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Microplastics and phthalate esters: presence and interactions in overlooked benthic anthozoans used as tracers of plastic contamination

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ABSTRACT

Plastic pollution is a planetary threat, affecting nearly every marine and freshwater ecosystem globally. Coastal waters worldwide are widely contaminated with different kinds of plastic, whose presence in aquatic ecosystems leads to various economic and social impacts and harmful effects on marine ecosystems. The study of effects of pollutants, especially microplastics and associated toxic substances, is relatively new, with an increase in discoveries in latest decades. Despite this, only recently occurrence, interactions and fate of microplastics have been investigated in certain environments, like coral reefs. Most of the studies on the interaction mechanisms and impacts between microplastics (MPs) and anthozoans have been conducted on stony corals, neglecting other important reef dwellers. To gain a better understanding of the microplastic influences on marine ecosystems, most studies assess their abundance, distribution and composition through different methodologies, like the use of marine organisms as bioindicators and the detection of plastic-associated contaminants in their tissues. Among plastic additives, phthalate esters (PAEs) are the most common class of plasticizers, which can easily leach from plastic debris into the environment. Due to their hydrophobicity, toxicity and bio-accumulative properties, these contaminants are a matter of worldwide concern and have been proposed as a possible tracer of marine organisms' exposure to plastic debris. This research seeks to improve awareness of microplastic-biota relationships, providing useful insights by investigating microplastics and phthalates occurrence and interactions in understudied anthozoan species, specifically, soft corals and sea anemones. At the same time, this research investigates the potential use of phthalates as a proxy to evaluate the exposition of such organisms to plastic litter present in the marine environment, assessing PAEs levels in soft-benthic cnidarian tissues through a potentially non-lethal alternative procedure (BioSPME-LC/MS). To evaluate the suitability of the above-mentioned hypothesis, this research

investigates MPs and PAEs interactions with different soft benthic cnidarian species across diverse conditions (i.e. in laboratory and natural environment), through three main steps. Firstly, at laboratory conditions, soft corals capacity to interact with MPs was assessed through feeding and adhesion tests, providing a first evaluation of some physical and physiological effects of microplastic exposure on the soft coral *Coelogorgia palmosa*. Thereafter, PAEs presence and bioconcentration factors were investigated using the BioSPME-LC/MS procedure in four different soft coral species. Once the capacity of soft corals to interact with MPs and PAEs was assessed at control conditions, the study moved at environmental conditions. MPs and PAEs occurrence and distribution were simultaneously detected in *Actinia equina* and *Anemonia viridis* sea anemones, showing patterns that mirror the environmental characteristics of the study area. Overall, the findings presented in this work highlight the ability of soft corals and sea anemones to interact both with plastic microlitter and phthalates, integrating existing literature on the uptake of microplastics by providing a scientific baseline on MPs occurrence and interactions in overlooked anthozoan species.

Italian Abstract

L'inquinamento da plastica è una minaccia che colpisce quasi tutti gli ecosistemi marini e d'acqua dolce a livello globale. Le acque costiere di tutto il mondo sono ampiamente contaminate da diversi tipi di plastica, la cui presenza negli ecosistemi acquatici porta a differenti impatti economici e sociali ed effetti dannosi sugli ecosistemi marini. Lo studio degli effetti degli inquinanti, in particolare delle microplastiche e delle sostanze tossiche associate, è relativamente nuovo, con un aumento delle scoperte negli ultimi decenni. Nonostante ciò, solo di recente la presenza, le interazioni e il destino delle microplastiche in alcuni ambienti, come, ad esempio, le barriere coralline, sono stati oggetto di studio. La maggior parte degli studi sui meccanismi di interazione e gli impatti tra microplastiche (MPs) e antozoi sono stati condotti sulle sclerattinie o coralli duri, trascurando altri importanti abitanti della barriera corallina. Per comprendere meglio l'influenza della presenza della microplastica sugli ecosistemi marini, la maggior parte degli studi ne valuta l'abbondanza, la distribuzione e la composizione attraverso diverse metodologie, come l'uso di organismi marini come bioindicatori e il rilevamento di contaminanti associati alla plastica nei loro tessuti. Tra gli additivi associati al materiale plastico, gli ftalati (PAEs) sono la classe più comune di plastificanti, che possono facilmente separarsi dalla plastica durante il suo invecchiamento ed essere rilasciati nell'ambiente. A causa della loro idrofobicità, della loro tossicità e delle loro proprietà bio-accumulative, questi additivi sono motivo di preoccupazione a livello mondiale e sono stati proposti come possibili marker dell'esposizione degli organismi marini alla plastica. Questa ricerca si pone come obbiettivo di contribuire alle conoscenze sulle relazioni tra microplastica e biota, fornendo nuove informazioni riguardo la presenza e le interazioni di microplastica e ftalati in specie di antozoi finora poco studiate, nello specifico coralli molli e anemoni di mare. Allo stesso tempo, questa ricerca indaga il potenziale utilizzo degli ftalati come traccianti per valutare l'esposizione di tali organismi

ai rifiuti plastici presenti nell'ambiente marino, valutando i livelli di PAEs nei tessuti di tali cnidari attraverso una procedura alternativa e potenzialmente non letale (BioSPME-LC/MS). In generale, per valutare l'idoneità dell'ipotesi sopra menzionata, questa ricerca studia alcune interazioni ed effetti di MPs e PAEs con diverse specie di cnidari bentonici molli e in diverse condizioni (in laboratorio e in ambiente naturale), attraverso tre fasi principali. In primo luogo, in laboratorio, è stata valutata la capacità dei coralli molli di interagire con le MPs attraverso test di alimentazione ed adesione, fornendo una prima valutazione di alcuni effetti fisici e fisiologici dell'esposizione alla microplastica sul corallo molle *Coelogorgia palmosa*. Successivamente, la presenza e i fattori di bioconcentrazione di alcuni ftalati sono stati esaminati utilizzando la procedura BioSPME-LC/MS in quattro diverse specie di coralli molli. Una volta valutata la capacità dei coralli molli di interagire con MPs e PAEs a condizioni controllate, lo studio si è spostato in ambiente naturale. La presenza di MPs e PAEs è stata rilevata simultaneamente negli anemoni di mare *Actinia equina* e *Anemonia viridis*, mostrando uno schema che rispecchia la contaminazione da plastica caratteristica dell'area di studio. Nel complesso, i risultati presentati in questo lavoro evidenziano la capacità dei coralli molli e degli anemoni di mare di interagire sia con le microplastiche che con gli ftalati, integrando la letteratura esistente sulle interazioni tra MPs e biota fornendo informazioni riguardo specie di antozoi solitamente poco studiate in questo ambito.

CHAPTER 1

1.1. Overview: marine litter, coastal regions and soft benthic cnidarian organisms

Marine litter is described as “any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment” (Jeftic et al., 2009). It consists of items that have been made or used by people and deliberately discarded into the sea, rivers or on beaches, brought indirectly to the sea with rivers, sewage, storm water or winds or accidentally lost (AWI-Litterbase 2023). Large amounts of litter have accumulated across all parts of our oceans in less than fifty years. Litter has thus become a serious threat to the marine environment and humankind, whose welfare is closely linked with ocean health. Research on marine litter is taking a great leap forward and has substantially increased our knowledge of the amount and composition of litter as well as its impacts on the marine environment, aquatic life and people (AWI-Litterbase 2023). Most of the marine litter items encountered are made of plastic, with larger litter items, but more and more reports on encounters with microplastics emerge (AWI-Litterbase 2023). Most studies on microparticles conducted at sea have been monitoring their distribution (Auta et al., 2017; Eriksen et al., 2014) or have focused on the interaction between marine organisms and marine litter, also by considering possible risks and harms to biota (Deudero & Alomar, 2015; Anastasopoulou & Fortibuoni, 2019; Camedda et al., 2022). Indeed, the tiny size fractions of debris are a major concern, since they are potentially bioavailable to a wide variety of organisms from different trophic levels and thus have the potential to impact the entire trophic web through bioaccumulation and biomagnification (Browne et al., 2008; Thompson et al., 2009; Carbery et al., 2018). At present, almost all of the world’s oceans, seas and coastal waters are widely contaminated with plastics, whose presence in aquatic ecosystems has been shown to produce a wide range of economic and social impacts and harmful effects on marine ecosystems. Plastic pollution in the coastal regions is a matter of concern as increasing population density, tourism, marine harboring and

coastal activities are contributing to a great extent to the release of complex and toxic contaminants, including daily used plastic items (Sharma et al., 2021). Indeed, coastal ecosystems, like coral reefs, are particularly impacted by plastic and microplastic pollution since the majority of these contaminants has land-based sources (Andrady 2011). Coral reefs are biogenic structures deposited by calcifying organisms that build extensive carbonate reef systems, constructing the framework that serves as a habitat for all other coral reef-associated species. They are located in a wide range of environments and constitute one of the most biodiverse and productive ecosystems in the world (Ortiz & Dove, 2011). Despite they occupy less than 1% of the global benthic environment (Burke et al., 2011), coral reefs are crucial to marine biodiversity maintenance, global climate mitigation, human harvesting of natural resources and livelihoods of more than 275 million people (Huang et al., 2021 and literature inside). For example, they protect the shorelines, they are important spawning, nursery, breeding and feeding areas for a multitude of organisms and are source of employment, providing recreational opportunities (e.g. diving and snorkelling), holding aesthetic value and attracting tourists from all around the world. Plastic pollution can be considered as an emerging threat to the coral reefs due to their complex interactions (e.g., entanglement/catches, covering/smothering) (Yoshikawa & Asoh, 2004; de Carvalho-Souza et al., 2018). In a field investigation in 124,000 reef building corals from 159 coral reefs across the Asia-Pacific oceans, Lamb et al., (2018) found that 11.1 billion large plastic items were “trapped” in coral reefs, with a predicted increase of 40% by 2025 (Huang et al., 2021). Regarding microplastics, only limited coral reef regions have been investigated (Figure 1). Current data show that microplastic abundance in the surface water of coral reefs generally ranges from zero to tens of thousands of items/m^3,

while these in sediments and corals are difficult to quantify due to lack of a relatively standardized unit or enough available data (Huang et al., 2020).

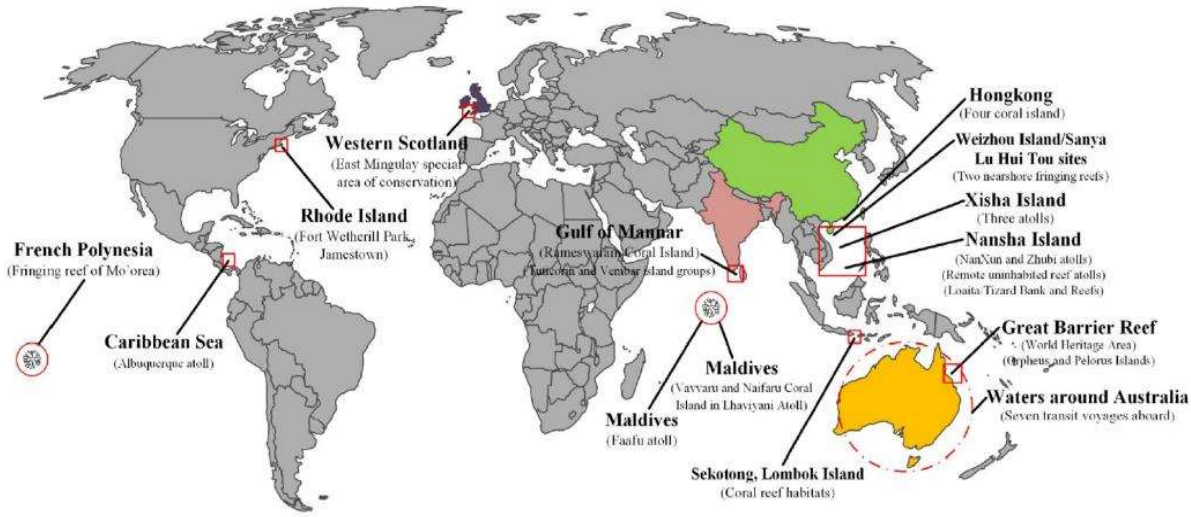


Figure 1: Worldwide coral reef sampling regions of microplastic investigations (Huang et al., 2020)

Because of its distinguishing semi-closed morphology, and different plastic waste generating activities originating from surrounding countries with dense population (~150 million), the Mediterranean Sea is highly vulnerable to plastic pollution (Figure 2) (Sharma et al., 2021). Indeed, it has been recognized as the sixth-highest accumulation hotspot for marine litter (Cozar et al., 2015). The Mediterranean Sea is a particularly fragile ecosystem since it presents an extremely high biodiversity together with a plethora of anthropogenic impacts (Deudero & Alomar, 2015; Everaert et al., 2020). Different shipping, fishing, industrial, touristic, and other coastal activities are also contributing largely to the Mediterranean plastic pollution. The Mediterranean basin is producing approximately 208–760 kg per year solid waste per capita (Alessi & Carlo, 2018), and tourist activities are one of the great contributors to this increased marine litter (Galgani et al., 2014). Moreover, the Mediterranean basin collects water from different highly populated river catchments (Nile, Rhone, Po) and is also connected by the Strait of Gibraltar to the Atlantic Ocean. Each year approximately 500,000 tons of macroplastics and 130,000 tons of microplastics penetrate in European sea (European

Commission, 2018), and an immense portion of these plastic fragments make their way to the Mediterranean Sea (Alessi & Carlo, 2018).

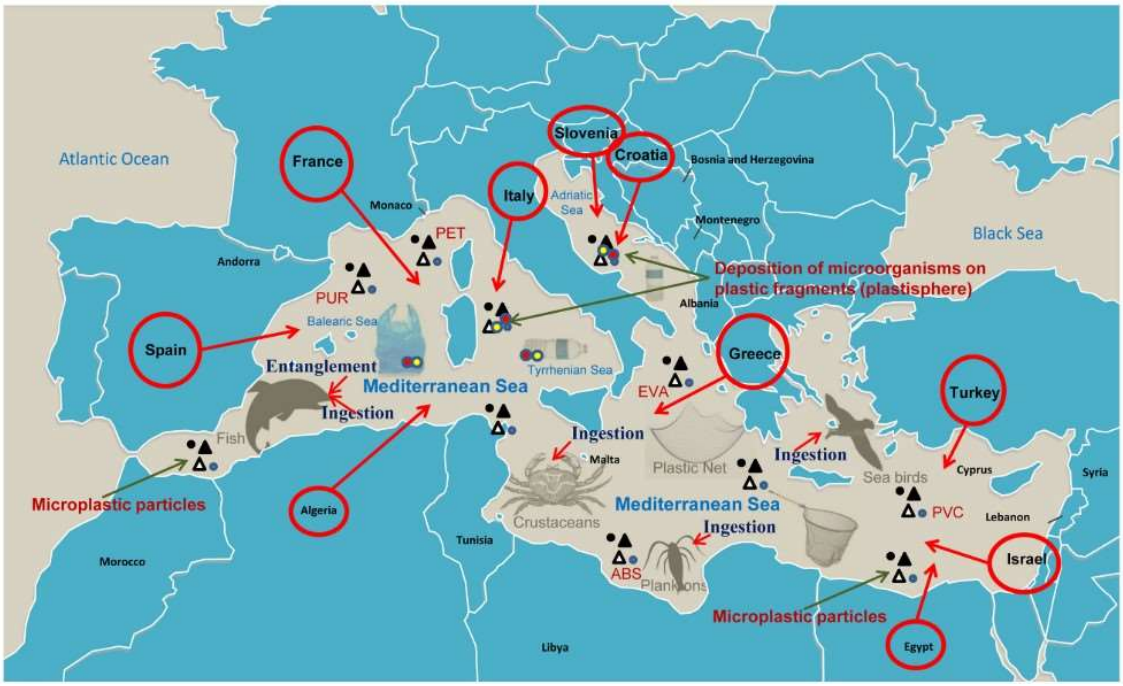


Figure 2. An overall representation of Mediterranean Microplastic pollution. The countries that contribute to the major amount of plastic litter and microplastics in the Mediterranean Sea are marked with red color. The plastisphere and the marine animals (inhabiting the Mediterranean Sea) affected due to entanglement and ingestion are shown separately (Map source: https://yourfreetemplates.com) (Sharma et al., 2021)

Plastic litter is one of the reasons for the loss of biodiversity in seas and oceans worldwide (STAP, 2012), and coral reefs and the Mediterranean Sea coasts are not an exception. The ingestion of microplastic fragments by aquatic biota is one of the main reasons for the loss of marine lives and over 800 marine species were contaminated with plastic litter either via ingestion or entanglement (Dias B. F. S., 2016).

Reef-building or stony corals (Phylum Cnidaria, Class Anthozoa) (Figure 3A, 3B) are clearly one of the most iconic organisms in coral reef ecosystems, since they are the builders of the calcium carbonate skeletons whose continued accumulation, over millions of years, forms the coral reefs (Ortiz & Dove, 2011). However, aside from stony corals, anthozoans include other underrepresented but fundamental invertebrates, e.g.

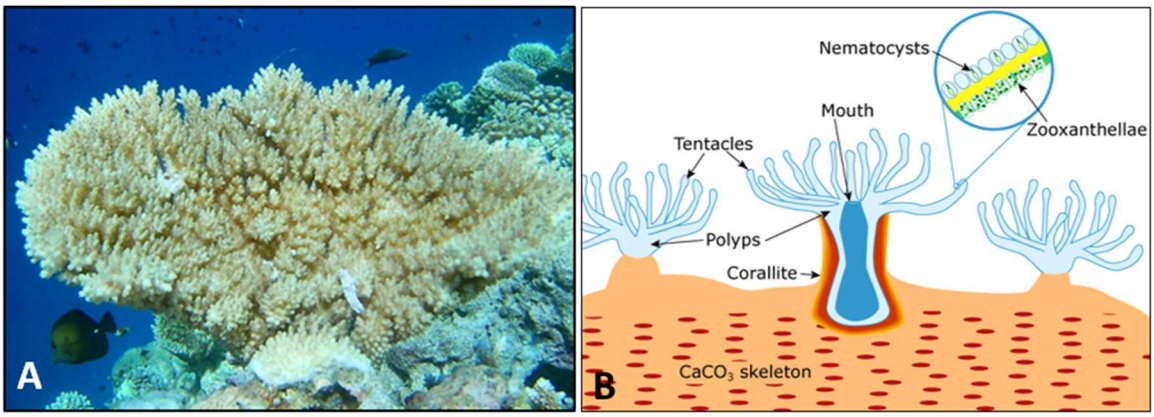


Figure 3. A) Picture of a reef building or stony coral; B) Anatomy of coral polyps. Source: US Geological Survey USGS website.

soft corals (Figure 4A, 4B) and sea anemones (Figure 5A, 5B) (Lusher, 2015). Both stony and soft benthic cnidarians are under increasing threat from both natural and human-induced disturbances, like climate change, disease, anchor and boat damage, and commercial collection (Loya et al., 2001; Precht et al., 2001; Santangelo & Abbiati, 2001; Goldberg & Wilkinson, 2004; Coma et al., 2006; Bruno et al., 2007; Thomas et al., 2015). Between such distrupting factors, there is plastic pollution, with plastic items that can result in the physical abrasions and injuries to the coral tissues, promoting the invasion of pathogens and ciliated protozoan and/or causing the coral diseases, such as skeletal eroding band (Page & Willis, 2008; Lamb et al., 2016). Both experiments and field studies show that scleractinian corals can interact directly with microplastics, by ingesting plastic particles suspended in the water (Hall et al., 2015; Allen et al., 2017) and indirectly, though adhesion of plastic particles and absorption of pollutants and contaminants carried by plastic items (Martin et al., 2019; Corona et al., 2020). However, studies on the interactions of plastic debris with anthozoans are still scarce and mainly focus on scleractinian corals, the main builders and major occupiers of space of reef frameworks. Indeed, those species that are not spatially dominant are often considered "non-primary" habitat and may be overlooked (Steinberg et al., 2020). Howether, in other shallow, tropical marine environments, or in the same habitats under different

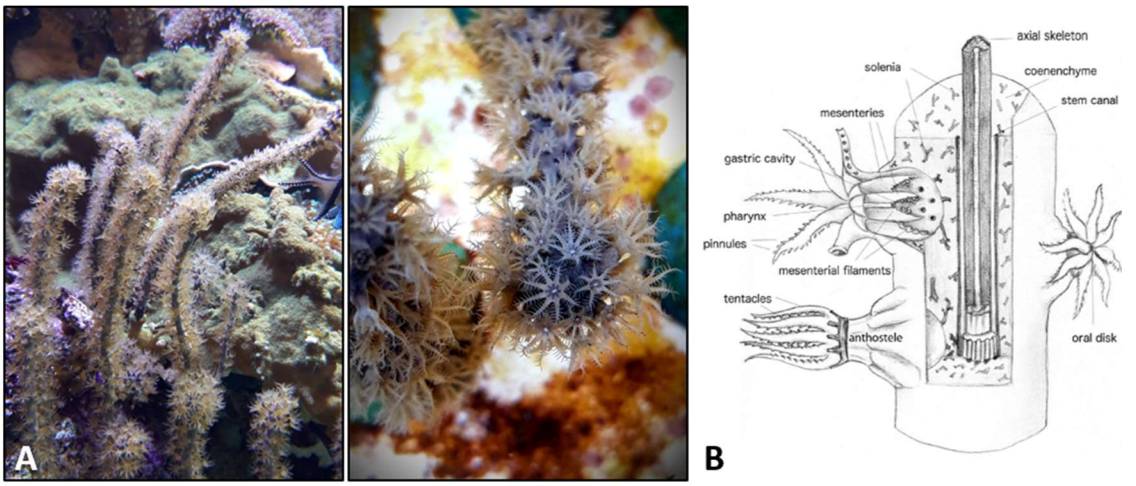


Figure 4. A) Picture of the soft coral *Coelogorgia palmosa.* B) Schematic representation of an octocoral. Diagram redrawn by Ellen Bigger Streeter (Bayer et al., 1983)

conditions, non-scleractinian anthozoans, typically zoanthids and octocorals, occupy comparable expanses of substratum (Fautin 1989). Likewise, some temperate and deep-water marine communities are dominated by anthozoans, generally actinians (Fautin 1989). Although these animals do not structure their communities physically, they are, in many respects, functionally comparable to reef-building corals. Thus, such anthozoans appear to comprise a group of ecologically equivalent (which is not to say interchangeable) benthic dominants (Fautin 1989). Octocorals and sea anemones have a world-wide distribution in marine and estuarine habitats, with species inhabiting all climate zones and habitat types (Verselveldt & Alderslade, 1982; Fautin, 1992; Heifetz, 2002; Fautin et al., 2013). They share a wide variety of physical and ecological traits (Steinberg et al., 2020), creating three-dimensional structures that provide suitable habitat and shelter for other organisms, contributing to increase the total reef biodiversity (Goh et al., 1999; Lau et al., 2019; Maggioni et al., 2020). Octocorals and sea anemones are soft-bodied, although octocoral tissues do contain calcified spicules and/or axes that increase tissue stiffness (Koehl, 1982; Fabricius & Alderslade, 2001; Sethmann & Wörheide, 2008; Fabricius, 2011). Octocorals primarily rely on secondary metabolites for defens, rendering them unpalatable and sometimes toxic, while

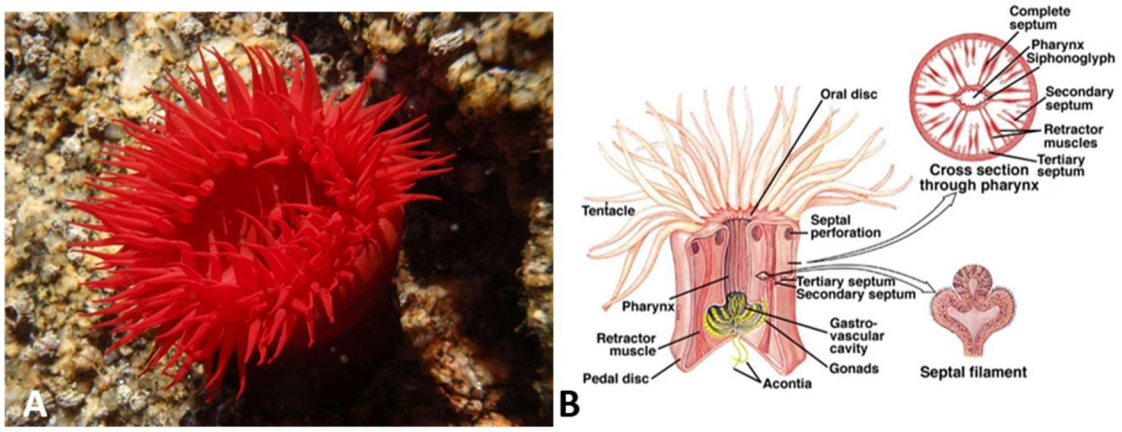


Figure 5. A) picture of a sea anemone specimen (A*ctinia equina*); B) structure of a sea anemone. Image from "Diversità Animale" (page 380), O. Coppellotti, L. Guidolin, 15th edition, McGraw-Hill, 2011.

anemones employ painful stings from venomous nematocysts (Changyun et al., 2008; Frazão et al., 2012). Octocorals are primarily colonial anthozoans that possess polyps with eight tentacles, often fringed with pinnules (Fabricius & Alderslade, 2001; Fabricius, 2011) (Figure 4B). Anemones are generally solitary, though they can also form colonies, with some species employing both life histories (Francis, 1979). A typical sea anemone is a single polyp animal with two tissue layers and a central gut cavity (Thangaraj et al., 2019) (Figure 5B), attached to a stony surface by its base, but some species live in soft sediments and a few float near the surface of the water. Essentially laminar organisms, two-dimensional epithelial construction has shaped both behavioural and physiological responses and led to great diversity in sea anemones (Shick 2012), as evidenced by their presence in all marine habitats, from the intertidal zone to deep-sea hydrothermal vents (Fautin et al., 2013). Regarding soft corals, usually they cover 2–25% of the substratum in tropical areas, but in some locations they are dominant, covering more than 80% of the available substrate (Fabricius 1997; Fabricius & Alderslade 2001). For example, in temperate waters, octocorals are critical habitat for various species and host epibenthic food sources, such as amphipods, for other organisms (Harasti et al., 2014; Harasti, 2016; Corry et al., 2018). In the subarctic, sea pens and other octocorals provide nursery habitat for larvae of commercially important fisheries species (Baillon et al., 2012). In

tropical and subtropical systems, anemones can form aggregations that house large anemonefish populations (Ricstonyson et al., 1997; Scott et al., 2011). Despite their potentially important ecological roles, to date there have been few studies of their specific threats and stressors, like plastic pollution. Furthermore, because of their sessile nature and their generally polyphagous and opportunistic feeding behaviour (Shick, 2012) they may be particularly affected by microscopic plastic particles consumption, which makes them an excellent potential study object to monitor micro-plastics contamination.

