et al., 2015), while their metabolism is rapid in organisms (Hu et al., 2016; Luís et al., 2021; Zhang et al., 2021), biomonitoring phthalates' metabolites (MPEs) has been accepted as a tool to assess biota exposure to parental phthalate congeners (Hu et al., 2016). However, the potential role of plastic debris interactions to the accumulation of PAEs in sea organism tissues is still not thoroughly understood (Saliu et al., 2020a). Because of the challenges associated with PAEs analyses (Paluselli et al., 2019), only a limited number of studies have examined PAEs exposure in aquatic wildlife samples (Hu et al., 2016; Sanjuan et al., 2023) and the contribution of MPs leaching into free-ranging marine organisms' PAEs content (Vered et al., 2019; Raguso et al., 2022). Despite this, the environmental link between plastics and phthalates presence and the fact that they do not biomagnify through the trophic web (Zhang et al., 2021), make it worthwhile investigating the suitability of PAEs as tracers of plastic interaction with marine wildlife. Since PAEs biodegrade rapidly (Hu et al., 2016), we hypothesize that monitoring their levels and their metabolites in organisms' tissues could be a useful way to indirectly assess short-term plastic-biota exposure. Thanks to a better metabolic efficiency, high trophic level organisms present PAEs content often lower than that of a lower trophic level organism (Net et al., 2015; Hu et al., 2016). Algae, cnidarians, molluscs, crustaceans, and fish are in order of increasing metabolic efficiency (Wofford et al., 1981), with organisms at the bottom of the trophic web with higher levels of PAEs expected.

Sea anemones (class Anthozoa; order Actiniaria) are worldwide-distributed cnidarians (Shick, 2012) that, thanks to their sedentary opportunistic polyphagous suspension feeding technique (Chintiroglou and Koukouras, 1992) are highly exposed to MPs litter (Galgani et al., 2015). They get in contact with MPs from the water column via ingestion and external tissue adhesion (Savage et al., 2022), and likely, interface with plastic additives. Moreover, considering their "simple tissue grade of construction" (Shick, 2012) and simple biochemical composition (soft tissue made up of 98 % water with collagen and lipids ranging from 9 % to 47 % of their dry weight, Yamashiro et al., 1999), sea anemones are particularly suitable for the phthalate detection methodology adopted here (Saliu et al., 2020a, 2020b). *Anemonia viridis* (Forsskål, 1775) and *Actinia equina* (Linnaeus, 1758) were proposed as target organisms since they are two well known, edible and widespread sea anemone species (González et al., 2001; Silva et al., 2017) consumed in some Mediterranean regions, like Sardinia (Italy) and across the Andalusian coast (Spain). The two species are omnivorous suspension feeders, which rely on slightly different feeding strategy: *A. viridis* actively searches for foods in the surrounding waters through its long tentacles (Chintiroglou and Koukouras, 1992); *A. equina* is less active respect to *A. viridis*, acting as a sit-and-wait predator that feed on whatever falls onto the tentacles and oral disc (Chintiroglou and Koukouras, 1992; Shick, 2012).

Using a potential non-lethal SPME-LC/MS method (Saliu et al., 2020b), this study investigates the occurrence of phthalate acid esters (PAEs) and microplastics (MPs) in wild-collected edible sea anemones, as well as the suitability of PAEs detection in sea anemone tissues for short-term assessment of plastic microlitter exposure. To this aim, we: 1) assessed and compared MPs uptake by sea anemones collected on-field, considering the ecological niches of the two species; 2) investigated the presence of PAEs and metabolites (MPEs) in *A. equina* and *A. viridis*, establishing baseline concentrations to investigate future trends in exposures and health risks associated with human consumption of such organisms; 3) evaluated whether sea anemones were suitable organisms for collecting data on both contaminants potentially on the site and in vivo conditions, examining for potential links between the occurrences of both these contaminants in sea anemones tissues.

2. Material and methods

2.1. Study area

This study was conducted along the coasts of the Sinis Peninsula (Western Sardinia, Italy). The sampling sites (Fig. 1) were located between Capo Mannu (40.1500° N, 8. 22,344° E) to the north and Seu (39.9080° N, 8.3910° E) to the south, including the island of Mal di Ventre (offshore the Sinis Peninsula). The region presents offshore circulation patterns (Olita et al., 2013) that favour the trapping of water masses in coastal areas (Treml et al., 2012), increasing the retention of planktonic aggregates (Cucco et al., 2006) and easily mix floating dispersed plastic items (De Lucia et al., 2018). Consequently, MPs remain trapped in the area due to the prevailing winds blowing from west and north, and the inflow of Modified Atlantic Water (Camedda et al., 2021; Palazzo et al., 2021).

2.2. Sample collection

Two operators collected the individuals in May 2022 by snorkelling. Samplings were conducted in 4 coastal sites: Mandriola (M) (Fig. 1A), Mal di Ventre North (MdVN) (Fig. 1B), Mal di Ventre South (MdVS) (Fig. 1C) and Seu (S) (Fig. 1D). Five specimens of each sea anemone species were randomly collected in each one of the 4 sites, for a total of 40 sampled sea anemones (20 *A. equina* and 20 *A. viridis*). At each of the four sites, specimens of both sea anemone species were collected simultaneously to allow statistical comparison. Our samples ranged in depth from 0 to 3 m to avoid differences in diet due to depth (Chintiroglou and Koukouras, 1992). Furthermore, considering the potential effect of body weight on MPs ingestion (Morais et al., 2020), only specimens with similar sizes were selected for the analyses (Table S1). The wet weight was 15.73 ± 1.3 g (mean ± SE) for *Actinia equina* samples and 20.54 ± 2.10 g (mean ± SE) in *Anemonia viridis*. The entire body of each anemone was removed from the field by gently detaching the pedal disc from the substrate by hand or with the aid of a metal spatula. Then, each specimen was stored in individual glass containers filled with pre-filtered seawater from the collection site and promptly placed in a field-cooler. At the same time, to evaluate the average concentration levels of phthalates in seawater, six aliquots of seawater (20 ml) were collected in triplicates from each site in glass vials.