1.2. Plastic pollution

The term "plastic" refers to a wide range of synthetic or semi-synthetic organic materials with a high molecular weight and a polymeric nature, that may consist of repeating identical units (homopolymers) or different sub-units in various possible sequences (copolymers). Those polymers that mainly derive from fossil fuel-based petrochemicals like natural gas or petroleum (Finch, 1985; Güven et al., 2017), can soften on heating, and can be moulded are generally referred as 'plastic' materials (GESAMP, 2015). Although the first synthetic plastic appeared in the early 20th century, widespread use of plastics outside of the military did not occur until after World War II (Geyer et al., 2017). Starting from the middle of the last century, the ensuing rapid growth in plastics production has been extraordinary, surpassing most other man-made materials (Geyer et al., 2017). Bakelite, viscose, rayon, nylon, polystyrene, polyvinyl chloride and polyethylene were the first plastic materials to become commonly used between the 1920s and the 1940s (Steffen et al., 2011), whereas polypropylene and expanded polystyrene foam were produced in the 1950s, while polyethylene terephthalate (better known as PET), one of the major component of plastic containers, was patented in 1973 (Zalasiewicz et al., 2015). The low cost, high versatility of these materials is leading to a continual increase in their global production, which, after a stagnation in 2020 due to

the Covid-19 pandemic, in 2021 rose 4% to more than 390 million tonnes, demonstrating the strong and continuing demand for plastics (PlasticEurope, 2022). The intense consumption and rapid disposal of plastic products (Figure 6) is leading to a visible accumulation of plastic debris into the marine environment. Indeed, plastics are long-lived on a human timescale since their degradation takes place very slowly either physically, chemically or biologically (Porta, 2021). Consequently, high production of these non-biodegradable materials and inadequate waste management make plastic a serious environmental problem (Moore, 2008). Today, plastic debris has become evident as a globally ubiquitous pollutant over the last decade, and comprises over 80% of all marine debris (Barnes et al., 2009; Bellas et al., 2016). Plastic waste is now so ubiquitous in the environment that it has been suggested as a geological indicator of the proposed Anthropocene era (Geyer et al., 2017). Indeed, when future geologists study the Anthropocene, fossilized plastics will be probably considered the key markers of the epoch in which we humans lived (Porta 2021). Nowadays, estimates range from 4.8 to 12.7 million metric tons of plastic discharged at sea every year (Jambeck et al., 2015) from land-based sources, ships and other installations at sea, and an increasing global production with a compound annual growth rate of 8.4% (Geyer et al., 2017). Thus, 12 billion tons of plastic waste are estimated to accumulate in natural environments by 2050 (Geyer et al., 2017). The most produced and dispersed polymers are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyamide (PA), polyethylene terephthalate (PET), polyvinyl alcohol (PVA) (Andrady, 2011). A global shift from reusable to single-use items has accelerated such growth. In particular polyethylene (PE) and polypropylene (PP) are mainly utilised to make disposable plastic objects (representing up to 50% of plastic products), including utensils, shopping bags and packaging (Plastics Europe, 2020). As an incredibly ductile and versatile material, strong but flexible, light and relatively inert, plastic has potential to take any form and be available for any use. These properties, which make such materials so useful, represent even a significant environmental threat, since, due to their durability they

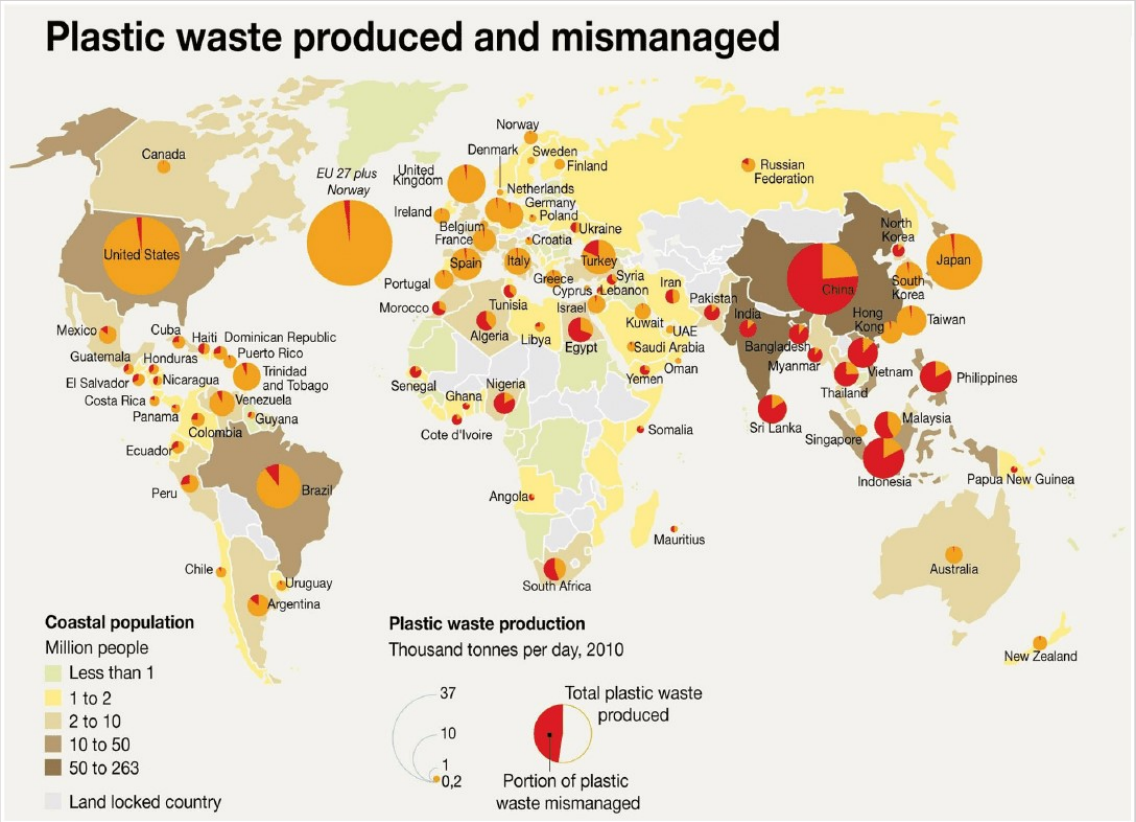


Figure 6. Global distribution of produced and mismanaged plastic waste (Lam et al., 2018).

persist in the environment for many years (Porta, 2021). Moreover, their low density allows them being readily dispersed by currents and winds, travelling thousands of kilometres from source areas (Ryan et al., 2009; Barnes et al., 2009), boosting their potential as main global environmental threats (Moore et al., 2009). Aside from creating substantial and various ecosystem impacts, plastic has also repercussions on the economy as well as on the well-being of the human society (Brouwer et al., 2017). For example, plastics productive process is responsible for conspicuous greenhouse gases emission into the atmosphere. Shen et al. (2020) predicts that plastic production will be responsible for the emission of 1.34 Gt per year throughout the next decade. Indeed, less than 10% of plastic items can be recovered (Geyer et al., 2017). In 2021, circular plastics represented about 9.8% of the World plastics production, of which 8.3% was recycled plastic and 1.5% were bio-based or bioattributed plastic (PlasticEurope, 2022).

Consequently, most of it ends up in landfills, where they may take a few hundred years to decompose (Cole et al., 2011). Plastic polymers can be classified in two main subsets according to their polymerization mechanism or engineering behaviour: thermoplastic and thermoset (Figure 7). Thermoplastic polymers such as polyethylene, polypropylene, polystyrene, polyvinyl chloride, nylon and PET, account for about 90% of the total

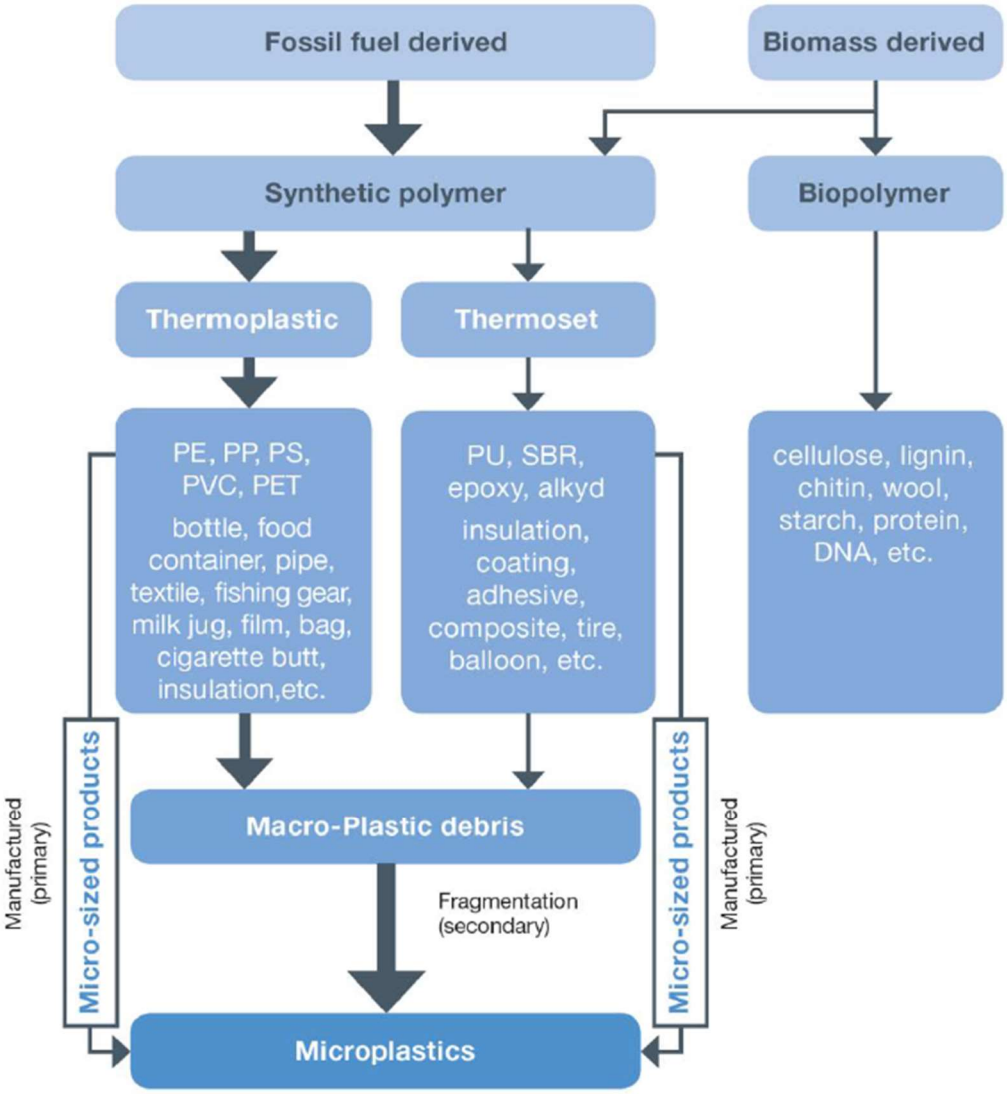
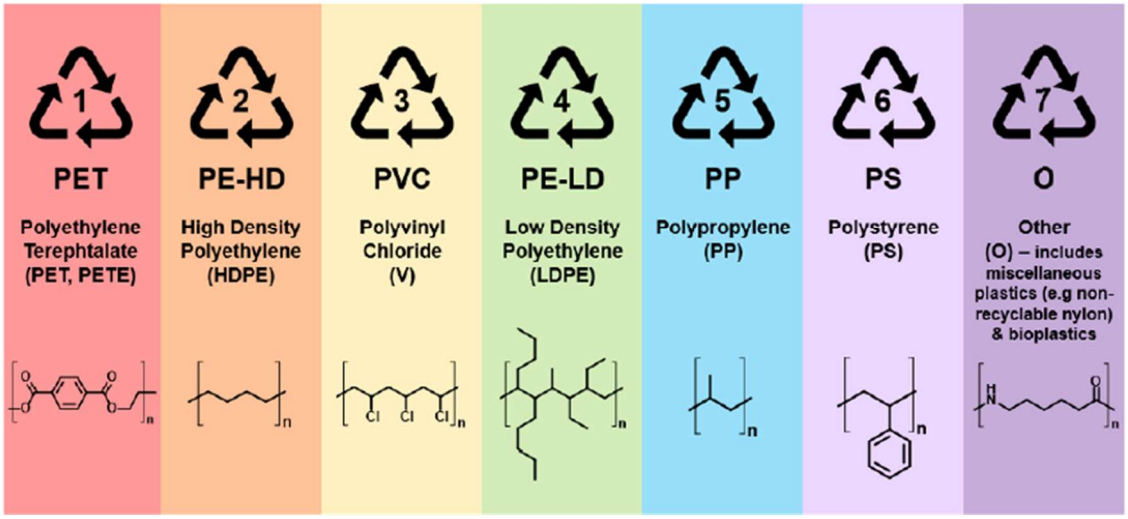


Figure 7. A generalised scheme of the most common artificial plastics and natural polymers, including some typical applications. Microplastics are manufactured for particular applications, such as industrial scrubbers or in personal cleaning products. All plastics can be subject to fragmentation into secondary microplastics on environmental exposure (GESAMP, 2015).

plastics produced and are chemically stable over a large range of temperatures. They are characterized by individual molecules separated from each other which flow past one another, preserving their mobility. These materials can be readily recycled by re-melting and re-shaping but, generally, they cannot be mixed together during recycling procedures, thus, they must be separated into the originating monomers (Porta 2021). Thermosets, such as polyurethane and melamine formaldehyde, as well as the epoxy and phenolic resins, are polymers characterized by high resistance to mechanical forces, heat and chemicals, and are thus unable to melt. Indeed, their individual chains have been chemically linked by covalent bonds during polymerization or by subsequent chemical or thermal treatment during fabrication and, when heated, undergo a chemical change, creating a three-dimensional network (Fried 2014). Consequently, they cannot be reprocessed upon reheating and are more challenging to be reutilized (Porta 2021). Besides thermoplastics and thermosets, the family of synthetic polymers also includes elastomers, polymers that are capable of high extension under ambient conditions, and synthetic fibers, suitable for textile application such as nylon and polyester (Fried 2014). Plastic items are also characterized by their chemical composition, even if some disagreement still exists on which polymers should be considered “plastics” (Hartmann et al*.,* 2019). The largest groups in total non-fiber plastics production are polyethylene, PE (36%), polypropylene, PP (21%), and polyvinylchloride, PVC (12%), followed by polyethylene terephthalate, PET, polyurethane, PUR, and polystyrene, PS (<10% each). Polyester, most of which is PET, accounts for 70% of all fiber production. Together, these seven groups account for 92% of all plastics ever made (Geyer et al., 2017). These polymers have different chemical and physical properties (Table 1), that can potentially lead to very heterogeneous fates and effect once they enter the environment. Plastic typically arrives in the ocean through water runoffs, direct dumping and loss of fishing and aquaculture gears (Andrady, 2011). Approximately 42% of “non –fiber” plastics are used for packaging, with PE, PP and PET as the most employed plastic polymers (Geyer et al., 2017). Land-based sources contribute about 80% of the plastic debris. About 18%

Table 1. Densities and common applications of plastics found in the marine environment (adapted from Andrady 2011).

Resin type	Common applications	Specific gravity
Polyethylene	Plastic bags, storage containers	0.91–0.95
Polypropylene	Rope, bottle caps, gear, strapping	0.90–0.92
Polystyrene (expanded)	Cool boxes, floats, cups	0.01–1.05
Polystyrene	Utensils, containers	1.04–1.09
Polyvinyl chloride	Film, pipe, containers	1.16–1.30
Polyamide or Nylon	Fishing nets, rope	1.13–1.15
Poly(ethylene terephthalate)	Bottles, strapping	1.34–1.39
Polyester resin + glass fibre	Textiles, boats	>1.35
Cellulose Acetate	Cigarette filters	1.22–1.24



of the marine plastic debris found in the ocean environment is attributed to the fishing industry (Geyer et al., 2017). Aquaculture can also be a significant contributor of plastics debris in the oceans (Hinojosa & Thiel, 2009). The rest is derived largely from land-based sources, including beach litter. In 2021, packaging and building & construction were the two largest world plastics markets. The third biggest end-use market is the automotive sector (Figure 8). Virgin resin pellets, a common component of debris, enter the oceans routinely via incidental losses during ocean transport or through run-off from processing facilities (Ogata et al., 2009; Doyle et al., 2011). After that the plastic litter would travel across the ocean following the system of winds and currents, the so-called "global

conveyor belt" (Broecker, 1992). Once in the environment, plastic debris are exposed to various mechanical, chemical, and biological degradation that lead to one of the most ubiquitous and dangerous kind of plastic pollution: the microplastics (MPs).

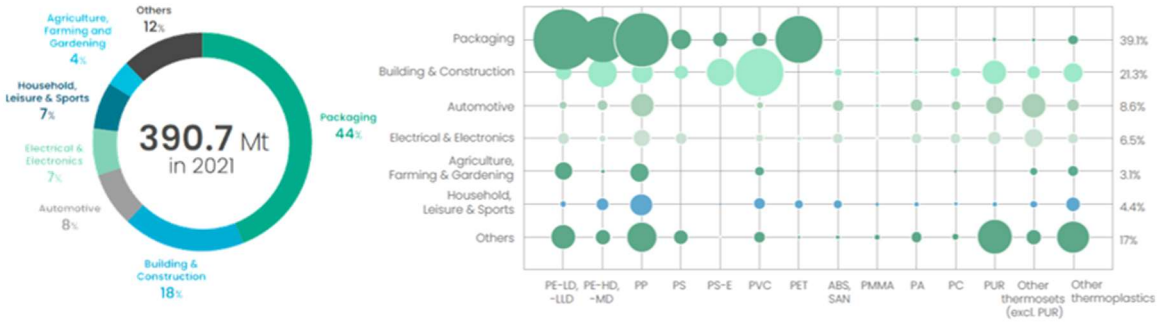


Figure 8. Distribution of the global plastics use by application (on the left) and European plastics converters demand by application and type (on the right) in 2021 (Plastics Europe 2022).

1.3. Microplastics

1.3.1. Definition, sources and occurrence

Microplastic litter is an omnipresent pollutant in marine systems across the globe that spread out from the water surface to benthic sediments. The term "microplastics" was first used in the year 2004 and is associated with a classification based on size (Thompson et al., 2004). Actually, there is no general consensus about a specific size nomenclature, although it has been suggested that microplastics should be defined as particles < 5 mm (Thompson et al., 2004; Arthur et al., 2009; Cózar et al., 2014; Horton et al., 2017). Microplastics (MPs) can be categorised in small microplastics (SMP, 25−1000 µm) and large microplastics (LMP, 1−5 mm), with generally SMP more abundant in natural environment than LMP (Poulain et al., 2019). The origins of the microplastics might be attributed to two main sources: direct introduction in the environment, usually through runoff, and weathering and breakdown of meso- and macroplastics debris (Andrady et al., 2011). Consequently, microplastics are subdivided into primary or secondary. Primary microplastics are the manufactured microparticles

of plastics used in consumer products (Maynard, 2006) and are introduced directly into the oceans via runoff. These include the micron-sized plastic particles typically used as exfoliants in cosmetic formulations (Gregory, 1996; Fendall & Sewell, 2009), and industrial manufactured pellets (beads of acrylic plastics and polyester) used in feedstock or plastic production (Cole et al., 2011). Secondary microplastics originate from the fragmentation of larger plastic items dispersed in the environments due to mechanical abrasion (e.g. breakdown by waves), hydrolyses, UV induced photolysis and biodegradation (Barnes et al., 2009; Browne et al., 2011; Eubeler et al., 2009; Ceccarini et al., 2018; Dawson et al., 2018; Liu et al., 2020). Microplastics are categorized also according to their morphology and physical characterization. The number of categories used to classify microplastics depends on the criteria of the respective study's authors, which can vary. According to Hidalgo-Ruz et al., (2012), the plastic items can be subdivided in: fragment, film, sphere, rope/filament, sponge/foam and fibre (Table 2). Hartmann et al., (2019) suggested to subdivide microplastics into five very similar types: spheres, spheroid, cylindrical pellet, fragment, film, and fiber. Generally, the shapes more commonly found in aquatic samples are fibers and fragments (Dusaucy et al., 2021). Microplastics in the oceans have been reported worldwide, from polar regions to

Table 2. Categories used to describe microplastics (Hidalgo-Ruz et al., 2012)

categories	
sources	consumer product fragments (e.g., fishing net) and raw industrial pellets
type	plastic fragments, pellets, filaments, plastic films, foamed plastic, granules, and styrofoam
shape	*for pellets*: cylindrical, disks, flat, ovoid, spheruloids
	for fragments: rounded, subrounded, subangular, angular
	general: irregular, elongated, degraded, rough, and broken edges
erosion	fresh, unweathered, incipient alteration, and level of crazing (conchoidal fractures), weathered, grooves, irregular surface, jagged fragments, linear fractures, subparallel ridges, and very degraded
color	transparent, crystalline, white, clear-white-cream, red, orange, blue, opaque, black, gray, brown, green, pink, tan, yellow, and pigmentation

the equator, from the intertidal zone to abyssal sediments (Lusher et al., 2015; Van Cauwenberghe et al., 2015). The abundance and distribution of microplastic particles can be extremely variable due to the wide number of variables like how plastic fragment, transport mechanism, hydrodynamic, and input of microplastic in given coastal areas (Huang et al., 2021). Microplastics mainly consist of polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), or polyethylene terephthalate (PET) (Andrady, 2011). Most common plastics range in density from 0.85 to 1.41 g/cm^3 (Table 1), where polypropylene and low/high-density polyethylene (LDPE, HDPE) plastics have densities lower than 1 g/cm^3, and polystyrene, nylon, polyvinyl chloride, and polyethylene terephthalate have densities higher than 1 g/cm^3. Due to their different specific weight, chemical composition and dimension, once released in the ocean, microplastics can be subjected to different fates: denser debris sink on the bottom and can be deposited within the sediment layer, lighter one remains suspended in the water column, or float on the oceanic surface (Wayman et al., 2021). Thus, the density of microplastics affects their distribution in the water column: for example, polypropylene (PP) and polyethylene (PE) are characterized by low density and float in the water, while polystyrene (PS), polyvinyl chloride (PVC), polyamide (PA) and polyethylene terephthalate (PET), which are characterized by higher density, deposit through the water column (Guo & Wang, 2019). Moreover, some properties of plastic items may change during residence at sea. For example, buoyancy and density of plastics may change due to weathering and biofouling, thus accelerating their sinking towards bottom sediments (Lobelle & Cunliffe, 2011). Indeed, the specific densities of many pelagic microplastics do not coincide with that of primary polymers (Andrady et al., 2011). The colonization of the plastic material by fouling organisms, for example, increases the density of the particles, allowing their vertical movement in the water column (Barnes et al., 2009; Brown et al., 2010) and their sinking towards the bottom, facilitating their deposition in the benthic environment (Bergmann et al., 2015), which can represent a long-term sink for these pollutants (Van Cauwenberghe et al., 2015).

Considering that the major sources of pollution are coming from the land, it would be expected that the plastic pollution issue would mainly affect the surface of seas and oceans. Nevertheless, it has been noticed that the global surface load of plastic is well below that expected from production and input rates. There is an important gap in floating plastic debris size smaller than 1 mm, suggesting that the surface waters are not the final destination for buoyant plastic debris in the ocean (Cozar et al., 2014). Due to their persistence, the current trends of microplastics accumulation and dispersion in the marine environment will enable these particles to remain there for centuries to come, making these plastics available for various interactions with the biota (Figure 9).

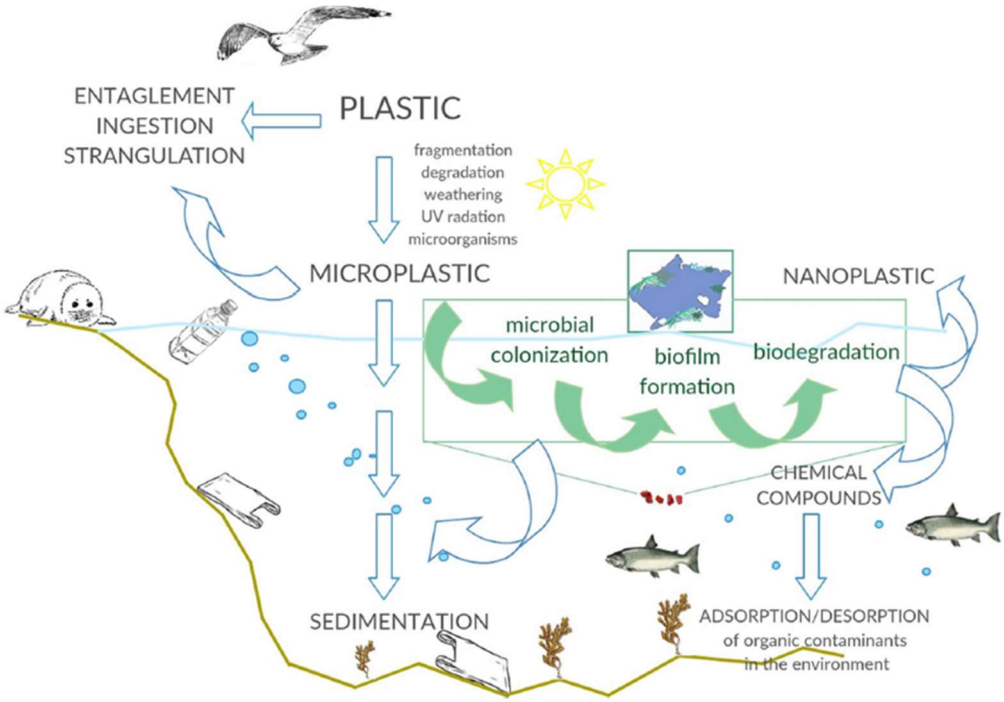


Figure 9. Examples of potential interactions between marine organisms and microplastics in marine environment (Urbanek et al., 2018).

1.3.2. Impacts on marine ecosystems & interaction with biota

Plastic pollution has been associated with a range of negative effects on the environment. Wildlife can encounter and interact with plastic debris and other anthropogenic materials in many different ways. Organisms interact mostly with larger litter items, but more and more reports on encounters with microplastics emerge. Ingestion is the most frequently observed interaction, followed by entanglement (e.g. ropes, fishing lines, heavy-duty sacks and industrial packaging sheet) (Schuyler et al., 2014; Domènech et al., 2019; AWI-Litterbase 2023). When floating litter is carried over long distances by ocean currents, associated rafters, like alien species, can invade new areas, and pathogens or contaminants may become available to a greater range of different organisms (AWI-Litterbase 2023). The small size of microplastics makes them particularly available for interaction with marine biota at different trophic levels, with various consequences on marine organisms and ecosystems (Graham et al., 2019; Okubo et al., 2020; Mendrik et al., 2021). Until now, interactions were reported for 4043 species of microbes, plants and animals (AWI-Litterbase 2023), with 1458 species recorded to ingest plastic debris (Monteiro et al., 2022). The potential ecological and human health risks of microplastics are relatively new areas of research, thus there is currently a large degree of uncertainty surrounding this issue. Risk is a function of hazard and exposure (dose), and evaluating the risks from microplastics requires knowledge of hazard (i.e. the potential of microplastics to cause adverse effects through plausible mechanisms), exposure levels (i.e. the quantities of microplastics detected in the environment, including in living organisms) and their effects (identification of dose-response relationships and threshold levels) (Law et al., 2017). Our knowledge about the uptake and the biological effects of MPs is only at the beginning in understanding all the possible consequences on the plethora of organisms inhabiting the marine environments. As previously mentioned, due to differences in shape and density, microplastics disperse diversely in various compartments of the marine environments

(water surface, water column and sediment), and this influences their availability to organisms at different trophic levels and/or occupying different habitats (Galloway et al., 2017). Of great concern is the possible trophic-transfer of MPs and associated contaminants, with the potential for bioaccumulation and biomagnification and thus adverse health effects at all trophic levels, from lower-trophic to the upper level of the food-chain, which may ultimately lead to contaminated seafood for humans (Smith et al., 2018; Vital et al., 2021). Previous studies on MPs interactions suggest that the role of the organisms in the food web may influence MPs uptake (Fossi et al., 2014; Wesch et al., 2016; Vered et al., 2019; Fang et al., 2021). In general, the effects of microplastics on marine life may arise from direct and/or indirect interactions between the organism and the microplastic particles. Marine fauna commonly interacts with MPs in a direct way through physical interactions and ingestion. Indeed, MPs can resemble marine organism's food in shape, size, and color, leading to an accidental consumption of anthropogenic material with or in place of the natural food items. Mistaken ingestion of MPs has been reported from zooplanktonic organisms (Desforges et al., 2015), to invertebrates (Wright et al., 2013), like ascidians (Vered et al., 2019), bivalves and crustaceans (Kamio & Derbi, 2017), cnidarians (Macali et al., 2018) up to vertebrates, like sea turtles (Camedda et al., 2014), fishes (Guerranti et al., 2016; Palazzo et al., 2021), and marine mammals (Fossi et al., 2012). Effects may result in potentially fatal injuries, such as blockages throughout the digestive system and the feeding appendages of aquatic invertebrates (Browne et al., 2008), abrasions from sharp objects and mechanical disturbance that may lead to behavioral responses (Costa et al., 2020), or even become embedded in tissues (Browne et al., 2008). MPs can indirectly interact with the organisms through adhesion patterns (Martin et al., 2019), by acting as a vector of alien rafting species and diseases (Hoeksema et al., 2018; Lamb et al., 2018), and by transporting and leaching toxic substances (Teuten et al., 2009; Koelmans et al., 2016;). Indeed, other effects of plastic pollution on marine organisms are related to the "cocktail of contaminants" (Bergmann et al., 2015) that the MPs may carry in the body

of the organisms (Bakir et al., 2014), and have the potential to be transferred into the food web (Gall & Thompson, 2015; Law, 2017). The substances that plastic absorbed and carry once in the environments may include persistent organic pollutants (POPs), like polychlorobiphenyls (PCBs), pesticides, and heavy metals (Andrady, 2011), chemicals that can interact with important biomolecules inside cells and disrupt the endocrine system (Teuten et al., 2009). Moreover, MPs can be themselves sources of pollutants (Hermabessiere et al., 2017). These include those substances that are ingredients of the plastic material (e.g., residual monomers or oligomers of the component molecules of the plastics) and additives, like polybrominated diphenyl ethers (PBDE), phthalates, nonylphenols (NP), bisphenol A (BPA) and antioxidants, referring as the most common plastic additives found in the marine environments (Hermabessiere et al., 2017). The concurrent presence of such chemicals, both embedded or absorbed by plastic items, and plastic polymers themselves may result in toxicity, endocrine disruption, carcinogenicity, and other negative effects which pose a risk to aquatic life health and survival (Wright et al., 2013; Piccardo et al., 2018; Mancia et al., 2020).

Aside from their coastal distribution and their sessile nature, the sensitivity of scleractinian corals, soft corals and sea anemones to microplastics exposure is also due to their polytrophic and opportunistic nature (Houlbrèque & Ferrier-Pagès, 2009; Shick, 2012; Savage et al., 2022). Indeed, besides from autotrophy, where they receive the energy from their endosymbiotic algae, most anthozoans are also active heterotrophs, ingesting organisms ranging from bacteria to mesozooplankton (Houlbrèque & Ferrier-Pagès, 2009). According to existing studies, the potential relationship between microplastics and corals include microplastic ingestion and exposure, combined effects of microplastics and associated chemical contaminants, coral disease induced by plastics, and impacts on coral zooxanthellae symbiosis (Huang et al., 2021). At present, studies highlight diversified possible interactions between corals and plastics, with different coral species that respond differently to different MPs exposures (Table 3). The

knowledge regarding the different effects of microplastics on coral species was summarized in table 3. Overall, researches highlight the ability of stony corals to interact with MPs actively through ingestion and passively through adhesion (Martin et al., 2019; Costa et al., 2020). As for suspension feeders and planktivorous animals, corals are susceptible to microplastic ingestion, both by capturing MPs instead of their prey because of shape and size similarity, and also indirectly, by feeding on zooplankton, amphipods, or copepods that have ingested MPs (Hall et al., 2015). Previous studies have demonstrated that, as generalist feeders, corals are non-selective towards the types of prey, but there is a preference for food particles which size are <400 µm (Hall et al., 2015; Corona et al., 2020; Palardy et al., 2008), that fall into the range size of microplastic particles. Moreover, the capacity of corals to discriminate plastic as an alien particle may be reduced if it is covered by biological material (Corona et al., 2020). Regarding the impacts of the ingestion of MPs, studies show that corals can ingest microplastics and retain them in the gut cavity for at least 24 h (Hall et al., 2015; Reichert et al., 2019), leading to potential effects on energetics, by reducing normal feeding on organic matter to spend the energy in egesting plastic (Rotjan et al., 2019). Besides, the long retention of the plastic particles in the mesenterial tissue enhances the danger of toxicity due to pollutants carried or leached by the plastic (Reichert et al., 2018). This potentially affects also the organisms that feed on corals, impacting the trophic-web by transferring those pollutants to differen trophic levels (Reichert et al., 2018). Adhesion to the coral surface seems to be the the dominant process of interaction between MPs floating in the water column and corals (Martin et al., 2019; Corona et al., 2020), supporting 40-fold greater removal rates than ingestion in stony corals (Martin et al., 2019). Specifically, the plastic particles are entrapped by the surface of the coral by the rugose skeleton structure or the mucus released by the coral itself (Reichert et al., 2019). The adhered particles entrapped may trigger a variety of physiological and behavioral responses, such as cleaning mechanisms (e.g. ciliary action, mucus production, or tissue expansion), and retention of particles through overgrowing or ingestion (Stafford-Smith

& Ormond, 1992). However, studies are still limited and mainly focus on scleractinian (stony) corals (Table 3).

Table 3. Impacts of microplastics on scleractinian corals (Soares et al., 2020).

Impact	Coral species	Microplastics concentration	Exposure time	Reference (s)
Reduced growth	*Acropora muricata*	~200 particles L^{-1} or 0.25 mg L^{-1}	6 months	Reichert et al. (2019)
	Heliopora coerulea	350 particles L^{-1}	2 months	Chapron et al. (2018)
	Lophelia pertusa	350 particles L^{-1}	5 months	Mouchi et al. (2019)
Significant decrease of detoxifying and immune enzymes	*Pocillopora damicornis*	50 mgL^{-1} or 9,0 × 10^{10} particles L^{-1}	6, 12, 24h	Tang et al. (2018)
Increases in the activities of antioxidant enzymes and chlorophyll content	*Pocillopora damicornis*	50 mgL^{-1} or 9,0 × 10^{10} particles L^{-1}	6, 12, 24h	Tang et al. (2018)
Alteration of coral metabolite profiles (increased levels of phosphorylated sugars and pyrimidine nucleobases)	*Stylophora pistillata*	5 particles/mL and 50 particles/mL	28 days	Lanctôt et al. (2020)
High production of mucus	*Acropora hemprichii*	53 – 500 μm; 13645 ± 139 beads L^{-1} or ~ 0.2 g L^{-1}	28h	Martin et al. (2019)
	Porites lutea	37–163 μm; ~ 4000 particles L^{-1} or 0,1 g L^{-1}	4 weeks	Reichert et al. (2018)
Symbiont chlorophyll content increased	*Pocillopora damicornis*	50 mgL^{-1} or 9,0 × 10^{10} particles L^{-1}	6, 12, 24h	Tang et al. (2018)
Reduction of fitness (impacted by plastic ingestion and adhesion)	*Danafungia scruposa*	212- 1000 μm, 2,996 ± 5 beads or 0.57 ± 0.0001 g per 1.5 L	2 days	Corona et al. (2020)
	Astroides calycularis	20 particles per 15L	30 min 90 min	Savinelli et al. (2020)
Significant decrease of detoxifying and immune enzymes	*Pocillopora damicornis*	50 mgL^{-1} or 9,0 × 10^{10} particles L^{-1}	6, 12, 24h	Tang et al. (2018)
Tissue necrosis	*Acropora formosa*	0.05 g. L^{-1}, 0.1 g. L^{-1}, and 0.15 g. L^{-1}.	14 days	Syakti et al. (2019)
	Pocillopora verrucosa	~200 particles L^{-1} or 0.25 mg L^{-1}	6 months	Reichert et al. (2019)
		37–163 μm; ~ 4000 particles L^{-1} or 0,1 g L^{-1}	4 weeks	Reichert et al. (2018)
	Pocillopora damicornis	37–163 μm; ~ 4000 particles L^{-1} or 0,1 g L^{-1}	4 weeks	Reichert et al. (2018)
	Acropora muricata	~200 particles L^{-1} or 0.25 mg L^{-1}	6 months	Reichert et al. (2019)
Coral bleaching	*Acropora formosa*	0.05 g. L^{-1}, 0.1 g. L^{-1}, and 0.15 g. L^{-1}.	14 days	Syakti et al. (2019)
	Acropora humilis *Acropora millepora* *Porites cylindrica*	37–163 μm; ~ 4000 particles L^{-1} or 0,1 g L^{-1}	4 weeks	Reichert et al. (2018)
	Acropora muricata	~200 particles L^{-1} or 0.25 mg L^{-1}	6 months	Reichert et al. (2019)
Lower fertilization success	*Acropora tenuis*	5 pieces L^{-1} to 200 pieces L^{-1}	2, 5 h and 24 h	Berry et al. (2019)
	Acropora humilis	37–163 μm;	4 weeks	Reichert et al. (2018)
Overgrowth on microplastics (energy costs)	*Acropora millepora* *Porites lutea* *Porites cylindrical*	~ 4000 particles L^{-1} or 0,1 g L^{-1}		
Ingestion, retainment within their gut cavity and egestion	*Dipsastrea pallida*	0.395 g. L^{-1}, 0.197 g L^{-1} (±0.2) and 0.24 g L^{-1} (±0.13)	48 h, 12 h and 3 h	Hall et al. (2015)
	Montastraea cavernosa	30 mg L^{-1}	48 h	Hankins et al. (2018)
	Orbicella faveolata	30 mg L^{-1}	48 h	
	Madrepora oculata	350 beads L^{-1}	5 months	Mouchi et al. (2019)
	Pocillopora verrucosa	37–163 μm; ~ 4000 particles L^{-1} or 0,1 g L^{-1}	4 weeks	Reichert et al. (2018)
	Astroides calycularis	20 particles per 15L	30 min 90 min	Savinelli et al. (2020)
Decreased feeding performance and food intake	*Astroides calycularis*	20 particles per 15L	30 min 90 min	Savinelli et al. (2020)
	Desmophyllum pertusum	350 beads L^{-1}	5 months	Mouchi et al. (2019)
	Montipora capitate *Pocillopora damicornis*	2 particles mL^{-1}	10 days	Axworthy and Padilla-Gamiño (2019)
	Astrangia poculata	0.1 g in 500 ml	15 min	Rotjan et al. (2019)
Changes on photosynthetic performance	*Acropora muricata* *Pocillopora verrucosa*	~200 particles L^{-1} or 0.25 mg L^{-1}	6 months	Reichert et al. (2019)
	Acropora formosa	0.05 g. L^{-1}, 0.1 g. L^{-1}, and 0.15 g. L^{-1}.	14 days	Syakti et al. (2019)
	Stylophora pistillata	5 particles/mL and 50 particles/mL	28 days	Lanctôt et al. (2020)
Decreased skeletal growth rates and calcification	*Desmophyllum pertusum*	350 beads L^{-1}	5 months	Mouchi et al. (2019)
	Heliopora coerulea	~200 particles L^{-1} or 0.25 mg L^{-1}	6 months	Reichert et al. (2019)

In order to gain a better understanding of the impacts of microplastics, most studies have focused on quantifying their abundance in the marine environment and on the assessment of their distribution and composition. To this aim, different sampling methodologies have been used to document microplastics' presence in seawater. Data from different studies are often difficult to compare due to the lack of standardized sampling methodologies, pre-treatment, quantification and identification (Ryan et al., 2009; Li et al., 2018). Results often vary between studies, but it is difficult to distinguish whether these dissimilarities are linked to different abundance and distribution of microplastics or to different methodological approaches. Moreover, data about microplastics are often reported using alternative reference units, as either the number (or mass) of microplastic particles per unit area (e.g., m^2) or per volume (e.g., m^3), making it difficult to compare research results (Horton et al., 2017). Despite this, continued method development is improving researchers' ability to identify microplastics and common practices have been established. Between these approaches there is the use of marine organisms as bioindicators, irreplaceable tool to assess distribution and composition of MPs in the sea since they measure microlitter levels in their environments in a way that is impossible to replicate by direct physical measurements, and the detection of plastic-associated contaminants in their tissues, knowing that microplastic fragments can function as vector of contaminants in various organisms. Consequently, measurements of microplastics in biota are key elements of exposure and risk assessments for this emerging environmental pollutant (GESAMP, 2015).

1.4. Plastic Additives

In many plastic products, the plastic is not the only component, but during processing and fabrication, additives are mixed with the polymer to arrive at a set of desired properties for the required products. Among these additives there are flame retardants,

stabilizers, pigments and colorants, reinforcements, bisphenol A and plasticizers, all chemicals that are considered persistent, bioaccumulative and toxic in the environment (Nakashima et al., 2012; Jang et al., 2016; Kim et al., 2017). Since most of them are not chemically bound to the plastic polymers, such chemicals can leach from the plastic litter and disperse into the environments. Thus, as a consequence of plastic accumulation and fragmentation in oceans, plastic additives could represent an increasing ecotoxicological risk for marine organisms (Hermabessiere et al., 2017). Between plastic additives, phthalate esters are the most commonly used plasticizers worldwide (Net et al., 2015; Paluselli et al., 2019; Paluselli et al., 2020).

1.4.1. Phthalate Esters

Phthalate esters (PAEs) (Figure 10) are a class of additives essential constituent of synthetic organic polymers and commonly blended with plastic polymers in high relative mass amounts (up to 60% of the total plastic product weight) (Net et al., 2015; Boll et al., 2020). In these materials, phthalates are either part of the polymeric structure, such as in polyethylene terephthalate (PET), or they are non-covalently dissolved as low-molecular-weight di-esters within the plastic polymeric structure to act as softeners/ plasticizers (Boll et al., 2020). They are abundant as plasticizers in polymers such as polyvinyl chloride, polystyrene, and polyethylene (Fan et al., 2018), and are generally used to enhance the flexibility, transparency, and longevity of plastic materials (Teuten

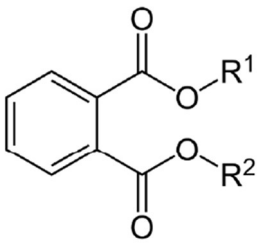


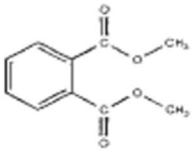
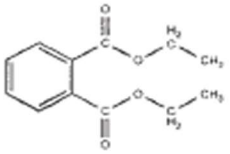
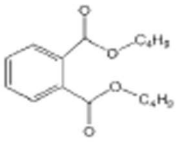
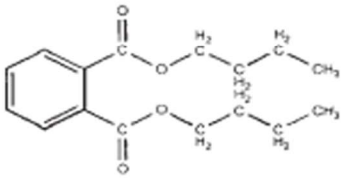
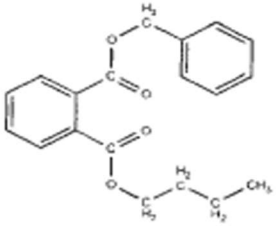
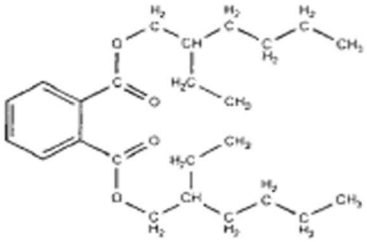
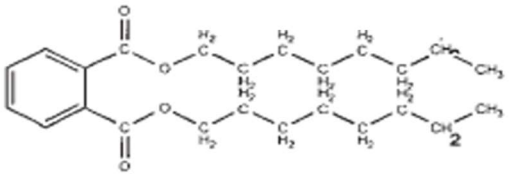
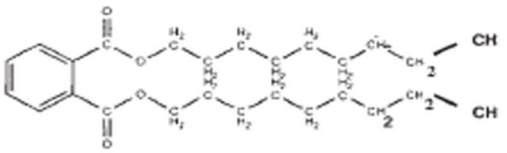
Figure 10. General structure of phthalates. The basic structure consists of a 1,2 benzenedicarboxylic acid with two side chains R1 and R2 (Ufficio federale della sanità pubblica della Confederazione svizzera, 2021)

et al., 2009; Net et al., 2015). Depending on the alcohol that makes up the alkyl chain, PAEs have a wide range of different properties for several applications. Short-chain PAEs, such as DMP, DEP, BBP, DnBP, and di-isobutyl phthalate (DiBP), are often used in non-poly (vinyl chloride) applications such as personal care products, paints, adhesives, and plastic bags (Net et al., 2015). Long-chain PAEs, such as DEHP, di-isononyl phthalate (DiNP), and di- isodecyl phthalate (DiDP), are primarily used in plastic polymers and applications such as building and construction materials, cables and wires, clothing, furnishings, toys, and also food-contact materials (Net et al., 2015). Since they are not covalently bound, they are easily released from the plastic polymers and leach into the environments (Paluselli et al., 2018; Schmidt et al., 2021), becoming bioavailable to different marine organisms. They enter into the environment through numerous sources, including industrial and municipal wastewaters, land application of sewage sludge, and leaching after the disposal of industrial and municipal solid waste (Paluselli et al., 2018; Abtahi et al., 2019). Although PAEs are not persistent and are biodegraded in the environment (Net et al., 2015), they result ubiquitous in the environment because of the continuous dispersion of plastic. Thus living organisms can be continuously exposed to PAEs (Mackintosh et al., 2004; Net et al., 2015; Baini et al., 2017). PAEs have low solubility in the water, characteristic that facilitate their leaching from plastic wastes at a steady rate (Fossi et al., 2014), and they are also high lipophilic, characteristic that makes them easily bioavailable for the aquatic life, with a strong tendency towards accumulation in living organisms (Mathieu-Denoncourt et al., 2016; Net et al., 2015). PAEs have been detected in various environmental matrices, such as air (Xie et al., 2005), water (Das et al., 2014; Xie et al., 2007; Zheng et al., 2014), sediments (Zheng et al., 2014), and biota (Fossi et al., 2012; Huang et al., 2008) on a worldwide scale (Net et al., 2015). Some PAEs and their metabolites have also been detected in human urine and other human tissue fluids, such as breast milk, blood, and saliva (Frederiksen et al., 2007; Guerranti et al., 2013). PAEs are associated with several adverse effects capable of enhancing the toxic effect of microplastics. One of the well-documented effect of

phthalates is their nature of endocrine disruptors, which may lead to toxic effects on fertility and the development of humans as well as on many aquatic and terrestrial species (Net et al., 2015). Moreover, in some cases, PAEs can cause oxidative stress and immunotoxicity (Oehlmann et al., 2009). These and other associated adverse effects led the United States Environmental Protection Agency (U.S. EPA) to categorize them as priority pollutants in 2012 (US EPA, 2015), while the European Union (EU) listed them as substances suspected of producing endocrine alterations in 2007 and limited or recommended values have been set for the most abundant and toxic PAEs (Net et al., 2015). The most common and studied PAEs plasticizers are: dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), diethyl phthalate (DEP), Bis(2-Ethylhexyl) phthalate (DEHP), and dimethyl phthalate (DMP) and mono ethylhexyl phthalate (MEHP) (Table 4). Among them, the most studied is dibutyl phthalate (DBP) due to its great environmental concentration (µg-level in marine water) and strong toxicity (Liu et al., 2016; Net et al., 2015b). Indeed, DBP has been reported to induce oxidative stress and produce reproductive and developmental toxicity to different aquatic organisms (e.g. *Xenopus laevis*, *Danio rerio*, *Daphnia magna*, *Karenia brevis*) (Li et al., 2015; Mu et al., 2018; Seyoum and Pradhan, 2019; Xu and Gye, 2018). In the marine environment, PAEs and their metabolites were detected from the top of food chain (plankton, algae) to predator organisms (fish, marine mammals) (Staples et al., 1997; Fossi et al., 2012), from not detected (nd) levels to few hundreds ng/g (Fossi et al., 2012; Baini et al., 2017). PAEs can be degraded by different biotic and abiotic pathways, as such they are not expected to be highly persistent in aquatic and terrestrial environments. PAEs in water can be eliminated by hydrolysis, photolysis, photooxidation, and biodegradation, with biodegradation activity that appears to be greater than abiotic degradation in surface waters (Net et al., 2015). Primary degradation half-life in water is expected to be on the order of less than 1 week, however, their extensive use and permanent emissions have resulted in their ubiquitous presence in the environment. However, there is a paucity of

data dealing with accurate description of degradation processes for the complete set of PAEs (Net et al., 2015).

Table 4. Chemical structure and acronyms of phthalate esters (Gobas et al., 2003)

Phthalate Ester	Abbreviation	Chemical Structure
Dimethyl Phthalate	DMP	
Diethyl Phthalate	DEP	
Di-iso-butyl Phthalate	DiBP	
Di-*n*-Butyl Phthalate	DBP	
Butyl Benzyl Phthalate	BBP	
Di(2-Ethylhexyl) Phthalate	DEHP	
Di-n-Octyl Phthalate	DnOP	
Di-n-Nonyl Phthalate	DnNP	

1.4.2. Phthalate's Metabolites

Although recently the level of phthalates can be determined in an organism, this measurement alone cannot be considered to be a sufficient indicator of the entire PAEs pollution, due to the quick metabolism of PAEs in the biota (Hu et al., 2016). The metabolic pathway of PAEs has been studied from microorganisms (Horn et al., 2004), to mammals, including humans. Phthalates normally follow a metabolic pathway in two steps: phase I, hydrolysis, and phase II, conjugation. In the first step, PAEs are hydrolyzed into their primary metabolite, phthale monoesters (MPEs). In phase II, it forms the hydrophilic glucuronide conjugate (Figure 11). From this knowledge of the metabolism and previous studies, the primary metabolites (MPEs) are likely to be used as biomarkers to predict exposure to both low molecular weight and high molecular weight PAEs (Frederiksen et al., 2007; Silva et al., 2007; Guo et al., 2011; Fossi et al., 2014). However, it remains uncertain whether MPEs can indeed be used as biomarkers to predict exposure to PAEs in aquatic biota, as phthalate metabolites have been scarcely documented in wild marine organisms (Blair et al., 2009; Fossi et al., 2012; Net et al., 2015; Valton et al., 2014). Primary metabolites of PAEs, especially mono butyl phthalate (MBP) and mono ethylexyl phthalate (MEHP) have been found in several aquatic organisms, including plankton, mussels, crab, and fish (Blair et al., 2009; Valton et al., 2014; de Lucia et al., 2014; Montano et al., 2020). Nevertheless, the mechanisms of bioaccumulation and metabolism of PAEs and MPEs in wild aquatic species remain unclear. The metabolism of phthalic acid esters appears to depend on both their chemical structure and species of biota (Liang et al., 2008; Staples et al., 1997). Studies show that phthalates with shorter ester chains, e.g., dimethyl phthalate (DMP), diethyl phthalate (DEP), and di-n-butyl phthalate (DBP), can be readily biodegraded and mineralized, whereas phthalates with longer ester chains, such as DEHP and di-n-octyl phthalate (DNOP), are less susceptible to biodegradation (Liang et al., 2008); this is due to the steric effect of phthalate' side ester chains, which hinders hydrolytic enzymes

from binding to phthalates, thereby inhibiting their hydrolysis (Net et al., 2015). Thus, investigated low-molecular-weight MPEs (short-branched) had shown to be quantitatively effective in reflecting parent PAEs contamination in wild marine organisms and useful as biomarkers of PAEs exposure, due to the several complex biotransformations they undergo during the metabolic activities (Frederiksen et al., 2007; Silva et al., 2007). Regarding the dependence of the kind of metabolic pathway upon the different species of the biota, results obtained in the study conducted by Hu et al., 2016 confirm that PAEs and MPEs undergo trophic dilution in the marine food web, which is likely to be the combined results of low assimilation efficiencies and

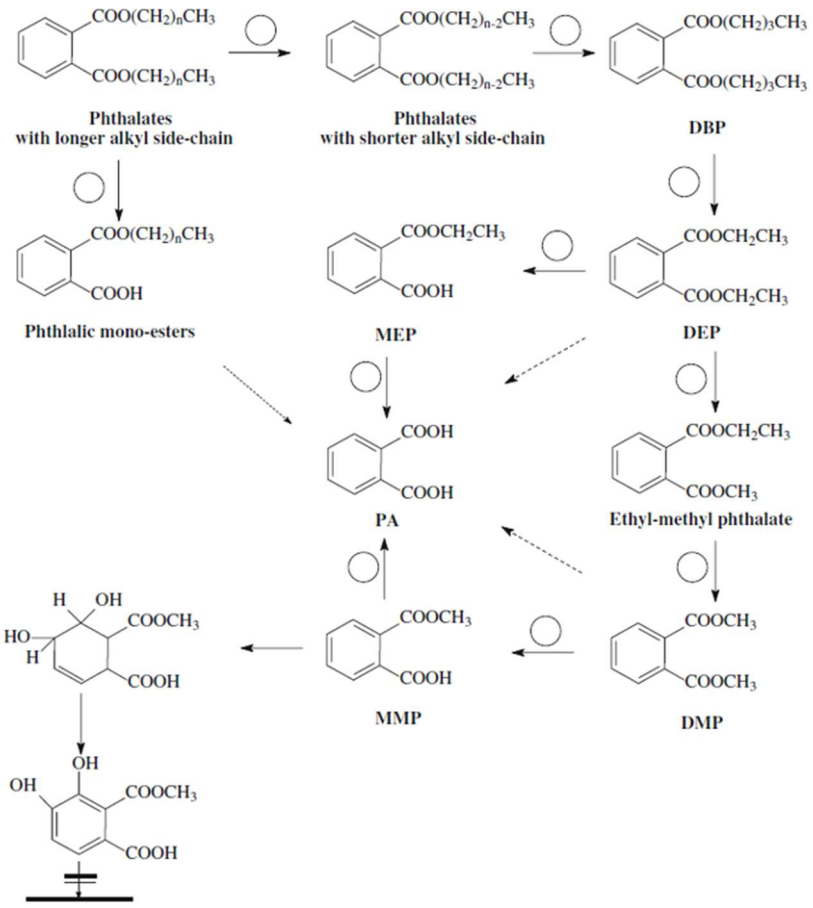


Figure 11. Phthalates degradation pathways and the enzymes involved. E1 DAP esterase, E2 MAP esterase (Liang et al., 2008).

effective metabolic transformations at higher trophic levels (Mackintosh et al., 2004). Indeed, the ability to biotransform PAEs among different species was found to be in the following order: algae < cnida rians < mollusks < crustaceans < fish (Net et al., 2015).