2.3. Sample processing

Upon arriving in the laboratory, each sea anemone was weighed (wet weight, second decimal point; g) and rinsed thoroughly with pre-filtered seawater onto a 50 μm sieve (Giuliani steel sieves) to collect potentially adhered microplastics. In order to collect any egested material that

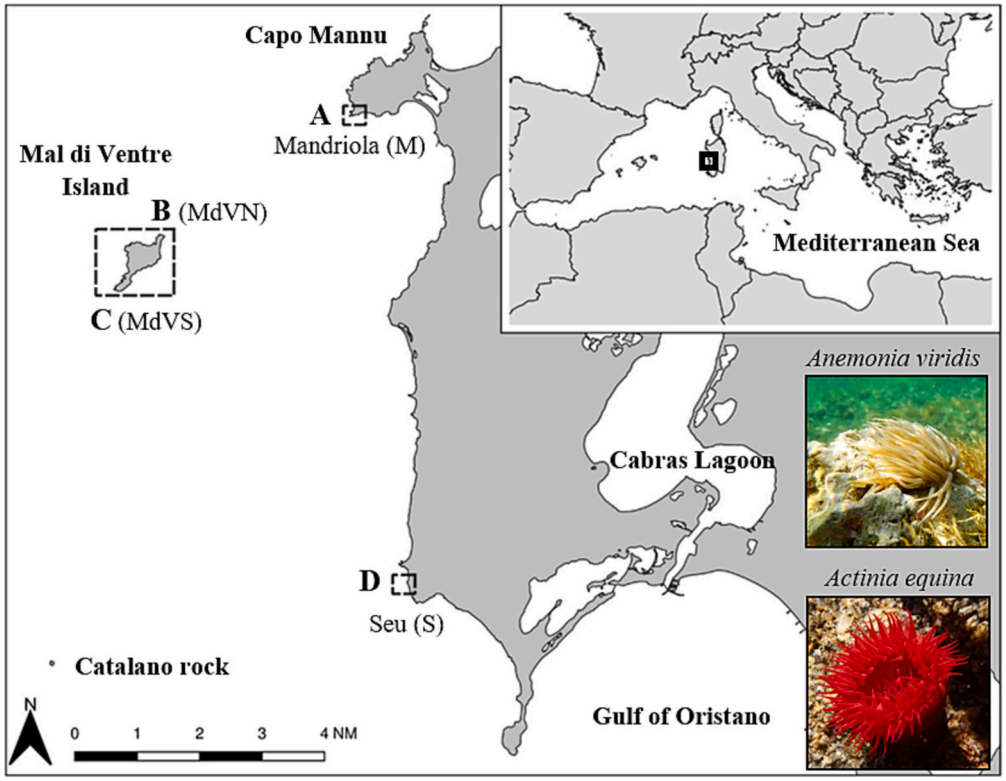

Fig. 1. Map of the study area showing the 4 sampled sites around the Sinis Peninsula (Sardinia); A) Mandriola, B) Mal di Ventre Island North, C) Mal di Ventre Island South, D) Seu. On the right, a picture of the target sea anemone species.

occurred during sample transfer due to the contraction of the central column and attached to tentacles or external tissues, the seawater of each glass container was filtered on the same steel sieve (Chintiroglou and Koukouras, 1992; Morais et al., 2020). For each sea anemone specimen, the 50 μm sieve was washed with pre-filtered water and potentially collected anthropogenic particles were concentrated into individual glass beakers (200 ml). Then, 2 g of tissues (body column, tentacles, and pedal disc) were removed from each sea anemone with carbon-steel scalpel blade and immediately stored in individual 5 ml clear glass vials at −80 °C for phthalates analyses. Water sample aliquots from each sample site were stored at −80 °C as well. The entire body of each specimen was then placed into individual 200 ml glass beakers (the same used to collect potential adhered and egested anthropogenic particles) and 15 % H_2O_2 was added to digest the organic matter (Bessa et al., 2019). Subsequently, each beaker was covered with an aluminium foil and kept at room temperature (~25 °C) until complete digestion of the organic material. Thereafter, each solution was filtered onto 50 μm sieve and positioned in single glass Petri dish to be observed under a binocular stereoscope (Carl Zeiss Microimaging GmbH, Germany) equipped with image analysis system (AxioCam ERc5s and Zen, 2011 Blue edition software). Fine-tipped steel tweezers were used to isolate the detected microplastic particles and place them onto individual Petri dishes. Microlitter particles were counted, photographed, and measured by means of image analysis. Detected microfibres were classified as plastic, natural or anthropogenic based (modified cellulose and cellulose combined with pigments, Lusher et al., 2020). Microlitter items were subdivided into the following typologies: pellet, fragment, fiber, film, rope/filament, microbeads, sponge/foam, and rubber, according to Bessa et al. (2019). Despite not being relevant to this study, the colours of the anthropogenic objects were noted for completeness of microplastic analysis, following the classification in Bessa et al. (2019).

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

Common practices to minimize both MPs and PAEs background contamination were adopted during sample collection and processing of the samples according to Bessa et al. (2019) and Isa et al. (2022), respectively. In particular, sampling, handling, processing and analysis were carried out in a closed room with restricted access during operations. All materials used during field collection, laboratory surfaces and equipment used for processing the samples were thoroughly washed and rinsed with ultrapure filtered Milli-Q water and ethanol. After each wash, the laboratory materials used were wrapped in aluminium foil until used. To reduce phthalates background contamination during sample collection and manipulation no plastic items were used and all glassware was pre-cleaned with acetone and baked at 300 °C before use. Other precautions were also taken during manipulation, extraction, sorting and identification, such as: wearing a cotton laboratory coat, covering the samples at all times and monitoring airborne contamination created during laboratory procedures.

2.5. Microplastics analysis

The chemical composition of a subsample of the anthropogenic items isolated from sea anemone specimens was analysed by using Fourier Transform Infrared Spectroscopy (FTIR). Particularly, each type of particles isolated from 2 specimens of each sampled site from both species were filtered onto single macroporous silicon membranes 1 mm^2 with 5 μm pore size (SmartMembranes GmbH). Spectra were collected by means of a Nicolet™ iN10 MX Infrared Imaging Microscope spectrometer in the range 4000–650 cm with 4 cm^{-1} spectral resolution and 4 scans in transmission mode and an area of 20 × 20 μm^2. Acquired spectra were compared to spectral libraries (Meyns et al., 2019).

2.6. Phthalates analysis

In both sea anemone and seawater samples, the presence and quantification of the 5 target phthalates congeners (butylbenzyl phthalate - BBP, dibutyl phthalate - DBP, di-2-Ethylhexyl phthalate - DEHP, diethyl phthalate - DEP, and dimethyl phthalate - DMP) and the 3 target metabolites, monoethylexyl phthalate or MEHP (monoester of DEHP), monobutyl phthalate or MBP (monoester of DBP) and monobenzyl phthalate or MBzP (monoester of BBP) were assessed by employing the SPME-LC/MS method described in Saliu et al. (2020a, 2020b) adapted to the sea anemone samples according to the steps described below. Employed bioSPME fibres are made of C18 functionalized silica particles (3 μm diameter) coated (45 μm thickness) onto a 200 μm metal fiber and embedded with a biocompatible binder. Briefly, the fibres were activated in 1 ml of methanol for 15 min. Then, the bioSPME fibres were inserted directly in the sea anemones tissues to enable the extraction step of the analytes at room temperature (25 °C). After 40 min, each fiber was removed from the samples and placed in a glass vial containing 1 ml of ultrapure water for the washing step and placed in 80 μl of pure methanol to perform the desorption step for 30 min. Then, each fiber was taken out from its respective glass vial and the final extract was submitted to LC/MS analysis. LC/MS analyses were conducted with a ThermoScientific TSQ quantum access max instrument, following the instrumental set up and applying the selected reaction monitoring (SRM) of the mass transitions described in Saliu et al. (2020a). We calibrated the system by drawing matrix matched calibration curves for each target analyte as described in Saliu et al. (2020a). Briefly, phthalate esters mix (EPA 506 Phthalate Esters Mix), the related mono-alkyl derivatives and labelled surrogates were purchased from Sigma Aldrich and used to prepare individual stock solutions in methanol at a concentration of 500 μg/ml in amber flask. After apposite methanol dilutions, the standards were used to spike a reference matrix sample prepared in house from agarose gel matrix as described in Saliu et al. (2020a). This reference was then used both for method optimization and validation since no reference material is commercially available. The matrix-matched calibration curves were drawn with 8 calibration points (0.5, 1, 5, 10, 25, 50, 100, and 250 ng/g.). Correlation coefficient (R^2) was >0.980 with randomly distributed residuals (<20 %) for all the investigated compounds. Accuracy was estimated from 12 replicated analysis of the QC sample prepared at 100 ng/g concentration level and by applying back calculation methods. Recoveries resulted comprised between 93 % - 107 % and precision 4–12 % (Relative Standard Deviation (RSD) on 12 replicated analysis). To determine the levels of background contamination a total of 14 procedural blanks was run during the batch analysis. Limits of detection (LODs) and limits of quantification (LOQs) were calculated from procedural blanks, by considering the blank mean plus three times and ten times the standard deviation, respectively. If no peak was detected at the retention time of the analyte, the LODs and LOQs were estimated as three and ten times the signal-to-noise ratio, respectively. This method is preferred for determining the LODs and LOQs of PAEs since PAEs are ubiquitous contaminants (Saliu et al., 2020a, 2020b). During sample analysis a positive identification was assumed when the following condition were assured 1) detection of the representative SRM transition at the exact *m/z* (unit resolution) for both the pre-selected qualifier and quantifier ions; (2) occurrence of the chromatographic peak within the interval of 15 s in respect to the mean retention times obtained the replicated analysis of the standard calibration mixture; (3) a signal-to-noise ratio mayor than 3. Peak areas were then integrated, and the concentrations of the target analytes were calculated using the response factors assayed from the matrix matched calibration (corrected with the labelled internal standard). Each sample was analysed in triplicate and the final value was accepted when RSD <4 %.

2.7. Statistical analysis

Univariate and multivariate analyses were applied to investigate any differences in MPs abundance and PAEs concentrations between *Actinia equina* and *Anemonia viridis* among sampling sites. Particularly, univariate two-way permutational analyses of variance (PERMANOVA) on square root transformed data were used to check for differences in: 1) wet weight; 2) the number of MPs detected; 3) total sum of the PAEs levels (Σ_8PAEs) detected; 4) sum of the parental PAEs (Σ_5PAEs); 5) sum of the metabolites (Σ_3MPE). Following, multivariate two-way PERMANOVA tests were run to investigate differences in detected levels of each one of the 8 phthalates between the two species of sea anemones and among sampling sites. For all the performed analyses, "species" was considered as a fixed factor with 2 levels (*A. equina*, *A. viridis*), while "site" was treated as a random factor with 4 levels (S, M, MdVN, MdVS). Univariate (Σ_8PAEs, Σ_5PAEs, Σ_3MPEs) and multivariate (single PAEs, square root transformed data) one-way PERMANOVA analysis were applied to investigate differences in PAEs levels detected in seawater among sampled sites ("site" = random factor with 4 levels). Analyses were run with the PRIMER6 statistical software (Plymouth Marine Laboratory, UK) complete with PERMANOVA+ package, based on Euclidean distance for univariate tests and Bray-Curtis distance for multivariate tests. Each term was analysed using 9999 random permutations and associated with a Monte Carlo test (Anderson et al., 2008). Pearson correlation tests were performed for both species to investigate associations between the number of MPs detected (total plastic items, only fibres respectively) and the levels of phthalates (Σ_8PAEs, Σ_5PAEs, Σ_3MPEs, respectively). These tests were executed using SPSS ver. 28 (IBM, New York).