1.5. BioSPME coupled to LC-MS

BioSPME followed by liquid chromatography (LC) coupled to mass spectrometry (MS) procedures has been recently proposed as a methodology for the determination of phthalates in marine invertebrates (BioSPME-LC/MS, Saliu et al., 2020a; 2020b). The relevance of this application relies on the current use of marine organisms as bioindicators for plastic contamination potentially through the detection of phthalates in their tissues. Such procedure takes in consideration the challenges that originate from the availability and/or possible restriction on the use of the biological materials (e.g. amount of biological matrices, need to study rare or protected species, etc…), the need for user- friendly procedures for simplifying and speeding up operations in the marine environments, and the ubiquitous presence of phthalates in the environments that may cause background contamination of the target samples.

1.5.1. Solid-Phase Micro-Extraction (SPME)

The solid-phase micro-extraction (SPME) was introduced by Arthur and Pawliszyn in 1990 (Arthur and Pawliszyn, 1990) and is a technique of extraction based on the equilibration of analytes between the sample matrix (gaseous, aqueous, and solid) and an organic polymeric phase usually coating a fused-silica fiber or a flexible metal alloy support, which enables simultaneous extraction and pre-concentration of an amount of the analytes that is proportional to the initial concentration in the matrix (Bojko et al., 2012). Since its introduction, SPME has been widely used to monitor residues of chemicals in a variety of environmental, biological, and food matrices (Spietelun et al., 2013). Most of the SPME applications were developed for GC–MS analysis in the

headspace mode, but also LC–MS/MS applications have recently gained attention, due to the introduction of biocompatible coatings (BioSPME), that allow direct extraction of analytes from biological matrices (Kennedy et al., 2010) and even *in vivo* (Togunde et al., 2012; Vuckovic et al., 2011), as it reduces the adhesion of proteins and other macromolecules, in favour of substances with lower molecular dimensions. The use of the polymeric phase as a solid phase differentiates this technique from the solid-phase extraction (SPE) and makes it possible to extract organic analytes directly from aqueous phases or the headspace of the sample. Moreover, this technique overcomes various problems linked to the liquid-liquid extraction (LLE) technique, like the formation of emulsions, the use of big volumes of solvents, with the later difficulty of waste management, and low recovery efficiency. Besides all these advantages, SPME is a fast and simple method of extraction that can be done even without solvents, with detection limits that can reach parts per trillion (ppt) levels for certain compounds. SPME also has a great potential for field applications and on environmental matrices (Poole, 2017), like organic tissues (Xu et al., 2016). Moreover, on-site sampling can be done even by not specialized but prepared stakeholders, without the need to have gas chromatography-mass spectrometry equipment at each location. Indeed, when properly stored, samples can be analysed days later in the laboratory without significant loss of volatiles. In general, the SPME procedure aim is to obtain a sample without interfering matrix and an adequate analyte concentration to be detected. The SPME technique consists of three main phases:

➢ Activation (or conditioning): in this step, the fiber must be activated to derivatize the adsorbing phase;

➢ Fiber exposure to the sample: once activated, the fiber is exposed to the sample, either pre-treated or not according to the samples;

➢ Desorption (or elution): this phase has the aim to put in solution the analytes that were absorbed by the fiber from the sample.

1.5.2. SPME fiber

As previously mentioned, the SPME methodology is an extraction and concentration technique for contaminants (even in traces) in homogeneous and heterogeneous mixtures, and it requires the use of specific fibers. The fibers used for the analysis performed during this thesis are C18-coated solid phase microextraction fibers in the needle probe format (SPME LC Tips C18 Pk/96 SUPELCO®, Figure 12). This fiber present a silicious atoms cylindrical fiber core of 200 mm diameter, a length of 40 mm, an extraction phase surface area of 8.1 mm^2 and was principally structured for the extraction of both polar and non-polar analytes, via desorption procedure in solvent and liquid chromatography application (LC). On sale, there are a lot of different types of fibers with different affinity to the targeted analyte and lower affinity to the components of the matrix, enhancing the values of the distribution constant. Nevertheless, all the fibers sold possess polymeric layers that have high hydrophobicity to avoid the absorption of the water in the absorption phase. This peculiar characteristic increases the extraction efficiency of the fiber towards the target analyte, as water is the major component of the matrix in a biological sample. The core of the fiber is made out of a metal alloy with titanium, that is flexible and is used as an inert support for the

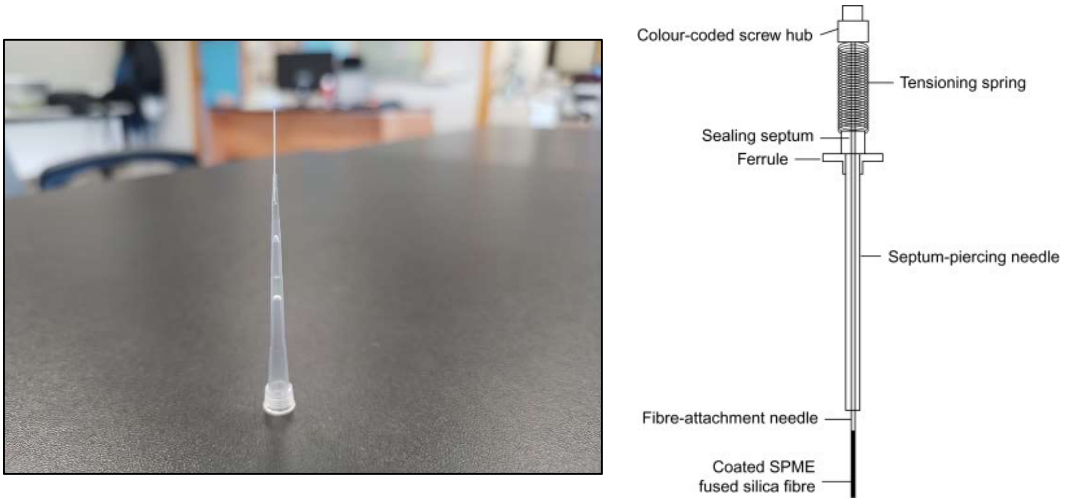


Figure 12. Design of the SPME LC fiber SUPELCO, C18 (Shirey, 2012).

stationary phase. This structure is fixed on a rigid support made out of plastic and with the shape of a tip to enable the use of the fiber with a Gilson pipette.

SPME advantages of use:

- It can perform measurement independent from the mass/volume of the sample;
- Easy to use and handy;
- Very low or almost "solvent-free" technique (low environmental impact);
- High execution velocity, as the equilibrium of partition, is rapidly reached (between 2 and 30 minutes on the base of the different analyte);
- Elevate sensitivity (even for tracers' analysis);
- Low cost, due to the knocking down of the expenses (solvent use and waste management, reusable fibers, etc.);
- Reduced dimensions;
- Possibility of *in vivo* and on-site applications (BioSPME) (Figure 13).

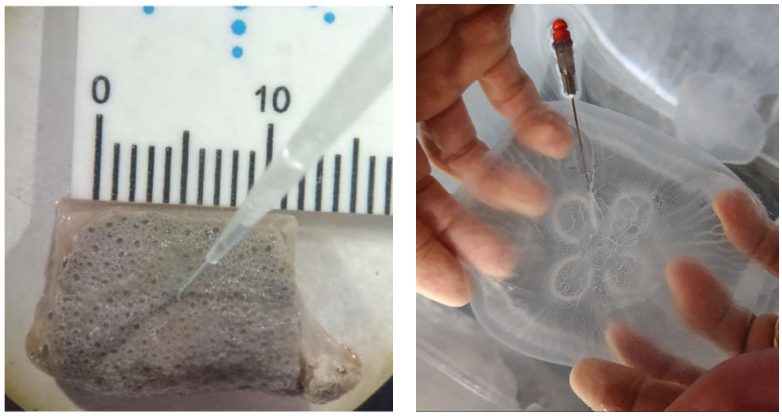


Figure 13. A) Picture at the stereomicroscope of a slice of the soft coral, *Sarcophyton sp.*, and B) a jellyfish specimen (*Aurelia aurita)* with the SPME fiber inserted.

1.6. Research objective

As described above, the pollution of the seas caused by the dispersion of litter is one of the most serious environmental emergencies worldwide. Various studies show that this litter consists primarily of plastics, mainly due to its continuously increasing global production and the fact that it is nearly immune to environmental degradation. Indeed, plastic items broke in smaller pieces called microplastics (MPs), that can threaten marine life in different ways, for example through physical interactions, and/or acting as vectors of plasticizers and contaminants. Phthalate esters (PAEs) are common plastic additives blended with plastic polymers that during ageing and under certain conditions, like the digestion process, can easily leach from the plastic debris, becoming available to marine organisms. Phthalates occurrence has already been reported in marine biota like zooplankton (Schmidt et al., 2021), marine invertebrates (Vered et al., 2019), and marine mammals (Baini et al., 2017). At the same time, a possible correlation between MPs exposure and PAEs presence was proposed in different marine organisms (Fossi et al., 2012; Baini et al., 2017; Vered et al., 2019). Consequently, the presence of phthalates was proposed as a marker to evaluate MPs contamination in marine environments and as a proxy of marine organisms' exposure to plastic debris (Fossi et al., 2012). Soft corals and sea anemones are overlooked benthic anthozoans characterized by similar physical traits and ecological roles. Despite their abundance and their crucial role in benthic communities, the effects of microplastics in coral reef environment have been mostly investigated only for scleractinian (stony) corals, neglecting other benthic reef dwellers. As sessile common organisms with generalist feeding behaviour and worldwide distribution, soft corals and sea anemones present the potential to describe interactions with plastic debris, mirroring on a short term the presence of contaminants (i.e. plastics and plasticizers contamination), shooting the interaction with anthropogenic pollutants in the particular environment where they are. Main goals of this work is to investigate the occurrence and interactions of MPs and associated phthalate esters in overlooked

soft-benthic anthozoans, exploring at the same time the possible use of PAEs as an assessment index of organisms' exposure to MPs. To do this, we suggest the application of BioSPME-LC/MS technique, using soft benthic cnidarians as marker of PAEs presence. This thesis is arranged into 6 chapters. This introduction, which provided an overview of the literature and the aims and motivations of the thesis, is followed by a collection of four papers (three of which already published and one in preparation), which investigate the research topic under very different conditions and with different soft benthic anthozoan species through 3 main steps:

I. At laboratory conditions, the capacity of the soft coral *Coelogorgia palmosa* to interact with MPs was examined through feeding and adhesion tests performed at different microplastics experimental concentrations; physical stress signals and the effects of the MPs exposure on its cellular physiology were evaluated (Chapters 2 & 3)

II. Then, PAEs occurrence and bioconcentration factors were assessed using the BioSPME-LC/MS technique with different soft coral species, all raised in the same microcosm environment, (Chapter 4).

III. Finally, we asked ourselves if PAEs could be useful marker in nature, where MPs occurrence and PAEs levels are lower and variable. For the on-field investigation, sea anemones of the species *Anemonia viridis* (Forsskål, 1775) and *Actinia equina* (Linnaeus, 1758) were proposed as target organisms to measure PAEs concentrations in a Western Mediterranean area (Sinis Peninsula, Sardinia, Italy) and investigate the use of these plasticizers as an assessment index of their exposure to MPs (Chapter 5).

In conclusion, Chapter 6 summarize what has been highlighted in the previous chapters and drawn some general conclusions about the results described. Moreover, future perspectives are highlighted.

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CHAPTER 2

2.1. Soft corals and microplastics interaction: first evidences in the alcyonacean species *Coelogorgia palmosa*

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Abstract

Microplastics pollution differently impacts coral reef systems, by threatening corals physically, through physiological distress and by increasing diseases. However, to date, most of the studies focused on scleractinian corals. The present work reports for the first time the patterns of microplastic ingestion and adhesion by the alcyonacean *Coelogorgia palmosa*. Feeding and adhesion tests were carried out with different concentrations of polyethylene microbeads. Results reported a wide range of surface adhesion, ranging from 3 to 1573 microbeads per coral fragment, suggesting that the adhesion driven by mucus is the main mechanism of microplastic's trap. The 60% of coral fragments ingested polyethylene, and the average value of ingested microbeads was much lower compared to scleractinian corals. Considering the ecological importance of soft corals in coral reef ecosystems, a specific attention about the microplastic pollution effects on this taxon is recommended.

2.2. Introduction

Plastic accounts for 80% of all accumulated ocean litter with an estimated global emission in 2010 to the oceans of 8 million metric tons (Mt) (Jambeck et al., 2015), an amount that has likely exponentially increased since then (Borelle et al., 2020). Lamb et al., (2018) reported that 11.1 billion plastic items were entangled on coral reefs across the Asia-Pacific, estimating that this number will likely increase 40% by 2025.

Plastic wastes gradually break into microscopic fragments (Huang et al., 2021), known as microplastics (< 5 mm in size). Recently, microplastic ingestion by scleractinian corals has been demonstrated and several studies have documented their negative effects on coral health (Allen et al., 2017, Reichert et al., 2018). The microplastics-coral interaction involves ingestion (Allen et al., 2017, Axworthy et al., 2019), egestion (Reichert et al., 2018) and surface adhesion (Martin et al., 2019). Laboratory studies demonstrated that microplastic exposure might adversely influence growth rate, health status and physiology of corals, with consequences for feeding behaviour, photosynthetic performance, skeletal calcification, tissue bleaching and necrosis (reviewed in Huang et al., 2021). Corals respond differently to microplastic stress, depending on the species (Reichert et al., 2018), the plastic size (Syakti et al., 2019) and the presence of microbial biofilm on the plastic (Allen et al., 2017). However, similar studies focused mainly on scleractinian species, while to date non-scleractinian anthozoans have been neglected. Currently, studies on the interaction between non-scleractinian anthozoans and microplastics are circumscribed to the Zoanthids, known as “button polyps” (Anthozoa: Hexacorallia: Zoantharia). In Rocha et al., (2020), *Zoanthus sociatus* showed an elevated sensitivity to polyvinylchloride microplastics, that caused a high epidermis adhesion, lipid peroxidation and antioxidant defences. Moreover, polyvinylchloride (PVC), polyethylene (PE), and polymethylmethacrylate (PMMA) microplastic adhesion and ingestion caused mucus secretion and bleaching in *Protopalythoa sp*. (Anthozoa: Hexacorallia: Zoantharia) (Jiang et al., 2020).

Alcyonaceans (Anthozoa: Octocorallia: Alcyonacea) represent a diverse component of coral reef communities and the second most common group of benthic animals on shallow reefs (Norström et al., 2009). They are fundamental in coral reef communities since they provide food, suitable habitat, shelter for reef dwellers and other services that underpin ecosystem biodiversity (Steinberg et al., 2020).

Coelogorgia palmosa Milne-Edwards & Haime 1857 (Order: Alcyonacea), a common soft coral in different countries of the Indian and West Pacific oceans, was chosen to explore for the first time the interaction mechanisms between microplastics and alcyonaceans in order to investigate their responses to the microplastics presence. In this study microplastic ingestion and adhesion were measured at two different microplastic concentrations and the alcyonacean health status was evaluated monitoring abnormal mucus production and polyp's extension.

2.3. Materials and methods

At the Genoa Aquarium, 13 *Coelogorgia palmosa* fragments of ~10 cm (average number of polyps in each coral fragment was equal to 190 ± 4.5) were collected with pliers from six different random colonies raised in the aquarium tanks. The fragments were promptly fixed on supports made by two-component epoxy resin. Subsequently, they were transported in the experimental tank for 48 h of acclimatation. After the first 24 h of acclimation, each fragment was transferred in single interaction chambers represented by 2L-capacity glass beakers, filled with 1.5 L of filtered seawater of the aquarium water system. Each interaction chamber was equipped with an air pump, to allow the circulation of microplastics and to imitate the motion of particles as occurring in nature (Martin et al., 2019). Chambers were allocated in a water bath aquarium's tank to maintain the temperature of 25 °C. Fragments were randomly assigned to two treatments with different concentrations of polyethylene (PE) fluorescent microbeads 0.98 g cc^{-1}, size range 180-212 µm (Cospheric LLC). Such microplastics size range has been chosen since it is similar to the dimension of the zooplankton provided to the corals

by the Genoa Aquarium (nauplii of *Artemia salina* and *Brachionus rotundiformis*) and it is a size range of microplastics common in other corals-microplastics exposure studies (Huang et al., 2021). PE has been chosen since it is one of the most common types of plastic present in the marine environment (Steinberg et al., 2020) and one of the polymer types most used in similar studies (de Ruijter et al., 2020, Huang et al., 2021). In the first treatment (T1), 0.013 g of microplastics were added in each chamber, corresponding to the concentration of 0.01 g /L. In the second treatment (T2), 0.1 g of microplastics were added in each chamber, corresponding to the concentration of 0.07 g /L. Since no reference studies were present for alcyonaceans, PE concentrations were chosen based on previous experiments on scleractinian and button corals (Hall et al., 2015, Jiang et al., 2020). For each treatment, five *C. palmosa* fragments were exposed in single chambers with microplastics for 48 h. In addition, for each treatment, one chamber with the air pump and the support but without the coral (blank) was set up to evaluate the loss of microplastics in the system. Moreover, three chambers, each one with a coral fragment but without PE, were used as controls to check the coral health status at experimental conditions (Martin et al., 2019). Three water aliquots of 2 ml were collected from each chamber at the beginning of the treatments (0 h) and after 2, 4, 6, 12, 24 and 48 h, in order to evaluate the variation of microplastic concentration through time. Subsequently, they were filtered using a 100 µm nylon mesh and the microbeads were counted under Paralux Stereomicroscope, equipped with Stereo Microscope Fluorescence Adapter with UV light head (NIGHTSEA) kit. At 0, 2, 4, 6, 12, 24 and 48 h, abnormal mucus produced by each fragment and the degree of polyps' extension were noted through visual inspection. We classified as abnormal mucus production the presence of mucus strings streaming off the alcyonaceans, while the surface mucus layer was considered as normal mucus. The degree of polyps' extension was classified as completely introflected (State 1), extroflected with closed tentacles (State 2) and extroflected with open tentacles (State 3). After 48 h of treatment, the microbeads adhesion and ingestion were assessed. Specifically, fragments were removed from their

chambers and accurately rinsed with salt water to count the number of microbeads adhered. Moreover, fragments were inspected under stereomicroscope (Paralux) integrated with UV light kit (NIGHTSEA) to ensure the absence of beads strongly attached to the coral surface. Finally, each coral fragment, controls included, was placed in a petri dish and dissolved in sodium hypochlorite for 2h, to allow the complete digestion of the coral tissue (Martin et al., 2019). We considered microbeads "adhered" when they were found attached to the coral surface, outside the polyps' mouth. We considered microbeads "ingested" when, once inspected under stereomicroscope, they were found inside the polyps' mouths or observed in the petri dishes after the complete dissolution of each *C. palmosa* fragment. Subsequently the solution was observed under stereomicroscope equipped with UV light and a yellow filter to count all microplastics ingested. The Mann-Whitney test was used to evaluate significant differences in microplastics ingestion and adhesion. The chi-squared test of homogeneity was performed to evaluate differences of abnormal mucus production among treatments, while the chi-squared test of independence was performed to evaluate the difference of the polyps' status between treatments. Kendall's tau-b non-parametric correlation test was performed to investigate associations between mucus presence and microplastic adhesion. All statistical analyses were performed in IBM SPSS 27.0 software (IBM Corp, Armonk, NY).

2.4. Results

2.4.1. Microplastic surface adhesion and ingestion

At the end of the treatments, all *Coleogorgia palmosa* fragments showed microbeads stuck to their surface (Figure 1A) and trapped by the produced mucus (Figure 1B). The highest adhesion value of polyethylene beads per coral fragment was observed in T2, with an average value 9 times higher than T1 (Figure 1C). Differences in microplastic adhesion between diverse PE concentrations were not statistically significant (U = 10, z

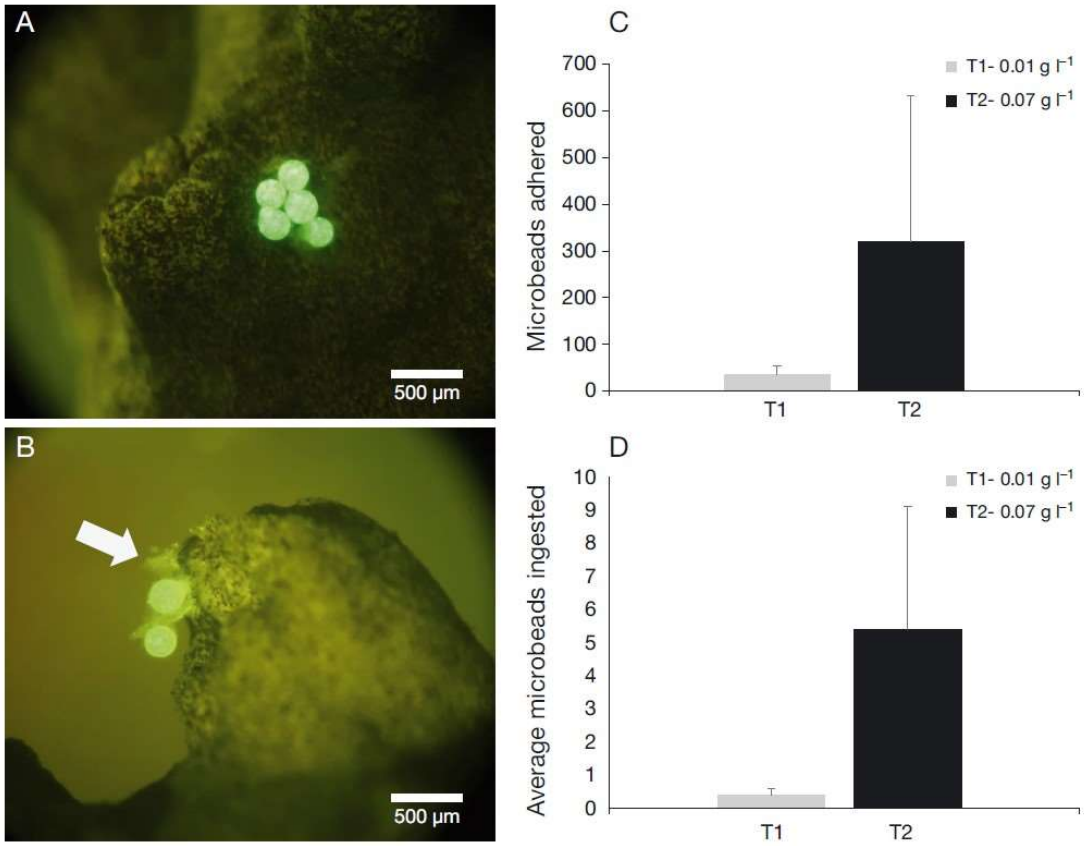

Figure 1. Microplastic interactions with *Coelogorgia palmosa*: A) adhered polyethylene (PE) beads on the coral surface next to a polyp mouth, B) PE beads trapped by coral mucus: the white arrow shows abnormal mucus coming from a coral polyp; C) adhered plastic (average n° of PE beads per coral fragment) in corals exposed to the treatments T1 and T2; D) ingested plastic (average n° of PE beads per coral fragment) in corals in T1 and T2. In panels C and D, bars indicate the Standard Error.

= 0.584, p = 0.686). By contrast, both T1 and T2 showed a statistically significant strong positive correlation between abnormal mucus presence and adhered microplastic number (Kendall's tau-b correlation test, τb = 0.550, p = 0.016).