3. Results

3.1. Microplastics assessment

Microscopic sorting coupled with FTIR analysis confirmed a 100 % frequency of occurrence (FO) of MPs in all the sampled specimen of both species, as well as among the considered sites. A total of 513 microlitter items in the size range 279–4657 μm were found (*Actinia equina*: $N = 247$; *Anemonia viridis*: $N = 266$) with an average concentration of 12.35 ± 2.06 MPs/ind. (0.79 ± 0.12 particles/g) in *A. equina* and 13.30 ± 1.68 MPs/ind. (0.79 ± 0.13 particles/g) in *A. viridis* (Fig. 2). Among *A. equina* specimens, the minimum number of plastic items was 1 (found in Mal di Ventre South) and the maximum number was 35 (found in Seu), while *A. viridis* specimens took up between 2 (found in Mal di Ventre South) and 33 (Seu) plastic items. Only fragments and fibres were isolated from sea anemone samples, while other microplastic types (Bessa et al., 2019) were missing. For both species, 94 % of the isolated items were microfibres and 6 % fragments. Across all sites, microfibres were the major component detected (Fig. 2A, B). In one site, both for *A. equina* and *A. viridis*, microfibers were the only plastic shape found (Fig. 2C). Colours assessment can be observed in Fig. 3. The analysis of variance on total MPs abundance and microfibres abundance did not show any significant differences between species or sites (Table 1). Given their low quantity, no PERMANOVA analysis was performed on fragments number. Items polymer composition was determined using FTIR analysis ($n = 190$, 37 % of total particles) (Fig. 4). Natural based microfibres (mainly of cellulose nature) represented the 21 % and 8 % of the suspected MP items detected in *A. viridis* and *A. equina* respectively. Anthropogenic based fibres (e.g., rayon) represented the majority of detected particles (*A. viridis*: 49 %; *A. equina*: 63 %)Synthetic polymers represented the 30 % and the 29 % of the detected items and comprised the following polymers: PU, polyurethane; PET, polyethylene terephthalate; PE, polyethylene; PVC, polyvinylchloride; PS, polystyrene; PP, polypropylene; PES, polyester, PA 6, nylon. Frequencies of polymers' type detected in each sea anemone species are visible in Fig. 4.

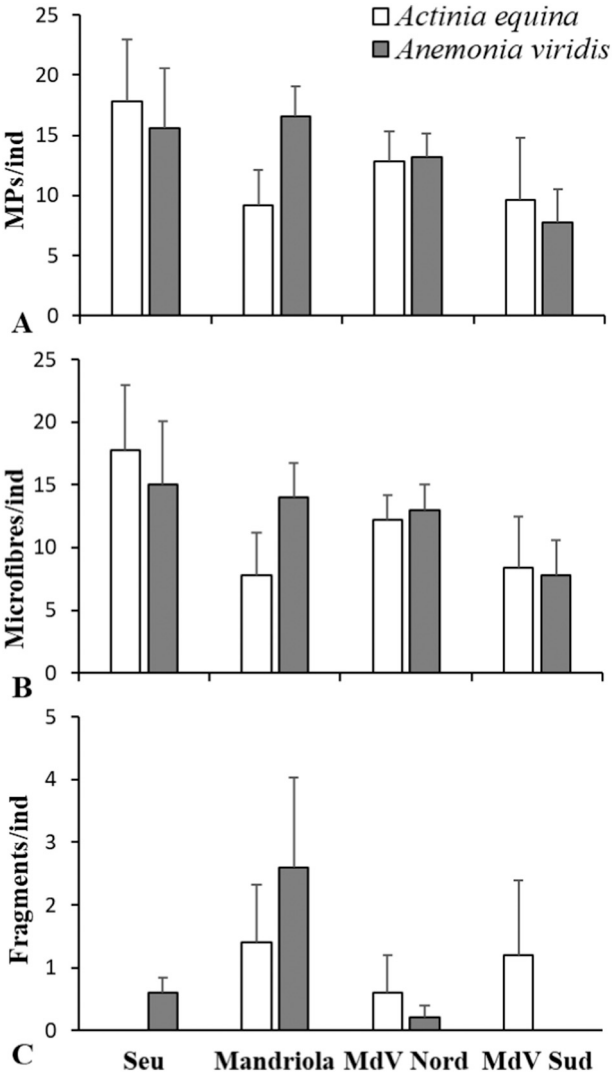

Fig. 2. Average concentrations of A) all the items, B) only microfibres and C) only fragments detected in *Actinia equina* (white) and *Anemonia viridis* (grey) according to the various sites considered. All the concentrations are reported with SE.

3.2. PAEs assessment by SPME-LC/MS

Table 2 reports the mean values of PAEs concentrations obtained through SPME-LC/MS assessed per sea anemone species and seawater samples among the different sites. In particular, phthalates were found in all the sampled sites and in the 70 % of all the sea anemone samples (*Actinia equina*, $N = 13$; *Anemonia viridis*, $N = 15$), with an average total concentration for Σ_8PAEs of 67.7 ± 13.3 ng/g and 61.8 ± 11.7 ng/g for *A. equina* and *A. viridis* respectively. In *A. equina* specimens, the maximum concentration value for the total sum of PAEs was 150.1 ng/g, with 90.3 ng/g as the highest value for the sum of the 5 congeners (Σ_5PAEs) and 119.9 ng/g as the maximum concentration for the sum of the metabolites (Σ_3MPEs). For *A. viridis* specimens, the maximum concentration value for the total sum of PAEs was 144.3 ng/g, with 116.7 ng/g as the highest value for the Σ_5PAEs congeners and 144.3 ng/g as the maximum concentration for the Σ_3MPEs. In general, for sea anemones, the metabolite component was more represented than the parental phthalates (Table 2). The short chain phthalate MBP was the most dominant among all the target PAEs for both sea anemone species, with an average concentration of 55.4 ± 13.0 ng/g for *A. equina* and 26.2 ± 9.9 ng/g for *A. viridis*, followed by DEP (*A. equina*: 7.8 ± 3.1 ng/g; *A. viridis*: 14.5 ± 6.2 ng/g) and DBP (*A. equina*: 4.5 ± 4.5 ng/g; *A. viridis*: 8.9 ± 6.1 ng/g) (Fig. 5A). Between the target PAEs and MPEs considered, BBP was the only phthalate ester not detected in any of the considered samples (Fig. 5). Among the *A. equina* samples, the phthalates DMP and DEHP were also not detected (Fig. 5A). Seawater samples showed an average total concentration for the sum of PAEs of 14.0 ± 3.8 ng/ml, with a higher concentration of Σ_5PAEs with respect to Σ_3MPEs in each sampled site (Table 2). In seawater, all the target phthalates, except for BBP, were detected. The long chain phthalate DBP was the phthalate most accounted (5.2 ± 2.8 ng/ml), followed by its metabolite MBP (4.1 ± 1.3 ng/ml) and the short chain phthalate DMP (Fig. 5B). PERMANOVA analyses did not show any significant variation in the distribution of the Σ_8PAEs, Σ_5PAEs congeners and for Σ_3MPEs among the two sea anemone species nor between the sites (Table 3A, B, C). No significant differences were highlighted among the concentrations of each phthalate congener and each metabolite between the two sea anemone species and among the different sampling sites (Table 3D). The distribution of phthalates detected in seawater samples was homogeneous based on PERMANOVA univariate (Σ_8PAEs: $F = 2.8577$, $p = 0.0614$; Σ_5PAEs: $F = 0.63754$, $p = 0.6026$; Σ_3MPEs: $F = 1.5216$, $p = 0.231$) and multivariate analyses (single PAEs: $F = 1.2537$, $p = 0.3062$).

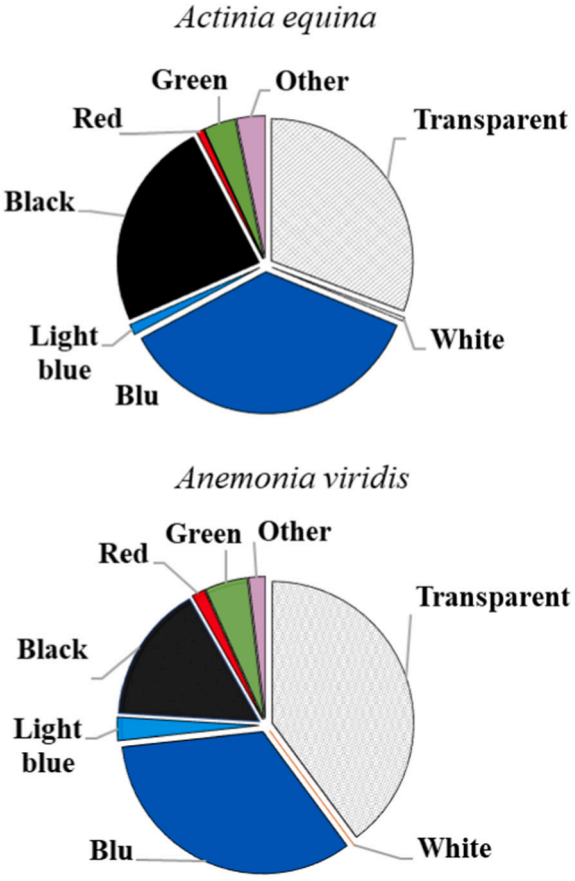

Fig. 3. Colour's categories of marine microlitter particles detected in A) *Actinia equina*, B) *Anemonia viridis*.

3.3. Correlations between MPs and phthalate's levels

Pearson's product-moment correlation tests highlighted a statistically significant, moderate positive correlation between the total number of fibres and the Σ_8PAEs ($n = 38$, $r = 0.316$, p-value <0.05), related to *Anemonia viridis* ($n = 18$, $r = 0.486$, p-value <0.05). Moreover, an increase in Σ_5PAEs levels was correlated with an increase in the total number of MPs items for the sum of sea anemone specimens ($n = 38$, $r =$

Table 1
Results of two-way PERMANOVA analyses of differences in A) total MPs abundance, B) fibres abundance between sea anemone species and among sampled sites (W = seawater; Ae = *Actinia equina*; Av = *Anemonia viridis*; S = Seu; M = Mandriola; MdVN = Mal di Ventro North; MdVS = Mal di Ventre South).

Analyses and variables	Source of variation	df	MS	Pseudo-F	p(MC)
A					
Univariate two-way PERMANOVA	Species (Ae, Av)	1	0.49906	0.49239	0.5262
	Site (S, M, MdVN, MdVS)	3	1.9721	1.9721	0.1389
	Sp × Si	3	1.0136	0.74887	0.5362
	Res	52	1.3535		
N MPs items	Total	63			
B					
Univariate two-way PERMANOVA	Species (Ae, Av)	1	0.63343	0.56742	0.503
	Site (S, M, MdVN, MdVS)	3	2.877	2.0876	0.1236
	Sp × Si	3	1.1163	0.80982	0.4935
	Res	32	1.3785		
N fibres	Total	39			

0.323, p-value <0.05). This result is explained by a strong positive correlation between the Σ_5PAEs and the MPs items in *Actinia equina* (n = 18, $r = 0.613$, p-value <0.01).

4. Discussion

This study detects both microplastics (MPs) and phthalate esters (PAEs) together in free-ranging and edible sea anemones, providing at the same time insights into the use of the detection of PAEs levels as a proxy for MPs exposure in short terms. The results show that MPs particles were always present across all sites considered, with a frequency of 100 % for the two target species. Particularly, MPs frequency of occurrence detected in this study is notable compared to other organisms known to ingest MPs (e.g., fish, Palazzo et al., 2021; Tsangaris et al., 2020), supporting the role of sea anemones as potential environmental plastic bioindicators (Morais et al., 2020; Savage et al., 2022). No differences were found in MPs amount and type between *A. actinia* and *A. viridis*, showing how, despite the different feeding strategies both species are similarly exposed to potentially ingestible microplastics. Considering the polyphagous opportunistic feeding pattern of sea anemones (Chintiroglou and Koukouras, 1992) and limited particle size and shape selection (Savage et al., 2022), the previous observation indicates that litter content in sea anemones is not a consequence of a particular feeding preference, but rather contamination conditions in the surrounding environment. In this work, the ubiquitous presence of MPs detected within sea anemones seems to reflect the environmental plastic distribution in the study area. In fact, De Lucia et al. (2014) report that despite the low population density, lack of large rivers, and scarcity of strong industrial activities, plastic microlitter is ubiquitous along the entire coast of the study area, with high densities of such pollutants. Additionally, as reported in our work, a higher number of fibres and fragments with respect to other MP typologies characterize the surface compartment of the water column, with fibres as the most abundant type of detected micro-litter category (Palazzo et al., 2021). Our samples' environmental uptake composition is consistent with this, since all sea anemones interacted with microfibers (94 % of total microlitter items), while fragments amount, the only other MPs type detected, was almost negligible. Both sea anemone species interfaced mainly with anthropogenic cellulosic fibres (*A. viridis*: 49 %, *A. equina*: 62 %) and plastic polymer particles (*A. viridis*: 30 %, *A. equina*: 29 %), with polyester, polystyrene and polyethylene being the plastic polymers with which the anemones interacted the most.