Coral polyps ingested microplastics in both treatments. *C. palmosa* in T2 reported the highest values of ingested PE beads per coral fragment (Figure 1D) but no statistically significant differences in microplastic ingestion between the treatments were detected (U = 4.5, z = - 1.433, p = 0.190). Under the fluorescent stereomicroscope, most of the ingested microplastics were found inside polyps' mouth (Figure 2), while others entered

the coral tissue. No leaching of the fluorescent dye was noted. No PE microbeads were found in the control fragments.

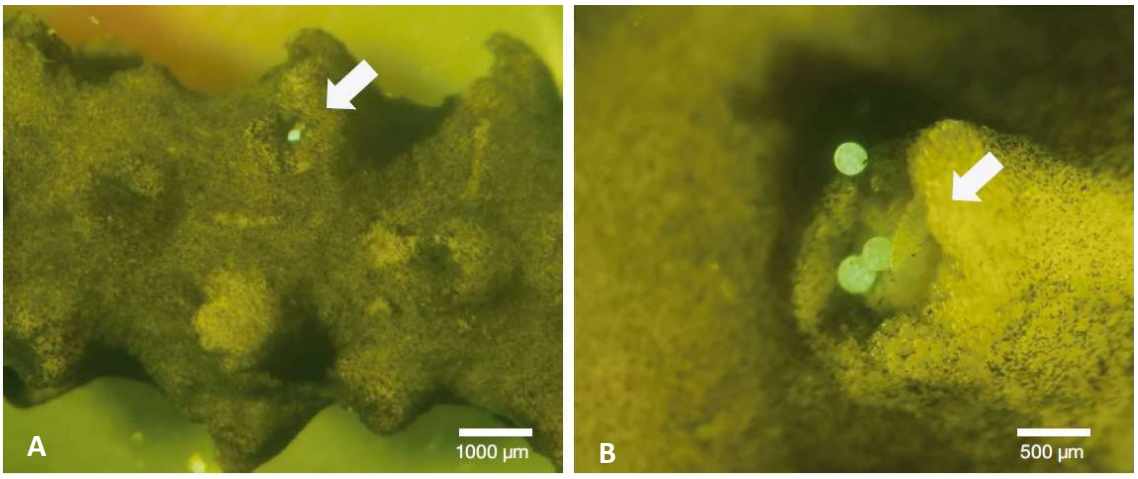

Figure 2. Polyethylene (PE) ingestion by *Coelogorgia palmosa*: A) a PE bead inside the polyp's mouth; B) beads trapped inside a polyp: the white arrow shows a polyp tentacle interacting with a bead.

2.4.2. Mucus production and polyp's extension

In both treatments, *Coelogorgia palmosa* fragments showed evidence of stress by an abnormal mucus production and the shrinkage of tentacles. During the experiment, 57% of the treated fragments had their polyps in State 2, while most of control fragments (64%) presented polyps in the healthier State 3 and never presented polyps in State 1 (Figure 3A). After 2 h of microplastics exposure, 40% of fragments already presented extra mucus filaments that remained through all the exposure time (Figure 3B). At the beginning there is an initial strong impact of the MPs on the coral fragments in both the treatments, followed by a slight decline within the next 6 h and a peak in the abnormal mucus presence (60% of coral fragments in both T1 and T2) around 12 h after the start of the experiment (Figure 3B). By contrast, control fragments did not show any abnormal mucus production. No statistically significant differences in the abnormal mucus occurrence between treatments (X^2_1= 0.583, $p > 0.05$, N=70) according to the diverse microplastic concentrations, were observed. However, when comparing T1 and T2 with

the control, statistically significant differences both in the abnormal mucus presence were found (X^2_1= 9.234, p < 0.05, N=91). The post-hoc test (z-test of two proportions) confirmed significant differences between each treatment and the control (X^2_1= 9.234, p < 0.05, N=91). Regarding the differences between the status of polyps, the chi-squared test of independence showed no statistically significant differences between T1 and T2 nor with the control (X^2_4= 0.230, p > 0.05, N=91).

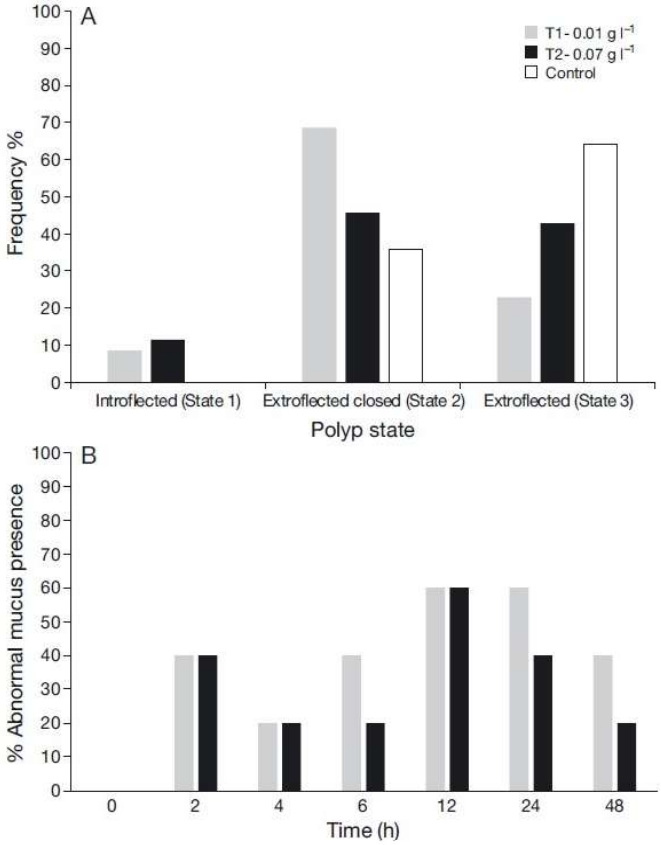

Figure 3. (A) Frequency of *Coelogorgia palmosa* polyps state during Treatment 1, Treatment 2 and in Control fragments after 48h of experiment conditions; (B) Frequency of the Abnormal Mucus Presence in *C. palmosa* fragments during Treatment 1, Treatment 2 and Control over the duration of the microplastics' exposure.

2.5. Discussion

Our results reported for the first time the microplastic ingestion and adhesion patterns of an alcyonacean. *Coleogorgia palmosa* control fragments did not exhibit signs of stress, while fragments exposed to microplastics showed a quick abnormal mucus production that generally persisted during the interaction time in all treatments. Moreover, they displayed a polyp contraction, with polyps that mostly remained extroflected but closed. Jiang et al., (2020) reported similar responses for the button coral Protopalythoa sp. interacting with microplastics at 0.05 mg/L. Our results showed that alcyonaceans can ingest microplastics, as already observed in scleractinian corals (Hall et al., 2015, Allen et al., 2017) and button corals (Jiang et al., 2020, Rocha et al., 2020). Conversely to other studies (Martin et al., 2019, Jiang et al., 2020), in this work we found a small number of microplastics ingested and not correlated to the microplastic concentration in the water. About the number of ingested microplastics, similar results were described by Rocha et al., (2020), where the average ingestion was equal to 1.0 ± 0.8 microbeads/coral, at polyethylene concentration 10 mg/L. The authors proposed that the low levels of microplastics observed in *Zoanthus sociatus* gut were due to low ingestion of these particles caused by a potential low heterotrophy need of *Z. sociatus* in short-term exposure. This hypothesis could be valid also for our observations, as *C. palmosa* is a zooxanthellate alcyonacean and it relies for energy and carbon source from zooxanthellae photosynthesis. However, it is even possible that, as already reported by Martin et al., (2019), the mucus occurrence here acted like a microplastic trap. In this case, microplastics on the alcyonacean surface may produce an involucre that might bury the polyps, preventing polyp's extension and external particles capture (Reichert et al., 2018). Since abnormal mucus production and polyp status resulted to be similar among treatments, this may suggest that the occurrence and intensity of these coral responses do not depend, as expected, on our microplastic concentrations, but it might depend on the time of interaction between *C. palmosa* and

microplastics. To date, microplastics are ubiquitous in marine environments, yet only limited coral reef regions have been investigated. Microplastic abundance in the surface water of coral reefs generally ranges from zero to tens of thousands of items/m^3, while in sediments and corals it is difficult to quantify due to lack of a relatively standardized unit or enough available data (Huang et al., 2021). At the present environmental microplastic concentrations it is possible to miss or underestimate an organism response resulting from the interaction with microplastics (Cunnigham & Sigwart 2019, Opitz et al., 2021). Therefore, when we set our experimental concentrations, we adopted a higher microplastic concentration range with respect to the environmental one. Still, our microplastic concentrations are similar to the ones of other microplastic-corals feeding trials, in order to observe reliable responses of these overlooked organisms to microplastic presence, maintaining the possibility to compare their responses with other peer-reviewed results. Moreover, it should be highlighted that temporary very high concentrations of microplastics in seawater have been recorded in the past, especially close to coastal areas, and also according to our results these contaminants can impact benthic fauna in a short time exposure (Sun et al., 2018, Everaert et al., 2020).

However, additional studies on realistic environmental microplastic concentrations and long-term exposure are required to get a clearer picture of the microplastic effects on alcyonaceans. Moreover, in order to better evaluate the intensity of the coral stress caused by the interaction with microplastic, it might be interesting also to assess quantitatively the abnormal mucus production. Recently, adhesion has been recognized as one of the dominant interaction mechanisms between microplastics and scleractinian corals, responsible for removing microplastics from the water column (Martin et al., 2019, Corona et al., 2020). Our results extend this hypothesis to soft corals. At 48 h of exposure, all fragments had similar numbers of polyethylene beads adhered to the surface, regardless of the microplastics concentration. This suggests that, in nature, the adhesion may not depend only on the microplastic concentration, but on the mucus production. Indeed, polyethylene beads attached to *C. palmosa* were mostly glued to

the mucus filaments produced by the stressed polyps. The positive correlation between mucus production and the number of microplastics adhered highlights the concept that the more *C. palmosa* creates mucus, the more microplastic will stick on its surface. Since corals produce mucus when subjected to stress (Brown et al., 2005), factors that induce the production of abnormal mucus may enhance the adhesion of random plastic present in the water column, promoting the adhesion and adding plastic pollution to every other coral stressing factor.

2.6. Conclusion

Alcyonaceans provide fundamental services to coral ecosystems (Steinberg et al., 2020), acquiring greater importance in the reefs of the future, since transitions from scleractinian-dominated to non-scleractinian dominated reefs have been already suggested (Bradbury & Mundy 1983, Bryce et al., 2018). Although conditions (PE shape and concentrations) and responses described here may not be representative of present natural reef environments, they may become more relevant in time, due to microplastic concentrations increase in the wild as the result of the ongoing input compounded with the further fragmentation of larger plastic debris (Cunnigham & Sigwart 2019). This study reports for the first time that soft corals are able to ingest microplastics, with results that provide an important first demonstration on how microplastics can have negative effects on soft coral species too. Relying on our observations, both laboratory experiments and in-situ studies could be carried on to assess on a finest scale possible different or similar reaction of soft corals to microplastic interactions. This might expand the research interest on these overlooked organisms, leading to a better understanding of resilience capacities in coral reef ecosystems affected by the increasing plastic pollution in the marine environment.

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Chapter 3

CHAPTER 3

3.1. Short-term microplastic exposure triggers cellular damage through oxidative stress in the soft coral *Coelogorgia palmosa*

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Abstract

Microplastics are a persistent and ubiquitous source of pollution in the marine environment, representing a severe threat to tropical coral reefs. The effects of microplastics on reef building (stony) corals have been documented (interference with normal digestion process, polyp retraction, oxidative stress, impairment of the photosynthetic machinery, bleaching). However, the impact of microplastics on soft corals, the second most abundant benthos of tropical reefs, remains to be thoroughly studied. In this work, we analysed the effects of a short-term microplastic exposure on the cellular physiology of the soft coral *Coelogorgia palmosa*. We found that samples exposed to >50 mg/l of microplastic showed significant increase in the activities of the antioxidant enzymes glutathione reductase, catalase, and superoxide dismutase, suggesting a rise in oxidative stress. Furthermore, exposure to microplastics increased lipid peroxidation, indicating oxidative damage. Overall, our results show that similar to stony corals, microplastic ingestion causes oxidative stress and cellular damage in soft corals. Our study provides a first assessment of physiological effects of microplastic exposure on the soft coral, *Coelogorgia palmosa*, highlighting the need for further investigations about these contaminants and their influence on marine benthic fauna. Such information is crucial to understand how different reef organisms respond to microplastic pollution and who the ecological winners or losers will be in an increasingly polluted marine environment.

3.2. Introduction

Microplastics are plastic fragments between 1 µm to 5 mm (Frias and Nash 2019) and are widespread contaminants in marine ecosystems worldwide. Increasing waste production and mismanagement, together with low biodegradation rates and the consequent long persistence in the water, contribute to a continuous accumulation of plastic polymers in the ocean (Moore 2008; Green et al., 2018). Marine microplastics are mainly generated by fragmentation of larger debris that enters the marine environment from terrestrial sources (Thompson et al., 2004). Fragile coastal ecosystems such as coral reefs are heavily impacted by this source of pollution (Fendall and Sewell 2009; Browne et al., 2010). In addition, coral reefs are often popular touristic locations and commercially important sites for fishing vessels and other recreational activities, making them particularly vulnerable to plastic contamination (Claessens et al., 2011; Hall et al., 2015). Microplastics impact both the biotic and abiotic components of marine ecosystems and affect marine life at different levels (Rice and Gold 1984; Teuten et al., 2007; Egbeocha et al., 2018). In particular, the marine benthic environment is becoming a reservoir of plastic microparticles (Egbeocha et al., 2018). Microplastics sink to the bottom as a consequence of different phenomena, such as microorganisms mediated fouling or through faecal pellets of zooplankton and other organisms that readily ingest and egest the micro-debris (Cole et al., 2016). Ingestion represents the most common way through which marine benthic organisms interact with microplastics (Lusher et al., 2016). For example, different species of scleractinian corals can actively, through ingestion, and passively, through adhesion, retain microplastics, even if they are fed with a natural food source at the same time (Hall et al., 2015; Martin et al., 2019). During ingestion experiments, debris is usually found deeply within the coral polyp, wrapped by mesenterial tissue, possibly interfering with food digestion (Goldberg 2002; Hall et al., 2015). The interaction of corals with microplastics hampers their health, causing for instance

tissue necrosis and bleaching (Reichert et al., 2018). In addition to mechanical damage to coral tissues, plastic ingestion could influence coral physiology by acting as a source of chemical contaminants (Browne et al., 2008; Balbi et al., 2017). For example, chemical substances leaching from polystyrene (PS) debris have been demonstrated to cause a significant polyp retraction in nubbins of the scleractinian coral *Stylophora pistillata* (Aminot et al., 2020). At the cellular level, short-term exposures to microplastics have been observed to cause a rise in the oxidative stress of the organism, and to impair photosynthetic rates, through species-specific and microplastic-specific processes (Tang et al., 2018; Mendrik et al., 2021). Furthermore, high concentrations of microplastics have been shown to disturb the initiation of symbiotic relationships between corals and dinoflagellates, possibly affecting the ability of the cnidarian host to adapt to future environmental changes (LaJeunesse et al., 2018; Okubo et al., 2018). The effect of microplastics has been mostly investigated in scleractinian (stony) corals. However, the impact of microplastics on other benthic reef dwellers, such as soft corals have stonyly been documented. Soft corals (octocorals) are key organisms of the coral reef benthos, ranking second in abundance after stony corals (Alderslade and Fabricius 2019; Garra et al., 2020). They usually cover 2–25% of the substratum, but in some locations they are dominant, covering more than 80% of the available substrate (Fabricius 1997; Fabricius and Alderslade 2001). Together with stony corals, soft corals play a crucial role in coral reef communities, they create three-dimensional structures that provide suitable habitat and shelter for other organisms, contributing to increase the total reef biodiversity (Goh et al., 1999; Lau et al., 2019; Maggioni et al., 2020). Soft corals lack a carbonate-protecting skeleton and are sessile organisms exposed to changes in their surrounding environmental conditions. They must rely solely on cellular and molecular mechanisms as a first line of defence against abiotic or biotic stressors (Kültz 2005; Mydlarz et al., 2010). The biochemical cellular homeostasis is fundamental for the ecological role of the animal. Given the ability of microplastic to impair different cellular processes and possibly

generate oxidative stress and damage, through the production of reactive oxygen species (ROS), soft corals can be particularly affected by this source of contamination (Wright et al., 2013; Galloway et al., 2017). The effect of microplastic on soft corals at cellular and molecular levels remain to be thoroughly studied. Overall, understanding how microplastic affect this important benthic group is crucial to understand how different reef organisms respond to microplastic pollution and who will be the ecological winners and losers of an increasingly microplastic-polluted marine environment that is likely to prevail in the future. Hence, in this study the cellular response of the soft coral *Coelogorgia palmosa* to an exposure to different concentrations of microplastic was investigated. The present study provides a first assessment of microplastic pollution impact on soft coral *C. palmosa*. In particular, the oxidative status of cells was evaluated through the analysis of antioxidant enzymes involved in ROS detoxification, and the cellular oxidative damage through the analysis of cellular lipid peroxidation. Moreover, the impact of microplastics on cellular protein homeostasis was also investigated, through the analysis of heat shock proteins (Hsps) and in particular of the mitochondrial Hsp60.

3.3. Materials and methods

3.3.1. Coral acclimatization and experimental plan

Experiments were performed with 20 fragments (~10 cm) of *C. palmosa* (Octocorallia: Alcyonacea) obtained from four large mother colonies raised in Acquario di Genova tanks. Prior to treatments, fragments were acclimatized under controlled conditions in a 400 litre tank (water turnover time 120 l/h) for seven days. Colonies were fed twice a week with a food mixture developed at Acquario di Genova, containing *Tetraselmis* algae and Rotifera. The tank was supplied with filtered seawater pumped from 50 m depth and temperature was set to 25°C through a single heater (NEWA therm, 300 W). Corals were illuminated by two 96 W metal halide lamps (Sylvania, Domilux) at an

irradiance of 170 ± 10 µmol photons m^{-2} s^{-1} (photoperiod was 10 h:14 h, light:dark). Following acclimatization, each fragment was moved into a separate 2 l interaction tank containing water from the acclimatization tank for a further acclimatization period of 24 h at the same conditions described above. Chemical and physical parameters (pH, ammonium, nitrite, nitrate, temperature, salinity) within these tanks were regularly checked. Polyethylene (PE) fluorescent microbeads (density 0.98 g/cc; size range 180–212 µm) were purchased from Cospheric LLC, Santa Barbara, CA, USA and used to expose five randomly selected coral fragments to different microplastic concentration treatments: control treatment without microplastic; treatment 1 (T1) with a PE concentration of 10 mg/L; treatment 2 (T2) with a PE concentration of 50 mg/L; and treatment 3 (T3) with a PE concentration of 70 mg/L. Polyethylene was chosen since it is one of the most common types of microplastic present in the marine environment (Vencato et al., 2021) and mostly used in microplastic-coral interaction studies (Martin et al., 2019). In each interaction tank, gentle water aeration was achieved through air pumps to maintain homogeneous microplastic circulation and to avoid aggregation of beads. Corals were exposed to microplastic enrichment for a period of 48 h. This time exposure to such PE concentrations has been reported to be sufficient for the adhesion and ingestion of microplastic beads by *C. palmosa* (Vencato et al., 2021). Water samples (2 mL) were collected from each interaction tank after 2, 4, 6, 12, 24 and 48 h, in order to check that concentrations of microplastic were constant over time. This was achieved by filtering water samples using a 100 µm nylon mesh and by counting microbeads with a stereo microscope (Paralux), equipped with Stereo Microscope Fluorescence Adapter with UV light head kit (NIGHTSEA). At the end of the experiment, coral fragments from each of the four conditions described above, were collected and immediately frozen to −80°C for further analysis.

3.3.2. Analysis of the antioxidant enzymatic activities

3.3.2.1. Protein extraction

Coral fragments were grinded using a pre-chilled mortar and pestle and homogenized in 750 µl lysis buffer (TrisHCl 50 mM, pH 7.4, NaCl 150 mM, glycerol 10%, NP40 detergent 1%, EDTA 5 mM) containing 1mM phenylmethylsulfonylfluoride (Sigma-Aldrich). After a first centrifugation step (5 min, 3000 rpm) to remove skeletal components, cells were broken by sonication (6×10 s pulse on ice, amplitude 10 µm, Soniprep 150, Sanyo). Samples were then subjected to a second centrifugation step (15 min, 14,000 rpm, 4°C) and the supernatant was immediately frozen (−80°C) until subsequent assays. Total protein content of each sample was determined through Bradford method using bovine serum albumin (BSA) as calibration curve.

3.3.2.2. Glutathione reductase activity assay

The enzymatic assay of glutathione reductase (GR) was performed according to Wang et al., (2001). The activity of GR was evaluated through the spectrophotometric detection of the absorbance at 340 nm (Varian Cary 50 Scan spectrophotometer, Agilent Technologies) of NADPH oxidation to NADP+ reaction, which occurs in conjunction with the glutathione reduction, and is proportional to the decrease in absorbance over time. NADPH reaction was initially measured in the reaction mix (containing 0.1 M potassium phosphate buffer pH 7.6, 0.16 mM NADPH, 1 mg m/L BSA and 4.6 mM oxidized glutathione), and subsequently adding different volumes of sample. GR activity was obtained from the difference of the two absorbance values. One unit of GR activity is defined as the oxidation of 1 nmol NADPH/min at 25°C. Results are expressed as units (U) of enzyme per mg of proteins.

3.3.2.3. Catalase activity assay

Catalase (CAT) activity was assessed by considering the peroxidative function of the enzyme. The method is based on the degradation of hydrogen peroxide (H_2O_2) by the

enzyme, as previously described in Bergmeyer and Grassl (1983). The reaction solution (containing 50 mM sodium phosphate buffer pH 7.5, 12 mM H_2O_2) was mixed in a 1 ml cuvette with different volumes of sample, and the decrease of H_2O_2 was followed spectrophotometrically at 240 nm (Varian Cary 50 Scan spectrophotometer, Agilent Technologies). Results are expressed as units (U) of enzyme per mg of proteins, and in this case, U refers to k, the first order kinetic constant (min^{-1}), as previously described (Aebi 1984).

3.3.2.4. Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was assessed according to Vance et al., (1972). As SOD competes with ferricytochrome c for oxygen radicals, its activity was detected as the ability to inhibit the reduction of ferricytochrome c by O_2- generated from the xanthine/xanthine oxidase system. For the reaction mix, the following reagents (purchased from Sigma-Aldrich), ferricytochrome c 0.01 mM, EDTA 0.1 mM, xanthine 0.01 mM and xanthine oxidase 0.0061 U were used in a final volume of 1 ml. Different volumes of each sample were tested and added to the reaction mix to determine the 50% inhibition of the reaction rate. The rate of reduction of ferricytochrome c was followed spectrophotometrically at 550 nm, 25°C, through a Varian Cary 50 Scan Spectrophotometer (Agilent Technologies). Under the above conditions, one unit of SOD was defined as the amount of enzyme inhibiting the reduction of ferricytochrome c by 50%. Results are expressed as units (U) of enzyme per mg of proteins.

3.3.2.5. Lipid peroxidation

Lipid peroxidation levels were assessed via malondialdehyde (MDA) contents determined using an MDA assay kit (Bioxytech LPO-586, Oxis International, USA). The method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C. Specifically, frozen coral apexes (approximately 1 g each) were ground with a pre-chilled mortar and pestle and homogenized in 1 ml of 20 mM

phosphate buffer, pH 7.4. To prevent sample oxidation, 10 µl 0.5 M butylated hydroxytoluene in acetonitrile was added to 1 ml of tissue homogenate. Following sample centrifugation (3000×g at 4°C for 10 min), an aliquot of supernatant was used for protein determination using Bradford method. The subsequent assay procedure (hydrochloric acid solvent procedure) was performed according to the manufacturer's instructions. The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998). Results are presented in µmol of MDA per µg of proteins.

3.3.2.6. Analysis of Hsp60 expression

3.3.2.6.1 Total protein extract preparation and Western Analyses

Hsp60 was selected as a biomarker to detect cellular stress after PE exposure as it has been shown to be involved in the maintenance of protein homeostasis under several different stress conditions, such as thermal, osmotic and physical stress (Chow et al., 2012; Seveso et al., 2013, 2014). Frozen coral fragments were ground with a pre-chilled mortar and pestle and homogenized in SDS-buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol) containing 1 mM phenylmethylsulfonylfluoride (Sigma-Aldrich), and complete EDTA-free protease inhibitors cocktail (Roche Diagnostic). Extracts were stored at −80°C until further processing. Aliquots were used to determine total protein concentrations through Bradford method using bovine serum albumin (BSA) as reference to build a calibration curve. An equal amount of proteins for each sample was separated by SDS-PAGE on 8% polyacrylamide gels (Vai et al., 1986), then run in duplicate using a Mini-Protean Tetra Cell (Bio-Rad Laboratories). After the electrophoresis, one gel was stained with Coomassie brilliant blue to visualize total proteins, while the other was electroblotted onto nitrocellulose membrane (Amersham Protran 0.45 mm) for Western blot analysis as previously described (Seveso et al., 2012). Filters were stained with Ponceau S Red (Sigma-Aldrich) to confirm correct protein transfer. The following primary antibodies were used: anti-Hsp60 monoclonal antibody (IgGI1 mouse clone LK-2, SPA-807, Enzo Life

Sciences) and anti-β-Actin monoclonal antibody (IgG1k mouse clone C4, MAB1501, Millipore). The primary antibodies were diluted as follows: 1:1000 in TBS-0.1% Tween 20 and 5% skimmed milk for Hsp60, and 1:3000 in the same solution for β-Actin, following Seveso et al., (2012). After being washed three times with fresh changes of TBS-0.1% Tween 20 (15 min each), filters were incubated with anti-mouse IgG polyclonal secondary antibodies conjugated with horseradish peroxidase (ADI-SAB-100, Enzo Life Sciences), diluted 1:10,000 for Hsp60 and 1:15,000 for β–Actin in TBS-0.3% Tween 20 and 5% skimmed milk. Western blots were developed using Pierce ECL Western Blotting Substrate followed by exposure of filters to Amersham Hyperfilm ECL.