The 70 % of the sea anemone specimens contained phthalates. Remarkably, Σ_8PAEs levels detected in sea anemone samples seem to follow the MPs pollution pattern, with a uniform occurrence and distribution between *A. viridis* and *A. equina* and a ubiquitous distribution in the four sampled sites, although the high variability between the single replicates. Our biota samples revealed the monoester MBP, primary metabolite of DBP, to be the most prevalent and frequent among target phthalates. To follow, DEP and DBP were the most present phthalate congeners detected in both sea anemone species. These two phthalate esters are manufactured chemicals that do not occur naturally, extensively used as a plasticiser (CAMEO Chemicals, 2022) and both indicated as some of the most detected phthalates in various marine organisms, such as fish (e.g., *Diplodus vulgaris*, *Oblada melanura*; Rios-Fuster et al., 2022), holothurians (e.g., *Holothuria poli*, *Holothuria tubulosa*; Rios-Fuster et al., 2022), ascidians (*Herdmania momus*, *Microcosmus exasperates*; Vered et al., 2019) and other anthozoans, like scleractinian (e.g., *Pocillopora verrucosa*; Montano et al., 2020) and soft corals (e.g., *Coelogorgia palmosa*; Isa et al., 2022). However, it is surprising the lower frequency of DEHP, and its primary metabolite (MEHP) herein observed compared to the phthalates above mentioned, since DEHP is generally one of the main components of ΣPAEs in seawaters and marine biota (Fossi et al., 2012). Despite this, diverse PAE patterns could be explained by differences in degradation rates in the environment, as well as local source-specific presence (Hu et al., 2016). For example, in an experimental study, Paluselli et al. (2019) show that DEP exhibits a major half-live ($t_{1/2}$ = 53 days) compared to DEHP half-life ($t_{1/2}$ = 6 days) in seawater at dark biotic condition, suggesting a faster biodegradation of DEHP respect to DEP at environmental conditions. Regarding the study area, De Lucia et al. (2014), while investigating the ratio between the concentrations of DEPH and MEPH in neustonic-planktonic samples collected in the Sardinian Sea, highlighted very low concentrations of DEHP respect to other Mediterranean Sea areas, which is also found in this study for all specimens collected at all sites. As expected, overall, both the sea anemone species sampled in the different sites, exhibited higher levels of phthalate monoesters (Σ_3MPEs) than those of parental PAEs (Table 2). Indeed, living organisms rapidly degrade PAEs into their primary metabolites, which is why MPEs are usually more abundant than PAEs in organisms' tissues (Hu et al., 2016). For the same reason, in biological tissues, PAEs levels alone are not considerate applicable indicators of PAEs contamination (Hu et al., 2016), while the presence of phthalates' metabolites indicates continuous exposure to their corresponding parental phthalate (Silva et al., 2007; Guo et al., 2011). These result in MPEs that qualitatively reflect contamination by PAEs (Hu et al., 2016), especially since some metabolites are detected in vivo only (Rocha et al., 2017; Rian et al., 2020).

The rapid metabolism of PAEs into the respective MPEs (Hu et al., 2016), the lack of biomagnification through the trophic web (Zhang et al., 2021), and the environmental link between plastic marine litter and PAEs occurrences (Paluselli et al., 2019) are key PAEs characteristics potentially useful in the perspective of a plastic monitoring program. According to this, we hypothesize that, by observing the ratio of PAEs and MPEs in sea anemone tissues, not only in terms of levels but also, in terms of frequency of occurrence and changing composition of PAEs into corresponding MPEs, the short-term exposure of sea anemones to MPs

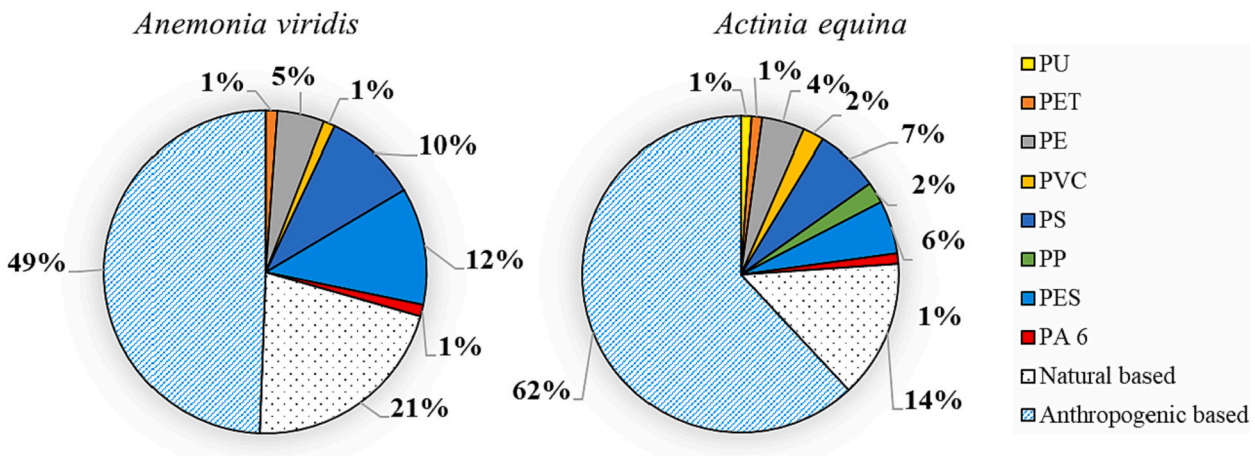

Fig. 4. Polymer types and respectively frequencies identified in *A. equina* and *A. viridis* by FTIR analyses. PU, polyurethane; PET, polyethylene terephthalate; PE polyethylene; PVC, polyvinylchloride; PS, polystyrene; PP, polypropylene; PES, polyester, PA 6, nylon. Natural and anthropogenic based percentage of fibres are also showed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Average concentration of phthalate ester congeners (Σ5PAEs), monoester phthalates (Σ3MPEs) and the sum of all the phthalates (Σ8PAEs) found in *Actinia equina*, *Anemonia viridis* and seawater samples collected in each sampled site (mean ± ES). All values referred to sea anemones are expressed as ng/g. Seawater values are expressed in ng/ml.

Sites	Matrix	Σ_5PAEs	Σ_3MPEs	Σ_8PAEs
Seu	*Actinia equina*	24.6 ± 17.6	46.5 ± 28.4	71.0 ± 30.4
	Anemonia viridis	14.2 ± 8.7	34.9 ± 23.3	49.0 ± 19.3
	Seawater	1.8 ± 1.8	0.4 ± 0.3	2.2 ± 2.1
Mal di Ventre North	*Actinia equina*	11.6 ± 7.1	58.9 ± 26.5	70.5 ± 30.5
	Anemonia viridis	36.4 ± 18.1	11.5 ± 11.5	47.9 ± 25.0
	Seawater	10.7 ± 4.2	5.6 ± 2.7	16.2 ± 4.5
Mal di Ventre South	*Actinia equina*	0	69.8 ± 28.5	69.8 ± 28.5
	Anemonia viridis	35.1 ± 22.8	6.9 ± 6.9	42.0 ± 21.1
	Seawater	9.1 ± 5.8	7.0 ± 3.0	16.1 ± 8.0
Mandriola	*Actinia equina*	13.1 ± 8.0	46.5 ± 28.5	59.6 ± 23.9
	Anemonia viridis	44.6 ± 19.9	63.6 ± 29.5	108.2 ± 20.5
	Seawater	15.9 ± 12.5	5.5 ± 2.5	21.4 ± 11.5

can be quantified. In areas with higher plastic contamination, biological tissues could reveal both parental and metabolite phthalates, with high parental PAEs occurrences. In better-preserved regions, lower presence, and frequencies of parental PAEs are expected, while metabolites could prevail. Thus, to investigate the potential differences in uptake of these contaminants and determine if environmental differences in the occurrence and distribution of plastic contamination are reflected in differences in phthalates pollution, it may be worthwhile to consider a broader study area characterized by defined differences in plastic pollution. In this context, we highlight the combination DBP-MBP occurrence as a ratio that deserves special attention among the various phthalate parental congeners and metabolites to consider. Indeed, it is noted that DBP and MBP, when detected, are never simultaneously present for both species and in almost all sites, and previous work highlighted a linear correlation between MBP and DBP concentrations in biological tissues (Hu et al., 2016), with MBP and its glucuronide being the major metabolites (90–95 %) of DBP (Silva et al., 2007). Previous studies found DBP associated to PE and PP in new plastic product and acrylic fibres in environmental collected marine debris in South Korea (Rani et al., 2015). Among 15 target phthalates, Tang et al. (2020) detected mainly DEHP and DBP in new preschool children's sampled clothing manufactured in seven Asian countries, phthalates that can easily leach by released fibrous microplastics. Fries et al. (2013) found that in sediment samples collected from a North Sea island DEHP and DBP were associated with polyethylene (PE) and polypropylene (PP) isolated microplastic particles as antioxidant additives, DBP and DEP with polystyrene (PS), and DEP for polyamide-6 (PA-

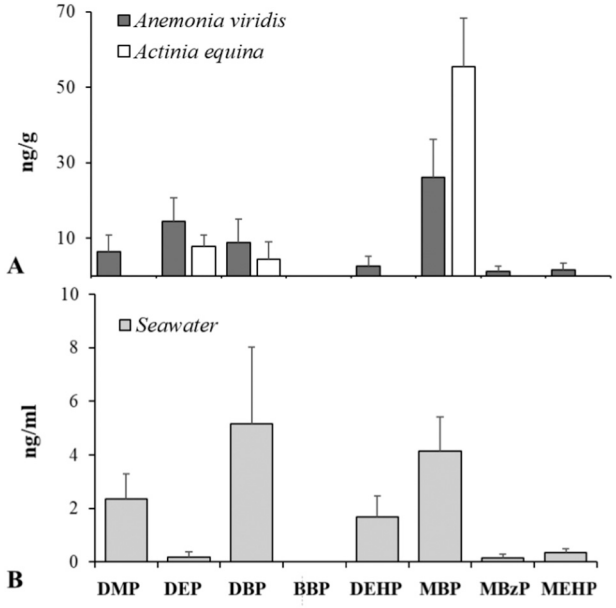

Fig. 5. Single PAE congeners (DMP, DEP, DBP, BBP and DEHP) and target metabolites (MBP, MBzP and MEHP) concentrations detected in A) *Actinia equina* and *Anemonia viridis* and B) seawater samples. Concentrations are reported in ng/g wet weight of sea anemone species and ng/ml for water samples.

Table 3
Results of PERMANOVA analysis to investigate A) Σ_8PAEs content, B) Σ_5PAEs, C) Σ_3MPEs, D) each phthalate ester levels between sea anemone species and among sites. Ae = *Actinia equina*; Av = *Anemonia viridis*; S = Seu; M = Mandriola; MdVN = Mal di Ventre North; MdVS = Mal di Ventre South.