3.3.2.6.2. Densitometric analysis

Densitometric analysis was performed as previously described by Seveso et al., (2013). Films were scanned on a Bio-Rad GS-800 calibrated imaging densitometer and the pixel density of the scanned bands were quantified with ImageJ free software (http://rsb.info.nih.gov/ij/) of the NIH Image software package (National Institutes of Health, Bethesda, MD). For each blot, the scanned intensity of the bands of Hsp60 was normalized against the intensity of the β-Actin ones, which was used as internal loading control since in all our experiments the β-Actin level did not display significant changes. The densitometric data were expressed as relative levels (arbitrary units).

3.3.2.7. Data analysis

Data normality was verified using Shapiro–Wilk test and where assumptions were violated, the data were corrected by transformations. To evaluate significant differences in antioxidant enzyme activities, MDA levels, and Hsp60 expression at different microplastic concentrations, separated one-way ANOVAs followed by Tukey's HSD post hoc tests were used. Analyses were performed using SPSS ver. 27 (IBM). Values were considered statistically significant at $P < 0.05$, and all data are presented as arithmetic means ± SE ($n = 5$, for each biomarker analysed), unless otherwise stated. A multivariate

analysis was performed using the statistical package PRIMER-E v.7 (Clarke and Gorley 2015) with the PERMANOVA+ add-on (Anderson et al., 2008) to investigate the modulation of all analysed biomarkers in different microplastic concentration treatments. In particular, data related to the levels of all the biomarkers were normalized and square root transformed to calculate a matrix based on the Bray–Curtis similarity. To test for differences in biomarker levels among treatments, a non-parametric permutational multivariate analysis of variance (PERMANOVA) was performed using 999 permutations with partial sum of squares and unrestricted permutation of raw data. Different microplastic concentrations were selected as fixed factors. Values were considered statistically significant at $P < 0.05$. Due to the restricted number of unique permutations in the pairwise tests, P-values were also obtained from Monte Carlo samplings (Anderson and Robinson 2003). To visualize similarities among responses of corals in different treatments a non-metric multidimensional scaling plot (nMDS) was done using Bray–Curtis similarity.

3.4. Results

3.4.1. Antioxidant enzyme activities

The antioxidant activity of GR, CAT and SOD was assessed in samples exposed to different concentrations of microplastic. Significant differences were found in GR activity among different treatments (one-way ANOVA, $F(3,12) = 6.702$, $P = 0.007$). *C. palmosa* samples exposed to microplastic concentration of 70 mg/L (Treatment 3) showed a GR activity significantly higher than corals in Control tank and corals exposed to 10 mg/L (Treatment 1). Similarly, GR activity of samples exposed to 70 mg /L of microplastic was higher than corals exposed to 50 mg/L of microplastic (Treatment 2), though not statistically significant (Figure 1A). Significant differences in CAT activity were detected in specimens exposed to microplastic concentration of 70 mg/L (Treatment 3). These results were significantly higher compared with corals exposed to other

treatments (one-way ANOVA, F(3,12) = 21.321, P < 0.000, Figure 1B). Considering SOD activity, significant differences were found among different treatments (one-way ANOVA, F(3,12) = 17.841, P < 0.000). Coral samples exposed to microplastic

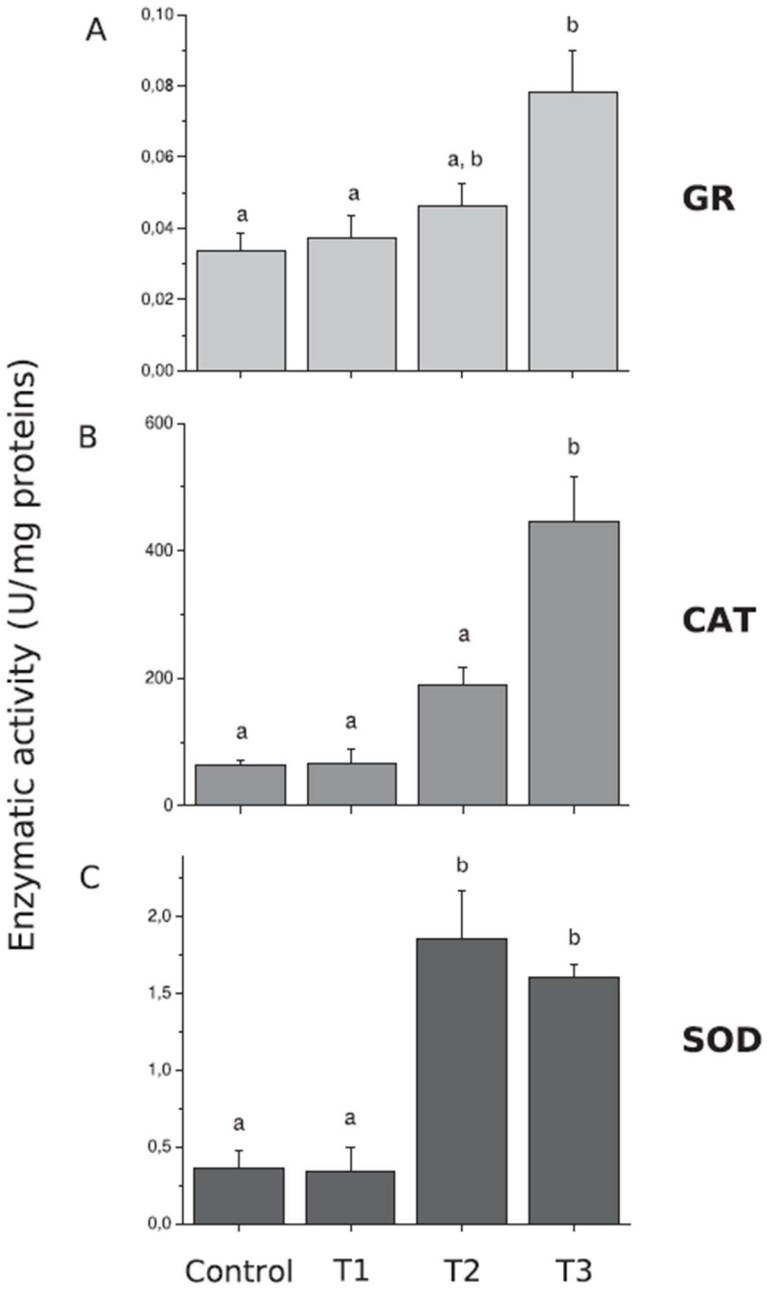

Figure 1. Enzymatic activity of GR (A), CAT (B) and SOD (C) detected in samples of C*. palmosa* exposed to different microplastic concentration treatments (Control; T1; T2; T3) after 48 h. Lower case letters indicate significant differences between corals maintained under different treatments.

concentration of 50 mg/L (Treatment 2) and 70 mg/L (Treatment 3) respectively, showed an enzymatic activity significantly higher than other treatments. The highest levels of SOD activity were recorded in corals exposed to Treatment 2 (Figure 1C).

3.4.2. Lipid peroxidation

Oxidative damage in *C. palmosa* samples exposed to different concentrations of microplastic was evaluated by analysing the lipid peroxidation levels through MDA production. Levels of MDA were significantly different among treatments (one-way ANOVA, $F(3,12) = 11.131$, $P = 0.001$, Figure 2). Samples exposed to the high microplastic concentrations, 50 mg/L and 70 mg/L respectively, showed significantly higher levels of MDA compared with samples subject to control condition and lower microplastic concentration of 10 mg/L (Treatment 1). Samples exposed to 70 mg/L of microplastic had the highest levels of MDA.

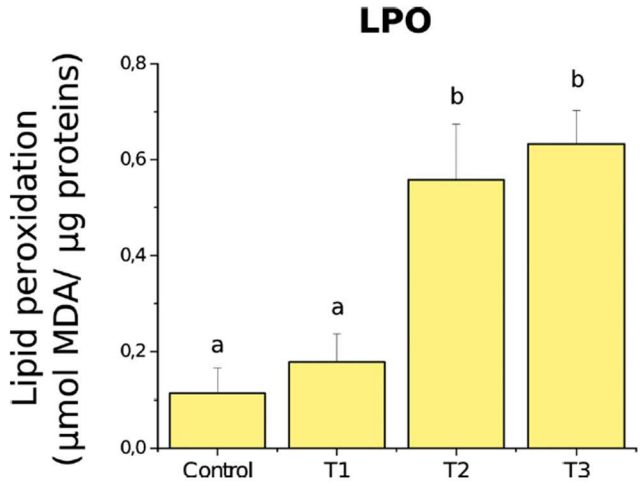

Figure 2. Levels of lipid peroxidation (LPO) detected in samples of *C. palmosa* exposed to different microplastic concentration treatments (Control; T1; T2; T3) after 48 h. The values related to MP concentrations for each treatment are reported in 'Material and Methods'. Data are expressed as µmol of MDA per µg of proteins and as mean ± SEM (n = 5). Lower case letters indicate significant differences between corals maintained under different treatments.

3.4.3. Hsp60 expression

In all samples, the monoclonal antibody anti-Hsp60 produced a single band (Supplementary Figure 1), whose molecular weight corresponded to 60 kD, as expected on the basis of the amino acid sequences (Seveso et al., 2020; Montalbetti et al., 2021). Hsp60 expression was higher in samples exposed to the lowest concentration of microplastic (Treatment 1), compared with control, Treatment 2 and Treatment 3. Samples exposed to microplastic treatments had higher expression of Hsp60 compared with samples in control treatment (Figure 3). However, no significant differences were found among different treatments and Control condition (one-way ANOVA, $F(3,8) = 0.672$, $P > 0.05$).

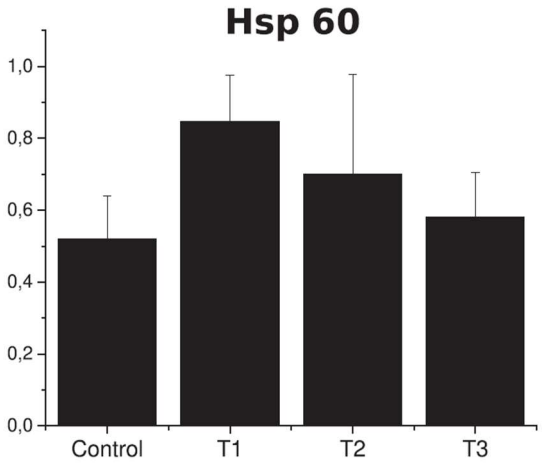

Figure 3. Levels of Hsp60 detected in samples of *C. palmosa* exposed to different MP concentration treatmnts (Control; T1; T2; T3) after 48 h. The values were determined by densitometric analysis as described under "Materials and methods". In the same section, the values related to microplastic concentrations for each treatment are also shown. Data are expressed as arbitrary units and as mean ± SEM (n = 4). For each biomarker, lower case letters indicate significant differences between corals maintained under different treatments.

3.4.4. Multivariate analysis

Multivariate analyses were used to investigate associations between enzymatic activities, cellular damage and Hsp60 expression. The PERMANOVA revealed significant differences in these biomarker levels among the different microplastic concentration treatments (Table IA). The biomarker levels in corals exposed to high microplastic concentrations, 50 mg/L (Treatment 2) and 70 mg/L (Treatment 3), were significantly different compared with those measured in corals from Control group and corals subject to low microplastic concentration (10 mg/L, Treatment 1) (Table IB). Similarly, the nMDS analysis showed that corals in Control group and corals subject to low microplastic concentration (10 mg/L, Treatment 1) had a similar cellular response that was different from the cellular responses of corals exposed to higher concentrations of microplastic (Treatment 2 and Treatment 3) (Figure 4).

Table 1. A) Results of the PERMANOVA testing the effects of different treatments (Control, T1, T2, T3) on the levels of GR, SOD, CAT, LPO, and Hsp60 obtained by permutations (perms) for each group. B) Results of the PERMANOVA pairwise comparisons among treatments obtained by permutations (perms) for each group. Significant P-values, both P (perms) and Monte Carlo P (MC), are in bold.

A. Source of variation	df	SS	MS	F	*P* (perms)	perms
Treatment	3	5154	1719.3	9.6426	**0.001**	999
Error	12	2139.7	178.31			
Total	15	7297.7				
B.	Groups	t	*P* (perms)	perms	*P* (MC)	
	Control vs. T1	1.1912	0.371	35	0.2955	
	Control vs. T2	3.274	**0.017**	35	**0.007**	
	Control vs. T3	4.4191	**0.013**	35	**0.002**	
	T1 vs. T2	2.7496	**0.029**	35	**0.014**	
	T1 vs. T3	3.8498	**0.027**	35	**0.003**	
	T2 vs. T3	1.7204	**0.035**	35	0.068	

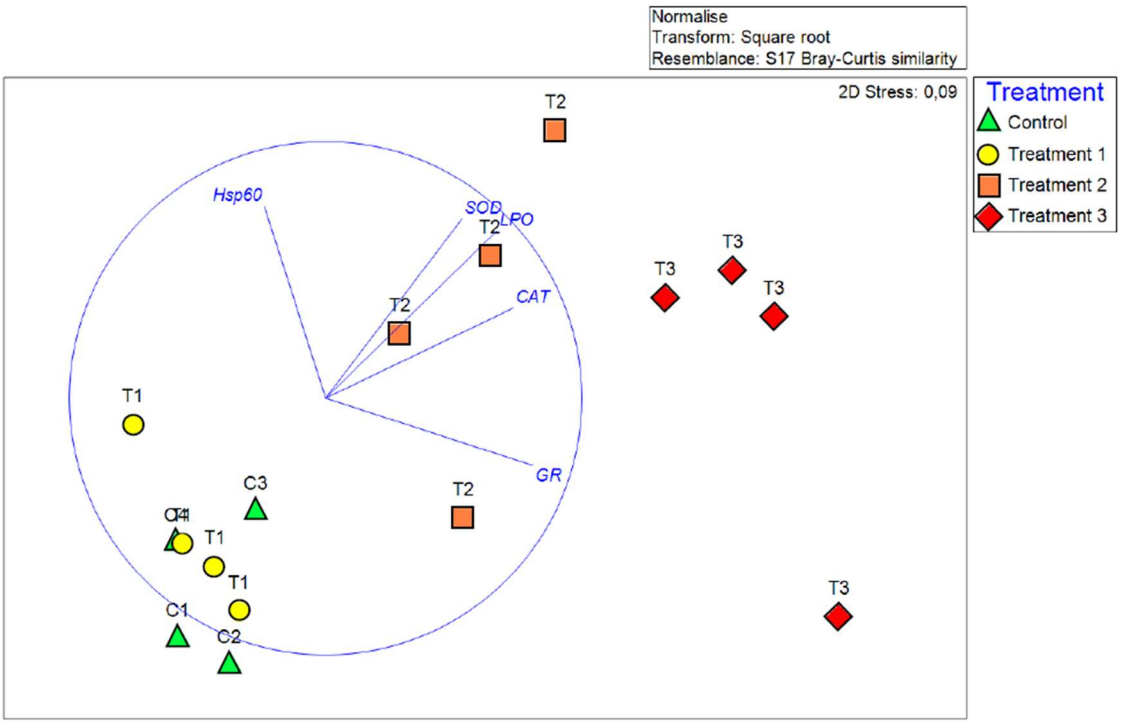

Figure 4. Non-metric multidimensional scaling plot (nMDS) showing the response pattern of all the *C. palmosa* biomarkers analysed by different treatments, with vectors (Pearson's correlations ≥ 0.8) representing the variables (biomarkers) driving significant similarities among experimental conditions.

3.5. Discussions

As coral reefs are currently subject to a growing number of anthropogenic stressors, it becomes particularly important to elucidate the effect of microplastic on the physiology of organisms inhabiting these ecosystems, in particular of those less studied groups, such as soft corals. In this study, we observed that microplastic contamination in soft corals can generate oxidative stress, cellular damage and possibly suppress protein homeostasis defensive mechanisms. The concentrations of microplastic used in this study were similar and even lower than those usually adopted in tank experiments, although these are still higher than those generally found in the marine environment (Lenz et al., 2016; Cunningham and Sigwart 2019; Martin et al., 2019; Cappello et al.,

2021). However, it should be highlighted that temporary very high concentrations of microplastic in seawater have been recorded in the past, especially close to coastal areas, and also according to our results these contaminants can impact benthic fauna in a short time exposure (Sun et al., 2018; Desforges et al., 2019). An increase of antioxidant enzymatic activity with exposure to increasing microplastic concentrations was recorded for all the enzymes analysed. The enzyme glutathione reductase is involved in maintaining and restoring the balance between oxidized and reduced forms of glutathione, a tripeptide able to react with different reactive oxygen species (ROS) forming a disulphide bond with another oxidized glutathione (Lesser 2006; Krueger et al., 2015). In our study, GR activity increased significantly after 48 h exposure to a concentration of 70 mg of microplastic. As a crucial phase II metabolic molecule involved in the detoxification process of all eukaryotes, glutathione in its reduced form plays an important role in detoxification from xenobiotics and unfavourable chemicals (Nicosia et al., 2014). Similarly, with the increase in GR activity following exposure to increasing microplastic concentrations, our results suggest that GR could be involved in the detoxication process of microplastic contamination. However, the effects of short-term exposure to microplastic on glutathione oxidation in benthic marine organisms are still not clear. Some studies also reported a decrease in GR following exposure to microplastic. For example, in the sludge worm (*Tubifex tubifex*) no significant differences were recorded in GR and glutathione peroxidase (GPoX) activities between samples exposed to a concentration of microplastic of 2 g/L and control samples after 24 h (Scopetani et al., 2020). Moreover, in the stony coral *Pocillopora damicornis*, the activity of glutathione-S transferase (GST) has been demonstrated to be significantly suppressed after 24-h exposure to 50 mg/L of polystyrene (Tang et al., 2018). Our results represent the first record of GR activity in soft corals following microplastic exposure. Further studies in reef organisms are needed to get a general GR regulation pattern in response to microplastic contamination. Similarly to GR, it was observed that the activity of catalase increased with increasing exposure to microplastic concentrations. CAT is one

of the main antioxidases in coral redox system (Levy et al., 2006). Previous studies have showed that microplastic exposure generates ROS at the cellular level in different benthic organisms (Jeong et al., 2016; Sutton et al., 2016; Rocha et al., 2020). Yet, this has not been investigated in soft corals to date. The increasing catalase activity observed in our experiments could hence be due to an increased production of ROS at higher microplastic concentrations, although this correlation should be better elucidated in these organisms. Similarly, in the zoanthid *Zoanthus sociatus*, a significant increase in CAT activity was observed following microplastic exposure (Rocha et al., 2020). The increase in catalase activity was however observed at lower microplastic concentration (10 mg /L) but the period of exposure (one week) was longer compared with the present study. The length of microplastic exposure may therefore play a role in the levels of antioxidant defences in benthic organisms. Even low concentrations of microplastic can become stressful to the organism following chronic exposure. A time-dependent disorder of metabolic functions, such as fluctuations in amino acids and osmolytes content, in response to exposures to high microplastic concentrations over a time period of 72h, has already been observed in the mussel *Mytilus galloprovincialis* (Cappello et al., 2021). Therefore, the effect of exposure time to low concentrations of microplastic should also be further investigated when investigating cellular oxidative stress of marine benthic organisms. The third enzyme analysed, superoxide dismutase is a major antioxidant component in coral physiology and is the first line of defence against ROS (Gardner et al., 2016). SOD propels the disproportion of O_2 − ion into H_2O_2 and molecular oxygen (O_2) and can be found in different cellular locations (Verma et al., 2019). Our results show that corals exposed to higher microplastic concentrations (50 mg/L and 70 mg/L respectively) had significantly higher SOD activity compared with corals of Control group and corals exposed to lower microplastic concentration (10 mg/l). Maximum SOD activity was recorded in samples exposed to 50 mg/l of microplastic. Corals exposed to 70 mg/l of microplastic had lower SOD activity. Though not significantly, this reduction in SOD activity at the highest experimental concentration could be due to the inhibition

of SOD activity when concentrations of microplastic exceed a certain threshold level, as previously observed in other studies (Tang et al., 2018). It is possible that SOD activity reaches a maximum of detoxification capacity, and the activity of the enzyme is inhibited at high microplastic concentrations (Sung et al., 2009; Silva Gomes et al., 2021). This could explain the lower SOD activities measured in samples exposed to the highest microplastic concentration. In order to assess the oxidative damage caused by exposure to microplastic, we measured the level of lipid peroxidation in samples, by estimating the malondialdehyde cellular content. LPO reflects the structural integrity of the cell membranes and production of LPOs indicates that levels of ROS are overwhelming the antioxidant pathways, accumulating and damaging cellular membrane lipids, thus signalling an ongoing oxidative stress (Lesser 2006; Weis 2008). Lipid peroxidation was significantly higher in corals exposed to the high microplastic concentrations of 50 mg/L and 70 mg/L. No significant difference in lipid peroxidation was noted between samples of control group and samples exposed to the low microplastic concentration (10 mg/L). These results suggest that a concentration of microplastic of 50 mg/L is sufficient to generate an oxidative damage in *C. palmosa* after 48 h of exposure. Results obtained in previous studies found a similar pattern, with a significantly high level of LPO recorded after 96-h exposures at 10 mg/L, showing the necessity to better elucidate the tolerance threshold for marine organisms to these contaminants (Rocha et al., 2020). A suppression or reduction of SOD activity can also be the cause of increased oxidative damage and high levels of lipid peroxidation (Downs et al., 2000; Marangoni et al., 2019). This hypothesis is supported also by findings of previous studies, in which a negative correlation between levels of SOD and LPO was found at short-term exposures to microplastic, suggesting that a suppressed SOD antioxidant activity could result in an enhancement of oxidative damage (Jiang et al., 2021). LPO levels have been demonstrated to increase in stony corals also in response to exposure to different chemicals, such as iron chloride and copper at different concentrations, indicating that oxidative damage could not only be triggered by a microplastic induced oxidative stress,

but also by compounds carried by microplastic (Teuten et al., 2009; Vijayavel et al., 2012; Bielmyer-Fraser et al., 2018). However, the commercial microplastic beads used in the present study did not contain any additive chemicals. It is highly probable that the oxidative stress observed is solely due to the exposure to microplastic material. No significant differences were found in Hsp60 expression in the different microplastic treatments, although a modulation pattern was observed, with the highest levels of biomarker found in corals subjected to the concentration of 10 mg/L of microplastic. Heat shock proteins (Hsps) are molecular chaperones, part of a cytoprotective mechanism able to mitigate the deleterious effects of stressors and have consequently often been adopted as cellular stress biomarkers in corals (Downs et al., 2000, 2005; Chow et al., 2012; Kenkel et al., 2014; Louis et al., 2017; Seveso et al., 2018). Studies investigating these biomarkers only focused on thermal, biotic or abiotic stress (Downs et al., 2002), but the possible Hsp response to microplastic contamination has stonyly been studied. Hsp expression is usually upregulated when organisms face conditions that may affect their cellular protein structure (Seveso et al., 2014, 2016). Hsp60 are also involved in maintaining protein homeostasis during oxidative stress (Montalbetti et al., 2021). Based on the results of our previous assays (LPO and antioxidant enzyme assays), that suggest oxidative stress in samples exposed to high microplastic concentration, our Western blot analysis does not reveal a significant increase in Hsp60 with increasing exposure to microplastic. Contrarily, the Hsp60 levels decrease with the increase of microplastic concentrations. This could indicate that microplastic impaired the expression of the Hsp60 protein. A suppression of antioxidant defensive mechanisms, such as GST and alkaline phosphatase (AKP), have already been observed in marine benthic organisms after a 24-h exposure to microplastic (Tang et al., 2018). The lack of upregulation of Hsp60 could hence be due to inhibition of their expression at high microplastic levels. Multivariate analyses showed significant differences between the high concentration treatments (50 mg/L and 70 mg/L) and low concentration treatment (10 mg/L) and controls. Indeed, according to the results of nMDS analysis, corals

subjected to high concentration treatments showed a similar response considering at least three of the biomarkers analysed: SOD, CAT and LPO. SOD and CAT are enzymes involved in direct detoxification of ROS, binding superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) respectively (Vance et al., 1972; Bergmeyer and Grassl 1983). As LPO showed similar concentration patterns to SOD and CAT in high concentration treatments (Figure 4), this suggests that the levels of oxidative damage due to microplastic contamination could be strictly related to the detoxification ability of ROS by antioxidant enzymes such as SOD and CAT, as also observed for other kinds of abiotic stressors (Lesser 2006; Weis 2008). In this context, other biomarkers such as Hsp60 and GR, being more related to protein homeostasis and regulation of the oxidative status of glutathione, could play a secondary role in protection from oxidative damage, although this aspect should be better elucidated (Figure 5). In conclusion, our results provide the

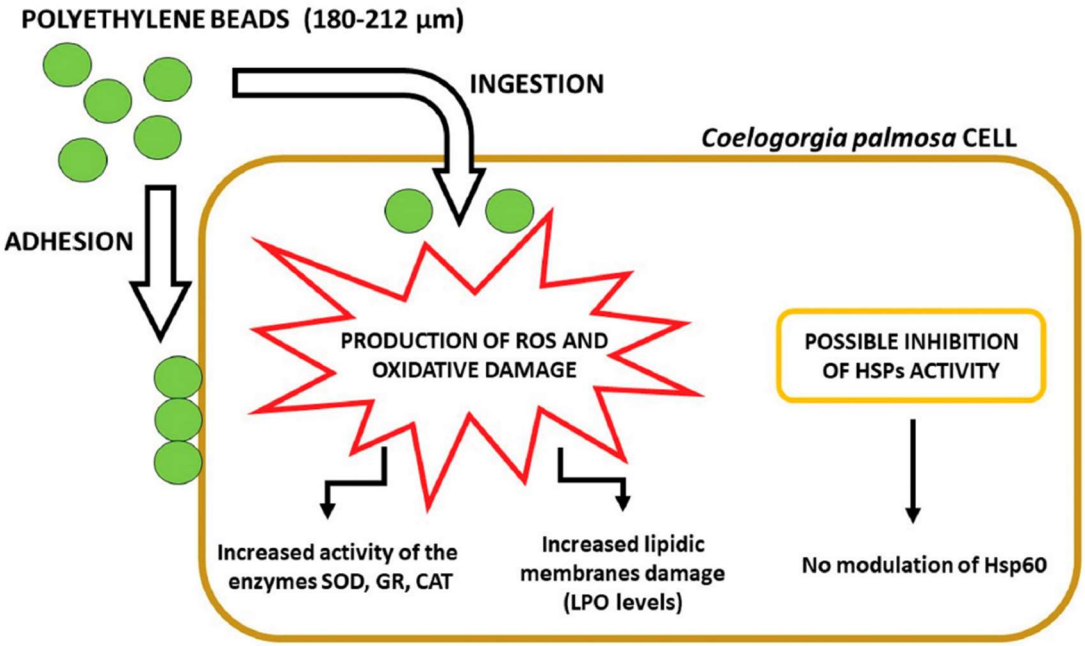

Figure 5. Schematic diagram showing the mechanism of action of microplastic contamination in the soft coral *Coelogorgia palmosa.*

first evidence of the effects of short-term microplastic exposure on a soft coral, at cellular level. *Coelogorgia palmosa* was found to be a good model during stress experiments in tanks and for laboratory analysis. Overall, a concentration of 70 mg/L of microplastic generated a significant raise in the activities of all the biomarkers studied, except for the expression of Hsp60. A concentration of 50 mg/L was sufficient to cause significant oxidative damage and an increase in SOD activity. Indeed, the multivariate analyses performed, indicated that cellular responses of corals exposed to high microplastic concentration were significantly different compared with samples exposed to lower microplastic concentrations. Furthermore, this study highlights a possible role of microplastic in suppressing cellular defensive mechanisms, as hypothesized here for Hsp60. This particular aspect must be further explored, as the threat represented by these contaminants for marine benthic life is predicted to worsen in the next decades, and their combination with several other stress sources could result in catastrophic outcomes for future coral reefs.