Analyses and variables	Source of variation	df	MS	Pseudo-F	p(MC)
A					
Univariate two-way PERMANOVA	Species (Ae, Av)	1	2.13E−02	1.52E−03	0.9712
	Site (S, M, MdVN, MdVS)	3	15.587	0.62695	0.6
	Sp × Si	3	14.053	0.56525	0.6404
	Res	32	24.861		
Σ_8PAEs (ng/g w.w.)	Total	39			
B					
Univariate two-way PERMANOVA	Species (Ae, Av)	1	48.717	4.8999	0.1183
	Site (S, M, MdVN, MdVS)	3	8.2926	0.60235	0.6102
	Sp × Si	3	9.9423	0.72218	0.5388
	Res	32	13.767		
Σ_5PAEs (ng/g w.w.)	Total	39			
C					
Univariate two-way PERMANOVA	Species (Ae, Av)	1	45.363	1.6815	0.2786
	Site (S, M, MdVN, MdVS)	3	4.7307	0.17718	0.9042
	Sp × Si	3	26.977	1.0104	0.4042
	Res	32	26.699		
Σ_3MPEs (ng/g w.w.)	Total	39			
D					
Multivariate two-way PERMANOVA	Species (Ae, Av)	1	944.3	1.2951	0.3405
	Site (S, M, MdVN, MdVS)	3	402.4	0.51224	0.8316
	Sp × Si	3	729.14	0.92818	0.4808
	Res	32	785.56		
Single PAEs and MPEs (ng/g w.w.)	Total	39			

6). Such patterns recall the composition of both MPs and PAEs detected in our study, since all these polymers were detected in sea anemone specimens, together with DBP and DEP. However, other phthalates should be monitored, like DEP, the most abundant phthalate congener herein detected. As usually most detected phthalate, DEHP should be studied as well, in relation to its secondary metabolites mEHHP and mEOHP, considered in vivo indicators of DEHP in animal tissues (Rian et al., 2020). However, to investigate in a more targeted way that plasticizers could interact with marine life, it should be noted which mixture of additives is released by specific plastic items. Leaching experiments of the most common plastic debris could be useful for the chemical identification of the released plastic additives (Rani et al., 2015). The correlation here found between MPs and PAEs contamination in both *A. equina* and *A. viridis* describes that increasing the MPs number corresponds to a moderate increase in PAEs levels, reinforcing the interest in developing a methodology where PAEs could be used to detect plastic-biota exposure in an indirect and less invasive way. Marine organisms are an important indicator of the MPs contamination of the marine environments, which makes this application important. To investigate the interaction of microplastics with biota, different extraction and analysis methods are used nowadays (Bessa et al., 2019), with many biomonitoring programs conducted on dead or hospitalized organisms (Baini et al., 2017). The availability and possible restriction of biological materials, particularly when considering rare and/or protected species, and the need for user-friendly procedures to simplify and speed up operations are challenges in marine environments.

Recently, the use of BioSPME coupled to LC/MS analysis has proven to be a suitable method for determining PAEs concentrations in Cnidaria, Porifera and Mollusca (Saliu et al., 2020a). It necessitates a small amount of samples (150 mg) and represents a practical working solution that does not immediately required fully equipped laboratory facilities. Furthermore, thanks to the introduction of biocompatible coating, SPME may be used in direct immersion mode with a biological matrix and even in vivo (e.g., *Tridacna maxima* and *Danafungia scruposa*; Saliu et al., 2020b). In addition, it is feasible to implement the procedure for operation in marine environments *on site* since it requires very little organic solvents and integrates sampling, sample preparation, and fiber handling in an efficient and easy-going manner (Saliu et al., 2020b). In the context of performing non-lethal analyses on living animals, sea anemones can be especially useful organisms. Indeed, they are model organisms easy to maintain and manipulate (de Orte et al., 2019) that can recover after the scission of some body parts and, potentially, the insertion of the SPME appropriate fiber format (Saliu et al., 2020b). As cnidarians, their "simple" biochemical composition (Yamashiro et al., 1999) makes them particularly suitable for the SPME application (Saliu et al., 2020b). In addition, most of the recommended criteria for selecting appropriate species for monitoring microplastics apply to sea anemones (Bessa et al., 2019). For example, they are species, which occur naturally with high abundance and wide geographic distribution, easy to sample and process in the laboratory and already proposed as bioindicators in other studies related with marine pollution (Morais et al., 2020; Savage et al., 2022). The result of this study indicates that *A. viridis* and *A. equina* can accumulate detectable PAEs levels even in a context like the Sinis coastal region, which has been proven to be less contaminated than other Mediterranean Sea areas (Sbrana et al., 2020; Valente et al., 2022). Recent studies describe the suitability of a multi-species monitoring strategy, though a micro-litter monitoring strategy has not yet been developed (Valente et al., 2022). In this context, and in the light of the results obtained in this study, we assert that sea anemones could be potential good candidates as one of these organisms, given even their ecological and human related importance. Indeed, some sea anemone species have been reported for human consumption (Silva et al., 2017), highlighting the health risk associated with human exposure to both MPs and phthalates through both *A. equina* and *A. viridis* ingestion.

5. Conclusions

The present study highlights the presence of microplastics and phthalate esters in free-ranging *Anemonia viridis* and *Actinia equina* specimens. Both contaminants were detected in the target species, demonstrating the suitability of the sea anemone as an organism capable

of detecting MPs and PAEs even in environments not strongly impacted by anthropogenic sources. Patterns of MPs and PAEs seem to reflect each other, mirroring the plastic pollution conditions of the area where the specimens were collected. The results suggest that the detection of both parental phthalates and their metabolites in sea anemone tissues could be used as a proxy for their short-term interactions with MPs. In view of the easy and rapid handling of the specimens and the SPME-LC/MS methodology, further studies are therefore recommended to investigate the potential role of PAEs-MPEs presence and levels as MPs contamination tracers.

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CRediT authorship contribution statement

Sara Vencato: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simone Montano:** Writing – review & editing, Supervision, Conceptualization. **Francesco Saliu:** Writing – review & editing, Supervision, Methodology. **Stefania Coppa:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Alessandro Becchi:** Methodology, Data curation. **Immacolata Liotta:** Writing – review & editing, Data curation. **Tommaso Valente:** Writing – review & editing, Data curation. **Mariacristina Cocca:** Writing – review & editing, Data curation. **Marco Matiddi:** Writing – review & editing. **Andrea Camedda:** Writing – review & editing, Investigation. **Giorgio Massaro:** Writing – review & editing, Investigation. **Davide Seveso:** Writing – review & editing. **Marina Lasagni:** Resources. **Paolo Galli:** Writing – review & editing, Funding acquisition. **Giuseppe Andrea de Lucia:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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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