3.6. Acknowledgments

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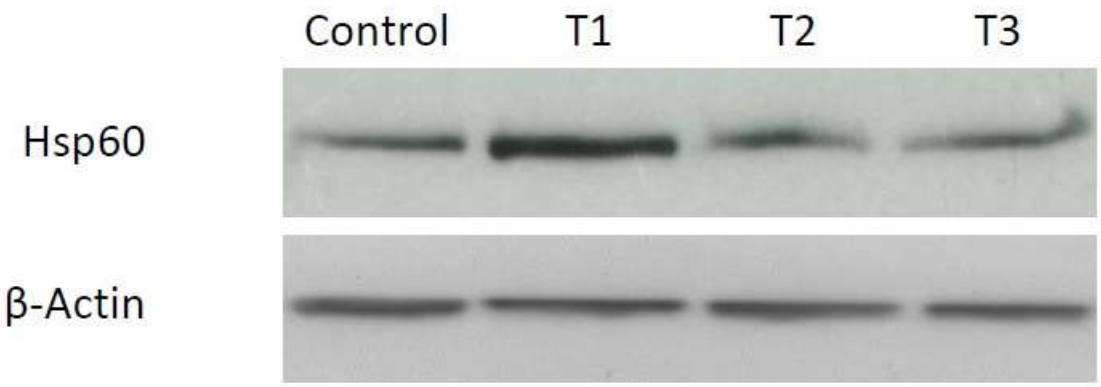
3.7. Supplementary



Supplementary Figure 1. Western blots representative of 4 experimental repeats showing the expression of Hsp60 and β-actin at different concentrations of microplastic.

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CHAPTER 4

4.1. Phthalates bioconcentration in the soft corals: Inter- and intra- species differences and ecological aspects

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Abstract

The bioconcentration of dimethyl phthalate (DMP) diethyl phthalate (DEP) dibutyl phthalate (DBP) butyl benzyl phthalate (BBP), di-(2-ethy hexyl) phthalates (DEHP), mono-butyl phthalate (MBP), mono-benzyl phthalate (MBzP), mono-(2-ethy hexyl) phthalate (MEHP) in the soft corals *Coelogorgia palmosa*, *Sinularia sp*., *Sarcophyton glaucum*, and *Lobophytum sp*. was investigated. Specimens were cultured in a microcosm environment built-up at the Genova Aquarium and analyses were carried out by in vivo SPME-LC/MS. The distributions of the phthalates among the four surveyed species resulted significantly different. Calculated bioconcentration factors (BCFs) showed values spanning over two orders of magnitude, from a minimum of log10 BCFDEP = 1.0 in *Sarcophyton glaucum* to a maximum of log10 BCFDBP = 3,9 calculated for *Coelogorgia palmosa*. Moreover, the calculated BCFs of the long chain phthalates resulted up to three orders of magnitude lower than theoretically predicted (from logKow), whereas BCF of short chain phthalates resulted higher. This, together with the detection of phthalic acid monoesters, suggests the presence of species-specific different metabolic transformation among the surveyed soft coral species that involve DEHP.

4.2. Introduction

Phthalates are currently the most diffused plastic ingredients added to polymer blends by manufacturers to enhance their plastic materials properties. They may be present in the formulation in high relative mass amounts, around 30–40% but even up to 60% (Teuten et al., 2009). Their global production has exponentially risen in the last decades together with the plastic production and today has reached 11 million ton/year (Fan et al., 2018). This poses concerns from an ecotoxicological standpoint since their action as endocrine disruptors (EDCs) is well documented (Pallotti et al., 2020). Moreover, there is evidence that phthalates do also trigger oxidative stress and immunotoxicity (Oehlmann et al., 2009). Nowadays, the presence, distribution and effect of phthalates in marine environments is a topical subject. Previous studies report the phthalates occurrence in different organisms, including zooplankton (Schmidt et al., 2021), marine invertebrates (Avisar et al., 2019; Horn et al., 2004; Vered et al., 2019) and marine mammals (Baini et al., 2017; Baini et al., 2012). However, such information for coral reef ecosystems are still scarce (Mendrik et al., 2021). Furthermore, no specific data regarding bioaccumulation, metabolism, and health effects of phthalates on both reef building and soft corals are available (Abdo et al., 2020; Livingstone, 1991). To date, the information about cytochrome p450 activity in cnidarians is very limited (Ertl & Winston, 1998; Heffernan and Winston, 1998a, 2000), but the effective presence of a cytochromeP450-dependent mixed-function oxidase (MFO) has been demonstrated in three scleractinian corals, *Favia fragum*, *Siderastrea* and *Montastrea* faveolata (Gassman and Kennedy, 1992; Ramos and García, 2007). For the best of our knowledge only a very recent study of Jafarabadi et al., (2021) surveyed the bioconcentration of phthalate esters (PAEs) in corals, highlighting how the soft corals with higher lipid content displayed higher phthalates levels than scleractinian coral species. The lack of data is mostly due to analytical difficulties, since background contamination may interfere highly during sample collection (Blair et al., 2009; Saliu et al., 2018). As

discussed in recent literature (Jafarabadi et al., 2021; Saliu et al., 2019; Montano et al., 2020) corals are exposed to phthalates from the surrounding environment and may get contaminated in their tissues by diffusion from seawater, by dermal contact with particulates and by ingestion of zooplankton. The levels of contamination in stony coral specimens collected from reef environments were found to range from 0 to 4 up to 210 ppb (Jafarabadi et al., 2021; Saliu et al., 2018). Taking into consideration the strong environmental correlation between plastic and phthalates pollution and aiming to provide a baseline assessment of the phthalates bioconcentration in different soft coral species, we measured the bioconcentration factors of dimethyl phthalate (DMP) diethyl phthalate (DEP) dibutyl phthalate (DBP) butyl benzyl phthalate (BBP), di-(2-ethy hexyl) phthalates (DEHP), mono-butyl phthalate (MBP), mono-benzyl phthalate (MBzP), mono-(2-ethy hexyl) phthalate (MEHP) in specimens of *Coelogorgia palmosa*, *Sinularia* sp., *Sarcophyton glaucum*, and *Lobophytum* sp. raised in the same microcosm environment.

4.3. Experimental materials and methods

4.3.1. Samples

The study was carried out by employing the soft coral species *Sinularia* sp., *Sarcophyton* sp., *Lobophytum* sp. and *Coelogorgia palmosa* cultured in a microcosm built up at Genoa Aquarium facilities (Genoa, Italy). A detailed description regarding the microcosm is provided in the Supplementary Information. The average concentration levels of phthalates in the tanks were monitored by taking 24 aliquots of 250 mL in triplicates, twice a week over one month. Determination of the phthalate concentration level in coral tissue was performed during the same month as the water survey by submitting 5 replicates for each coral species to the analysis, as indicated in the next section.

4.3.2. Phthalates extraction and analysis

The analysis of phthalates in the soft corals was carried out by employing *in vivo* SPME following a method previously described in Saliu et al., (2020). The analysis of water

samples was carried out by employing SPE with HLB sorbent and eluting with ethyl acetate according to the method described by Paluselli et al., (2019). LC-MS analyses were carried out by using a Thermofisher 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. (2020).

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

To reduce background contamination during sample manipulation no plastic items were used. All glassware was baked at 300 ◦C and pre-cleaned with acetone before use. The calibration curves were drawn for all the analytes with concentrations ranging from 0.2 ng/g to 150 ng/g. The regression coefficients of calibration curves were >0.99. The LODs ranged from 0.4 ng/g of DMP to 1.1 ng/g of DEHP. 12 procedural blank samples were processed together with the samples and used to calculate LOQs as an average plus six times the standard deviation (Fierens et al., 2012; Saliu et al., 2020). Specifically, the LOQs for DMP, DEP, DBP, BBP, DEHP, MEHP, MBzP and MZP were 0.6, 0.9, 1.9, 2.4, 3.1, 1.8, 2.0, and 1.4 ng/g, respectively. Accuracy was estimated in pre-spiked matrix samples as reported previously (Saliu et al., 2018) and ranged from 88% to 103%. The presence of artifacts that is stated in current literature as main problem in studies dealing with the evaluation of phthalates bioaccumulation factors (Staples et al., 1997), in the here described experimental set up may be ruled out since the corals were healthy (this is the main benefit of running the experiment in the microcosm), the concentration of phthalates in water were within their solubility values (thus particulate aggregation may be ruled out), the exposition of the soft corals to the phthalates was maintained for extended time (28 days), and the volume of water recirculated among the tanks was high enough to ensure a fair partition of phthalates among the coral tissues causing any significant variation of the phthalate concentration in the water (as verified by the water monitoring).

4.3.4. Determination of bioconcentration factors (BCFs)

BCF are used to evaluate the inclination of aquatic organisms to accumulate chemicals from their ambient environment and are calculated by considering the ratio of the concentration of a target analyte in biota to that of the surrounding water (Jafarabadi et al., 2018, Mckay, 1982). The experimental BCFs related to phthalates water-coral tissue partition were calculated for each phthalate considering the concentration measured in the water and in the selected soft coral according to the formula:

$$BCF=Cb/Cw$$

Where Cb ($mg\cdot^{kg-1}dw$) and and Cw ($mg\cdot/L$) are the phthalate concentrations in biota (normalized to lipid content), and seawater, respectively. These values were then plotted against the expected accumulation factors, calculated by considering the octanol-water partition coefficient (Log K_{ow}) recovered from the European Agency for Chemical Substances official website (https://echa.europa.eu/).

4.3.5. Statistical analysis

Statistical analyzes on the collected data were performed using the software SPSS ver. 27 (IBM, New York). More details are reported in the Supplementary.

4.4. Results

4.4.1. Phthalate distribution in the water and in the soft coral tissues

Analysis of the water recirculated in the aquarium tanks (Supplementary, Table S2.2) showed an average concentration of 135 ng/l (SE = 7) for the sum of phthalates, which is consistently lower than the values reported for the Liguria Sea (Fossi et al., 2012), the source where the water used in the tanks is collected and subsequently treated. DEHP resulted the most represented phthalate with an average concentration of 86 ng/l (SE = 7). The analysis carried out on the soft corals (Supplementary, S2, Table 2.3) showed an

average of total phthalates of 19.2 ng/g. The most represented phthalates were found to be the medium/long chain phthalates DBP and DEHP and short chain phthalate DMP. Considering the analysis of variance (Supplementary, S3) monoalkyl phthalates appeared significantly lower than dialkyl phthalates. DBP, the most abundant phthalate in terms of average concentration (7.8 ng/g, SE = 1.2) resulted significantly higher than DEHP, BBP and all the monoalkyl phthalates. DEHP, the second most abundant phthalate (5.0 ng/g, σm = 1.5) resulted significantly higher than DEP (Mann Whitney *U* test, p = 0.004), BBP (Mann Whitney *U* test, p = 0.034), and the monoalkyl phthalate MEHP (Mann Whitney *U* test, $p < 0.001$). DMP (5.1 ng/g, σm = 2.0) resulted significantly higher than MBP (Mann Whitney *U* test, p = 0.011), MBzP (Mann Whitney *U* test, p = 0.023) and MEHP (Mann Whitney *U* test, p = 0.014). Considering distributions among the species, no significant differences were displayed for the sum of phthalates (Kruskal Wallis H test, p = 0.532), the short chain phthalates DMP (Kruskal Wallis H test, p = 0.096) and DEP (Kruskal Wallis H test, p = 0.895), the monoalkyl phthalates MEHP (Kruskal Wallis H test, p = 0.769), MBP (Kruskal Wallis H test, p = 0.773) and MBzP (Kruskal Wallis H test, p = 0.773). Also, the lipophilic DBP and BBP displayed no significant difference among species (Kruskal Wallis H test, p = 0.455 and p = 0.062 respectively). It is noteworthy that DEHP resulted significantly higher in *Sarcophyton glaucum* with respect to *Lobophytum* sp. (Mann Withney *U* Test, p = 0,0036) and Coelogorgia *palmosa* (Mann Whitney *U* Test, p = 0,0036). Calculated bioconcentration factors varied from log10 BCF = 1.0 of DEP in *Sarcophyton glaucum* to log10 BCF = 3,9 calculated for DBP in *Coelogorgia palmosa*. Overall, the BCF of long chain phthalates resulted as equal to four order of magnitude lower than the predicted BCFs (Figure 1A) whereas the short chain phthalates showed experimental BCF from equal to four order of magnitude greater than predicted, with some differences among the surveyed species (Figure 1 B–C-D-E). Finally, no correlation was found between the ecological volume occupied by the sampled specimens and the concentration of phthalates, considering the sum and each

phthalate (DMP, DEP, DBP, BBP, DEHP, MBP, MBzP, MEHP, was highlighted (Supplementary S3).

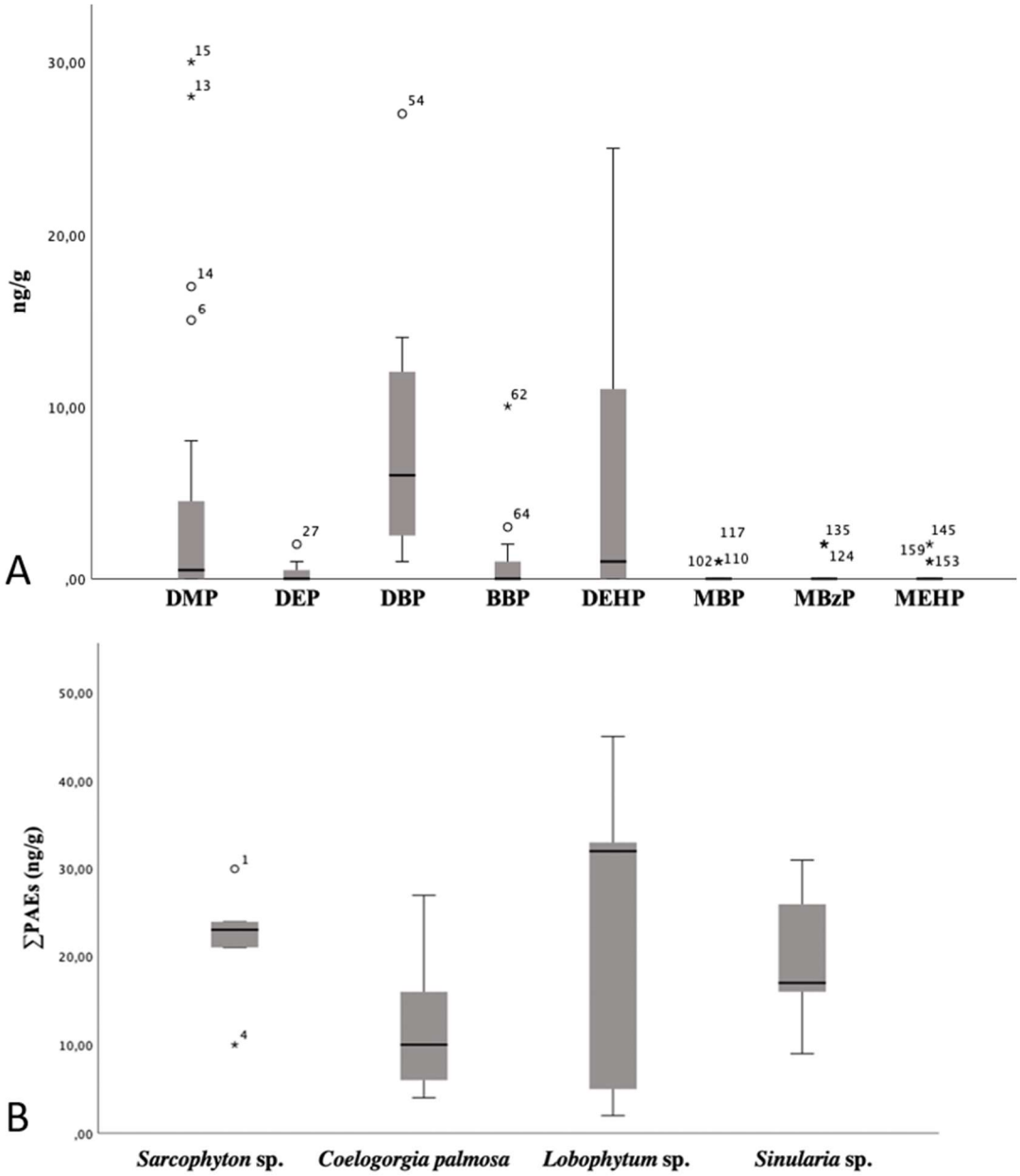

Figure 1. A) Box plot reporting the distribution of phthalates among all the surveyed soft coral species. B) Box plot reporting the concentration of total phthalates for the different soft coral species. For both the box plots: Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers.

4.4.2. 2Hints of the metabolic activity

The results presented here indicate that the short chain phthalates DMP and DEP display higher levels of accumulation in the soft coral tissue than theoretically expected, while the larger phthalates BBP and DEHP display levels of accumulation lower than expected. Indeed, the observation that lowest molecular weight phthalates esters display bioaccumulation factors greater than predicted from a lipid-water partitioning model and that higher molecular weight phthalate esters are below those expected was reported in previous lab and field studies involving other aquatic organisms (Staples, 1997). This together with evidence of trophic dilution of the high molecular weight phthalates is generally considered a proof of metabolic transformation (Baar et al., 2003). Strong differences were also observed, with bioconcentration factors in invertebrates generally higher than those in vertebrates. This is considered an indication of species-specific differences in metabolic transformation (Oehlmann et al., 2009). Similar consideration may be applied to our finding which indicates a possible metabolic pathway involving the transformation of long chain/high molecular weight/more lipophilic phthalates into shorter chain and monoalkyl phthalates before excretion. Moreover, the significant differences in the concentration of DEHP, indicates that the surveyed soft corals display differences in their metabolic pathways and/or efficiencies. Since monoalkyl phthalates are obtained from hydrolyzation as the first step of metabolic pathways of phthaltes in many organisms (Barr Dana et al., 2003; Blair et al., 2009; Horn et al., 2004; Silva et al., 2007). Therefore, the fact that we detected monoaklyl phthalates in the tissues of the examined soft corals (which we did not in the water) should be considered a proof of the activation of these metabolic pathways in soft corals as well. Interestingly, the average BCF of DEHP observed in our study for soft coral equal to 120 is lower than 2500 reported for the mussel *Mytilus edulis* and comparable with values reported for different fish species. In this respect, it should be pointed out that the extraction of phthalates by using SPME involves only the

unbounded molecules present in the tissue of the soft coral, and phthalates “adherent” to the external surface of the tissue and ingested with particulates are not sampled. Thus, the level of short chain phthalates found in the coral tissues might be viewed as signs of an active metabolism of DEHP exerted solely in the coral tissue. Previous observations regarding the lower susceptibility of DEHP to microbial attack in respect to the short chain phthalates confirms the hypothesis of polyp involvement (Chang et al., 2004). Finally, the fact that no significant correlation was found with the ecological volume is in agreement with previous indications about the metabolic rates of polyp colonies (Fabricius and Klumpp, 1995) with peculiarity among the taxonomic groups (Sorokin, 1991), and due to the dependency from zooxanthellae (Wagner et al., 2001). However, the detoxification action, as indicated, should be related to the cytochrome P (450) MFO system located in the polyp.

4.5. Conclusion

This preliminary study showed that phthalates do bioconcentrate in soft corals. Experimental BCFs differ from those predicted by considering Kow, and the lipid content of the corals. It is suggested that the longer chain di-alkyl phthalates such as DEHP are degraded into shorter chain di-alkyl phthalates and mono-alkyl phthalates by a metabolic pathway metabolic pathway within the tissue of the polyps. Moreover, this phthalates metabolism appears to be species specific and non-related to the colony size.

4.6. Aknowledgment

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4.9. Supplementary

4.9.1. S1_Description of the Microcosm at the Genoa Aquarium facility

The soft coral colonies sampled come from the Genoa Aquarium tanks, where the water system collects seawater 50m in depth from 200m outside the Foranea dam of the port of Genoa. The collected water is pumped through the filtration system made by 2 sand filters and one UV filter, used for disinfection. After the filtration the seawater is stored inside 4 accumulation tanks (200m^3 each one). If analyses show that the chemical-physical parameters (Ph, salinity, ammonia, nitrites, nitrates and phosphates) are optimal for the aquarium, the seawater of one accumulation tank is pumped into a mixing basin, where the water is kept in constant motion. After further UV filtration, the water is pumped from the mixing basin to all the tanks of the aquarium. During the day, from 8:00 to 16:00, the water is pumped from the mixing basin to the aquarium tanks with a flow of 1 liter every 30 seconds, so the tanks are considered as a semi-open system (the tanks are considered as a closed system from 16:00 to 8:00). All the aquarium piping system is composed of PVC. In this way the considered colonies are supposed to be exposed to the same average concentration of phthalates present in the tank's water. In the tanks, used for the sampling (3x1x0,7 m, 2400 l, composed by acrylic and glass resin) in which the experiment was carried out, the water is uptaken by a pump (Astralpool, Victoria Plus) with a 24- hour flow rate of 8m^3/h (to ensure a complete water change every about 30 minutes) and reinserted into the tank after passing through the filtration system. The filtration system is composed by a sand filter (Astralpool Artic, filtering particles from 0.4 to 2mm), a protein skimmer and a UV filter (Panaque 750 s AB 4 lamps of 40W). The water passage through the UV filter is instantaneous since water passes with a flow equal to 8m^3 per hour. Inside the tanks there are structure used to sustain the coral colonies. These structures are composed by 3 PVC grids (60x40x2 cm) sustained by 4 nylon ropes (50-65 cm in length and 0,5 cm diameter).

Moreover, there are other materials inside the tanks made by glass fiber and PVC. At the bottom of the tank there is one layer of coarse sand (3 cm thick), made of calcium carbonate (the sand is produced by smashing the skeleton of dead corals). 2 L of water containing a solution of the algae *Tetraselmis sp.* and zooplankton belonging to the Phylum Rotifera (the average concentration of zooplankton is 250 individuals /mL and the average dimension is 0,5mm) are placed daily inside the tanks in order to feed the corals. Both algae and zooplankton are farmed inside 80L cylindrical tanks made by plexyglass. Furthermore, twice a week 20g of food mixture are given in the tanks to feed the corals: such mixture is composed by 70% of silverside fishes (5cm in length) and 30% carrots, while the next day, the mixture is composed by 70% of mussels and 30% of courgettes. In order to facilitate the calcification of the coral skeleton 50l of water containing 500g of calcium hydroxide. The tanks used for the experiment displayed a volume of 2400L (2,0 L/g of colony) and were pumped (Astralpool, Victoria Plus) with a flow rate of 8m^3/h to ensure a complete change of the seawater in 30 minutes. Continuously chemical-physical analysis (Ph, salinity, ammonia, nitrites, nitrates and phosphates) ensure that the seawater in the accumulation tanks meet the requirements of the aquarium. Since the water of Ligurian Sea contain levels of phthalates, with concentration up to 170 ng/g in the neuston (Fossi et al., 2012) and plastic is also widely used in the facility (piping and tanks.), important levels of background contamination were expected in the recirculated water.

Table S1.1 Mass of materials and biological components in 1 m^3 of water.

Compartment	**total mass (kg)**	**relative mass to coral (g/g of coral)**
Total mass of water	2400	218
PVC grid	0,8	0,07
Nylon rope	0,6	0,05

Fiberglass grid	2,4	0,2
sediment	70	5,9
coral	11,7	1

Table S1.2 Water chemo-physical parameters.

DATA	pH	Salinity (ppt)	N-NH4 (mg/l)	N-NO2 (mg/l)	$NO3^-$ (mg/l)	$PO4^{3-}$ (mg/l)
05/10/2020	7.95	35.6	0.01	0	0	0
12/10/2020	7.95	35.1	0.02	0	0	0
19/10/2020	8.23	35	0	0	0	0.07
26/10/2020	8.17	35.1	0	0	0	0
02/11/2020	7.91	35.2	0.02	0	1	0
03/11/2020	7.94	34.8	0.02	0	1	0
09/11/2020	8.19	35.6	0.01	0	0	0
16/11/2020	8.18	35.1	0.01	0	0	0
23/11/2020	8.18	35.2	0	0	0	0
30/11/2020	8.27	35.3	0	0	0	0

4.9.2. S2_Samples analysis and results

The lipid content in coral tissues was determined following the procedure described by Jafarabadi et al., 2018. The determination of phthalates in the water samples was carried out by employing SPE with HLB sorbent and eluting with ethyl acetate according to the method described by Paluselli et al. (2019). Briefly, 250 mL of samples were filtered onto glass fibers filters prebaked at 450 °C for 12 h. The glass cartridges packed with 200 mg of Oasis HLB were preconditioned by sequentially passing through dichloromethane, acetone, and methanol, followed by deionized water. Subsequently, the sample was loaded, and the cartridges were dried under vacuum. Elution was done with 6 mL of ethyl acetate. The eluates were evaporated to dryness, reconstituted in methanol (1 mL), and transferred to an injection vial for analysis by LC/MS. Prior to extraction, the water samples were spiked with 10 µL of the internal standard solution (5 µg/mL). The analysis of phthalates in the soft corals was carried out by employing *in vivo* SPME following a method previously described in Saliu et al. (2020). Briefly, SPME fibers were purchased from Sigma Aldrich (part no. 57234-U) and used in direct immersion mode, for a 10 min time of total exposition. After that the analytes were eluted by 100 µl of methanol directly into a glass vial insert for the LC/MS analysis. LC-MS analyses were carried out by using a Thermofisher 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. (2020). Ultra-grade methanol (MeOH) from Sigma Aldrich (Sigma Aldrich, Darmstadt, Germany). Ultrapure Water (resistivity, 18.2 MU cm) was produced on a Milli-Q Plus apparatus (Millipore, Milan, Italy). The phthalate ester standard mix was purchased from Sigma Aldrich (EPA 506 phthalate ester mix). It contains: dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), bis(2- ethylhexyl)adipate (DEHA), each component added at 500 mg/ml in methanol. Bis(2-ethylhexyl)phthalate-3,4,5,6-d4 (DEHP 4, 98%) and monoethyldihexyl

phthalate (MEHP, 98%) monobutyl phthalate (MBP), mono benzyl phthalate (MBzP) were also provided by Sigma Aldrich and were used to prepare individual stock solutions in methanol at a concentration of 500 mg/ml. All the standards were stored at 4°C nd used for spiking and calibration. The chromatographic separation of the analytes was obtained by using a Thermo Scientific Accucore C-18 aQ column (100 mm _ 2.1 mm I.D., 2.6 mm). Chromatographic elution was carried out with a binary system comprising water with 0.1% of acetic acid for pump A and methanol for pump B. A gradient was applied from 80 to 96% of B at 0.6 ml/min for 15 min, post-column switching was applied to prevent insource contamination. Mass spectrometry was carried out by applying the spray voltage at 3500 V. The vaporizer temperature at 350°C and capillary temperature at 270°C, sheath gas 50 a.u. e auxiliary gas 15 a.u., sweep gas 2 a.u. collision gas pressure1.0 mTorr and cycle time of 0.6 s. The injection was performed in partial loop of 20 ml. Mass transition were followed in time segmented selected reaction monitoring. Linearity and limit of detections were evaluated by using matrix-matched calibration curves considering relative area against the labelled internal standard. Calibration points were prepared at 0.5, 1, 5, 10, 25, 50, 100 and 250 ng/g. Correlation coefficient (R2) resulted comprised within 0.992 and 0.998 with randomly distributed residuals (<20%). LODs for coral tissues analysis resulted comprised. The LOD for the phthalates in seawater resulted between 2 and 21 ng/g and within 0.8 ng/g and 1.5 ng/g dw for coral tissue samples. Analytical results less than the LODs were considered to be zero. The recoveries varied from 83 to 112% in the seawater samples, from 87 to 116 % in the coral samples.

To avoid contamination with PAEs during sample handling, several precautions were applied. The laboratory glassware and tools were rinsed with acetone 3 times and heated at 400 °C overnight. Then, they were rinsed with the same ultrapure solvents that were to be applied for the procedural steps (ultra-pure methanol or water or ethyl acetate). No plastic labware was used in the sample collection/handling/preparation

processes, and the sample extraction and vial preparation steps were carried out in a clean air flow cabinet. Field blanks and procedural blanks were treated identically to the real samples. Solvent blank and standards were run every 5 samples to check for any carryover, background contamination, and instrument drift. Specifically, a total of 12 procedural blanks were analyzed on six different days and showed averages of 3.5 ng/g for the sum phthalates and with 1.6 ng/g standard deviation. Finally, accuracy was evaluated considering estimated values for back-calculation by using homogenized tissue (4.0 ±0.2 g) spiked with 100 ml of 4 mg/l native standard solution. The obtained mean values ranged from 92 to 103%. Precision for inter-day assays displayed RSDs under 13%.

Table S2.1. List of the soft coral individuals employed in the experiments with the related ecological volume

ID Sample	Species	Ecological Volume (cm^3)	Estimated lipid content (mg/g)
I1_I	*Sarcophyton sp.*	1236	29.7
I2_I	*Sarcophyton sp.*	2309	30.5
I3_I	*Sarcophyton sp.*	3925	28.2
I4_I	*Sarcophyton sp.*	213	29.1

I5_I	*Sarcophyton sp.*	855	31.3
J1_I	*Coelogorgia palmosa*	2939	22.4
J2_I	*Coelogorgia palmosa*	785	24.1
J3_I	*Coelogorgia palmosa*	2289	19.7
J4_I	*Coelogorgia palmosa*	2000	22.8
J5_I	*Coelogorgia palmosa*	12590	18.4
K1_I	*Lobophytum sp.*	1697	25.3
K2_I	*Lobophytum sp.*	708	28.4
K3_I	*Lobophytum sp.*	3629	20.9
K4_I	*Lobophytum sp.*	454	24.3
K5_I	*Lobophytum sp.*	265	19.7
L1_I	*Sinularia sp.*	2806	19.4

L2_I	*Sinularia sp.*	5049	26.7
L3_I	*Sinularia sp.*	706	22.5
L4_I	*Sinularia sp.*	760	23.1
L5_I	*Sinularia sp.*	2687	17.4

Table S2.2. Mean concentration of phthalates detected in the Genoa Aquarium tanks employed for the microcosm experiment

	DMP	DEP	DBP	BBP	DEHP	**SUM**
05/10/2020	9	23	15	11	87	145
05/10/2020	11	22	21	9	112	175
05/10/2020	8	19	19	8	102	156
12/10/2020	4	19	3	3	67	96
12/10/2020	7	24	6	6	14	57
12/10/2020	6	22	4	4	112	148
19/10/2020	0	24	13	13	135	185
19/10/2020	3	21	16	16	63	119
26/10/2020	11	19	14	14	73	131
26/10/2020	10	17	13	13	15	68
26/10/2020	9	11	11	11	56	98
02/11/2020	4	12	4	4	153	177
02/11/2020	4	11	0	0	147	162
03/11/2020	5	11	11	11	56	94
09/11/2020	11	24	21	21	99	176
09/11/2020	12	18	18	18	102	168

09/11/2020	11	14	14	14	102	155
16/11/2020	7	18	18	18	98	159
16/11/2020	7	22	16	16	86	147
23/11/2020	6	19	10	10	75	120
23/11/2020	5	16	9	9	91	130
30/11/2020	6	21	6	6	78	117
30/11/2020	5	27	7	7	75	121
05/12/2020	7	25	25	25	67	149
Mean (ng/l)	7	19	12	11	86	135
SE	1	1	1	1	7	7

Table S2.3. Phthalates' contamination detected in the soft coral samples.

SAMPLE	DMP	DEP	DBP	BBP	DEHP	MBP	MBzP	MEHP	∑PAEs
I1_I	0	0	14	0	16	0	0	0	30
I2_I	0	1	12	10	0	1	0	0	23
I3_I	1	0	12	0	11	0	0	0	24
I4_I	0	0	7	3	0	0	2	0	10
I5_I	0	0	9	0	12	0	0	2	21
J1_I	15	0	12	0	0	0	0	0	27
J2_I	0	2	8	0	0	0	0	0	10
J3_I	8	0	5	1	2	0	0	0	16
J4_I	1	0	3	0	0	0	2	0	4
J5_I	0	0	3	0	3	1	0	0	6
K1_I	0	1	1	0	0	0	0	0	2
K2_I	1	0	1	1	2	0	0	0	5
K3_I	28	0	5	0	0	0	0	1	33
K4_I	17	0	27	0	1	0	0	0	45
K5_I	30	0	2	0	0	0	2	0	32
L1_I	0	0	14	1	11	0	0	0	26

L2_I	1	1	13	0	1	1	0	0	16
L3_I	0	0	4	2	25	0	0	0	31
L4_I	1	0	2	0	14	0	0	1	17
L5_I	0	1	1	1	6	0	0	0	9
Mean	5,2	0,3	7,9	0,9	5,2	0,2	0,3	0,2	19,2
SE	2,2	0,1	1,5	0,5	1,6	0,1	0,2	0,1	2,7

4.9.3. S3_Statistical Analyses

Variations in PAEs contamination among the 4 target soft corals species were analyzed in the first place with the descriptive statistics, such as histograms. After that, since the data are not normally distributed, the resulted concentrations among samples were tested using a non-parametric Kruskal Wallis test. Then, a Spearman test was performed to explore a possible correlation between the ecological volume occupied by the soft corals' colonies that were sampled, and the phthalates contamination of the corresponding fragments. For determination of the ecological volume of the sampled colonies we considered the formula described by Abdo et al. (2020):

$$V = \pi r2h \text{ and } r = (1+w)/4$$

Where (V) is the Ecological Volume. (h) the Height of colony. (l) the Length of fragment. (w) the Width of fragments.

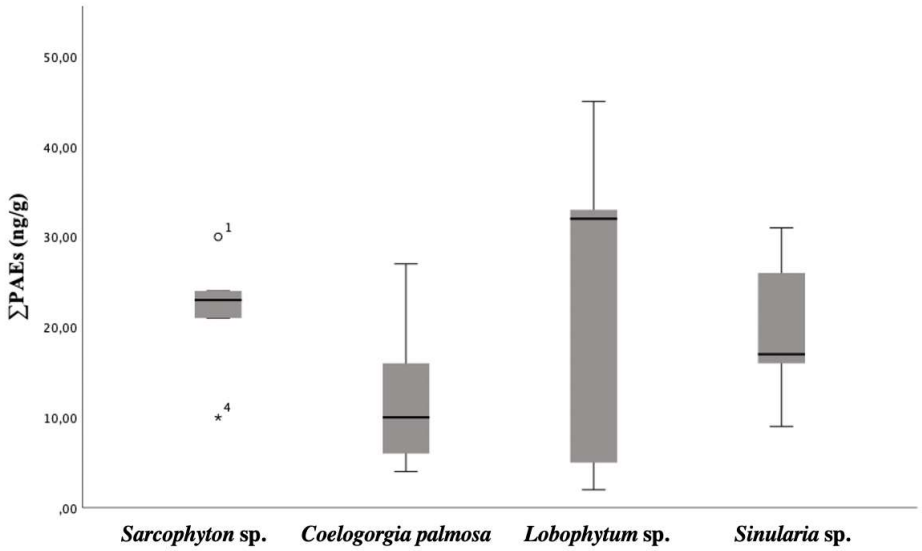

Figure S3.1. Box plots reporting the concentration of sum of phthalates for the different soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers.

∑PAEs no significant differences among species (Kruskal Wallis H test, p = 0.532).

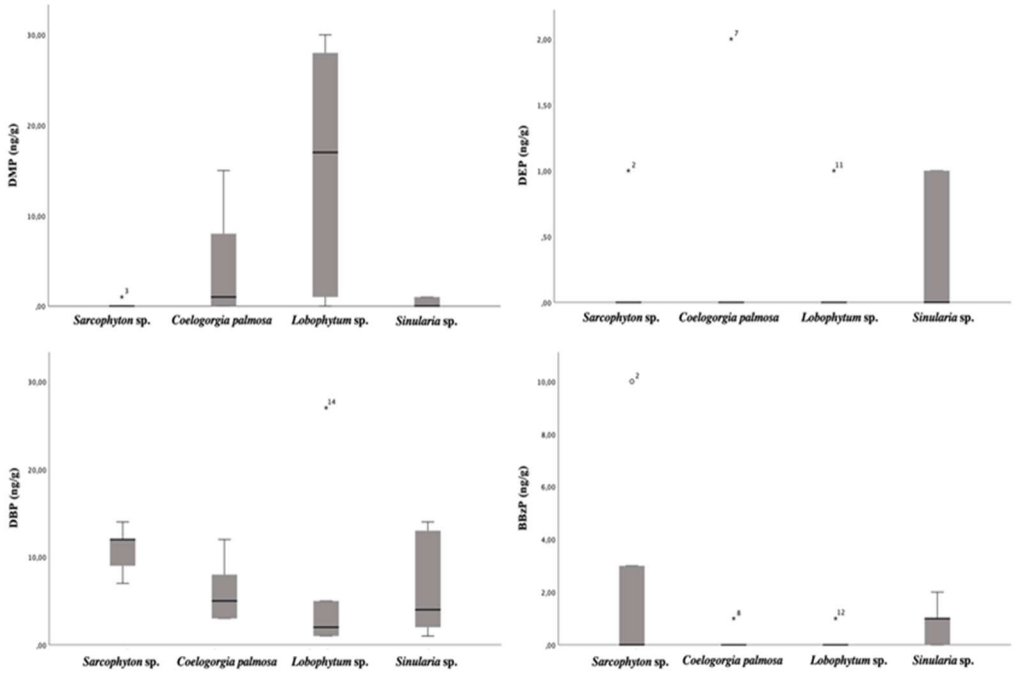

Figure S3.2. Box plots reporting the concentration of DMP, DEP, DBP and BBP for the different soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers.

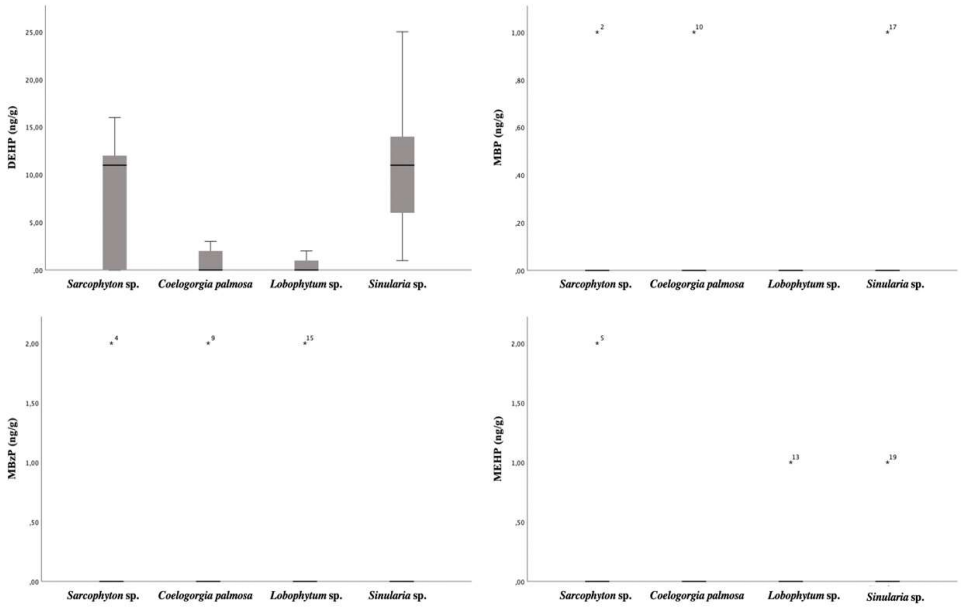

Figure S3.3. Box plots reporting the concentration of DEHP, MEHP, MBP and MBBP for the different soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers

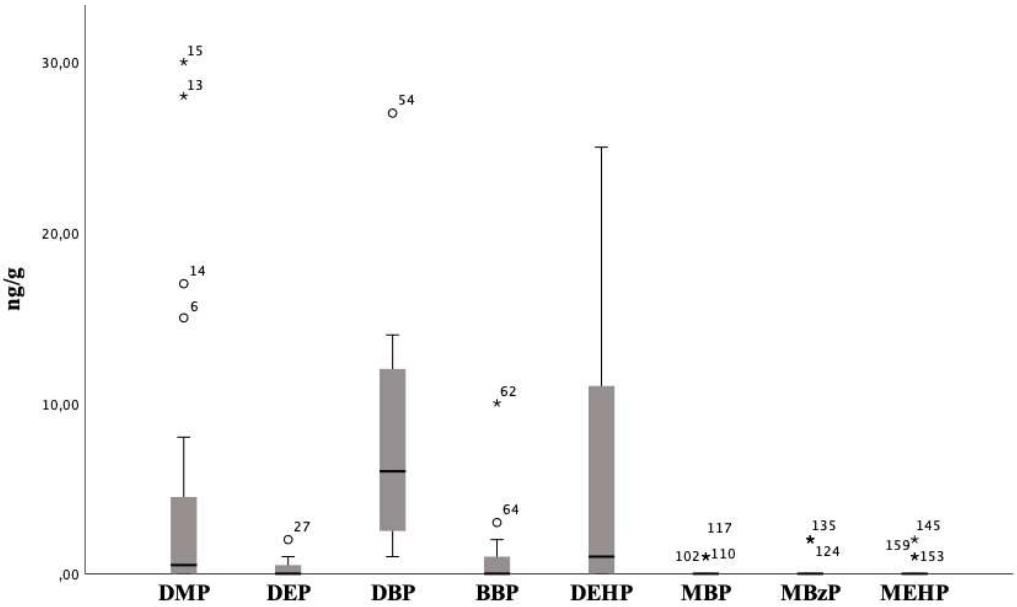

Figure S3.4. Box plots reporting the concentration of the phthalates for different soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers.

DMP significantly higher than MBP (Mann Whitney U test, p = 0.011), MBzP (Mann Whitney U test, p = 0.023), MEHP (Mann Whitney U test, p = 0.014).

DBP significantly higher than DEP (Mann Whitney U test, p < 0.001), BBP (Mann Whitney U test, p < 0.001), DEHP (Mann Whitney U test, p = 0.026), MBP (Mann Whitney U test, p < 0.001), MBzP (Mann Whitney U test, p < 0.001), MEHP (Mann Whitney U test, p < 0.001).

DEHP significantly higher than DEP (Mann Whitney U test, p = 0.004), BBP (Mann Whitney U test, p = 0.034), MBP (Mann Whitney U test, p < 0.001), MBzP (Mann Whitney U test, p = 0.002), MEHP (Mann Whitney U test, p < 0.001).

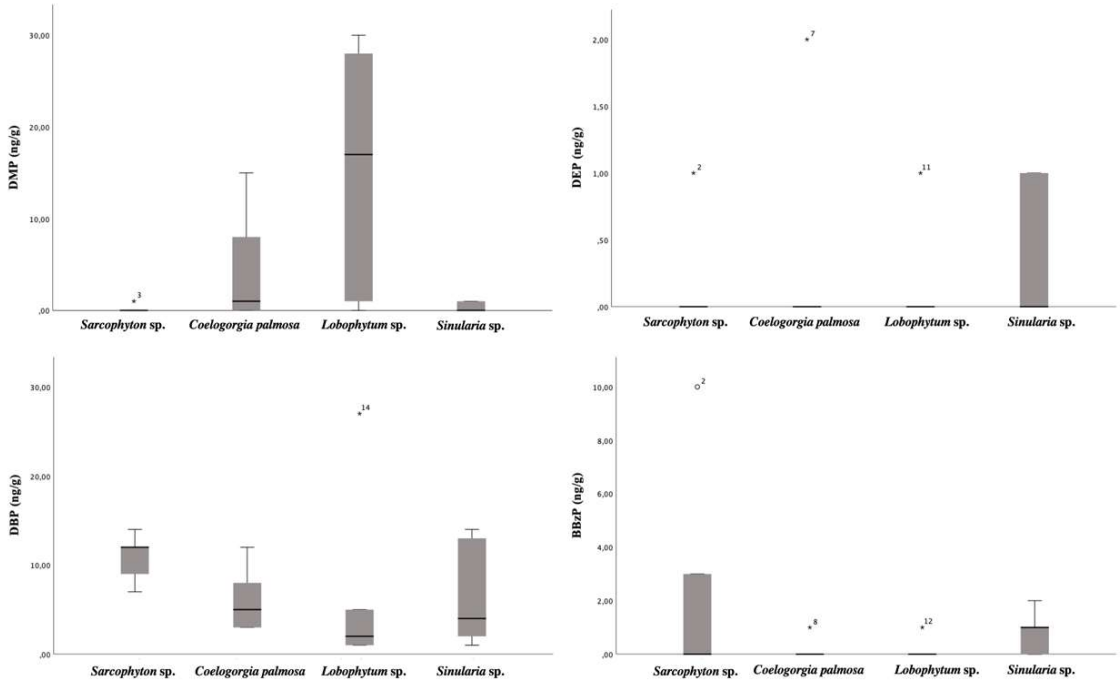

Figure S3.5. Box plots reporting the concentration of each phthalate for each soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers

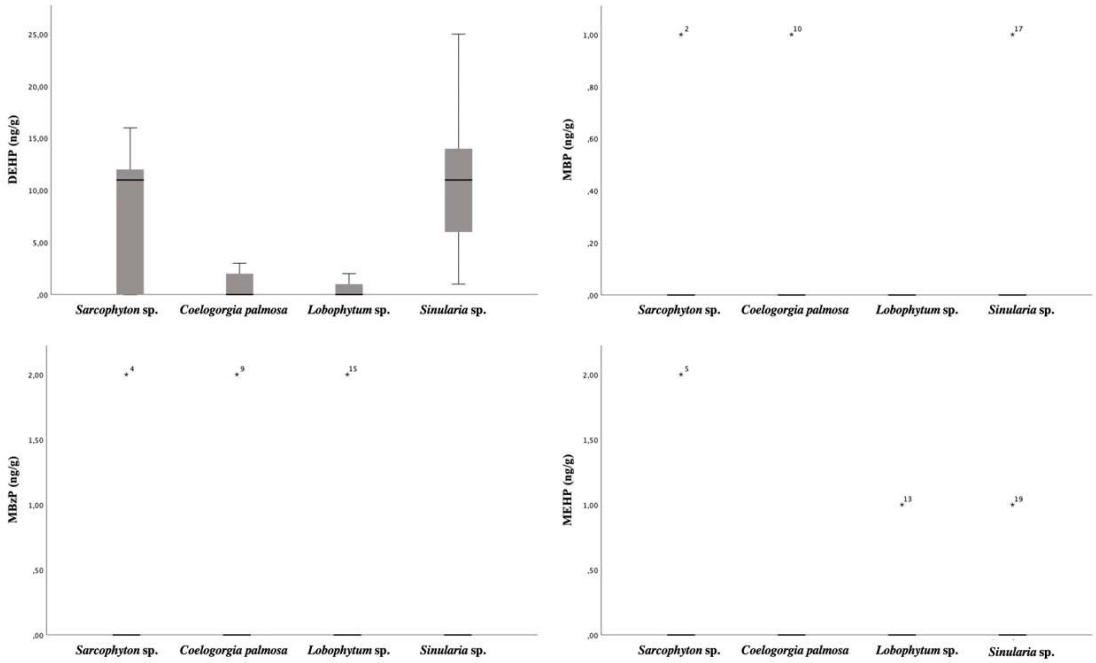

Figure S3.6. Box plots reporting the concentration of each phthalate for each soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers

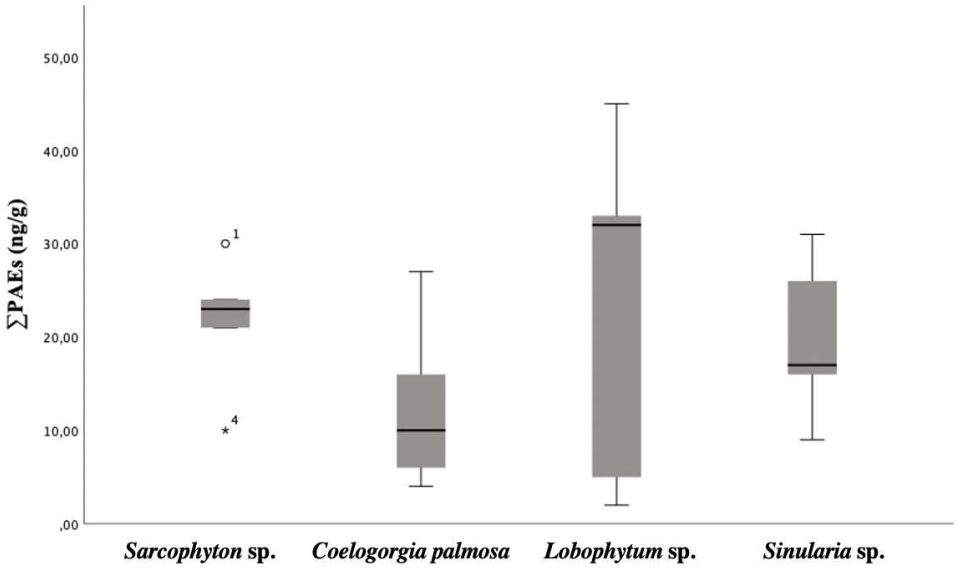

Figure S3.7. Box plots reporting the concentration of the sum of phthalates for each soft coral species. Line in box = median of sampled concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers

∑PAEs no significant differences among species (Kruskal Wallis H test, p = 0.532)

4.9.4. S4_Correlation Analyses

Correlazioni

			DMP	Vecological
Rho di Spearman	DMP	Coefficiente di correlazione	1,000	,707
		Sig. (a due code)	.	,182
		N	5	5
	Vecological	Coefficiente di correlazione	,707	1,000
		Sig. (a due code)	,182	.
		N	5	5

Figure S4.1. Correlation analysis

In *Sarcophyton sp.* DMP and the ecological volume resulted to be correlated but not significantly (Spearman coefficient, ρ = 0.707, p = 0.182).

Correlazioni

			ecologicalvol	DEHP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,051
		Sig. (a due code)	.	,935
		N	5	5
	DEHP	Coefficiente di correlazione	,051	1,000
		Sig. (a due code)	,935	.
		N	5	5

Figure S4.2. Correlation analysis

In *Sarcophyton sp.* no correlation among DEHP and the ecological volume (Spearman coefficient, ρ = 0.051, p = 0.935) were found.

Correlazioni

			ecologicalvol	DBP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,667
		Sig. (a due code)	.	,219
		N	5	5
	DBP	Coefficiente di correlazione	,667	1,000
		Sig. (a due code)	,219	.
		N	5	5

Figure S4.3. Correlation analysis

In *Sarcophyton* sp. DBP and the ecological volume resulted to be correlated but not significantly (Spearman coefficient, ρ = 0.667, p = 0.219).

Correlazioni

			ecologicalvol	TOTFTALATI
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,700
		Sig. (a due code)	.	,188
		N	5	5
	TOTFTALATI	Coefficiente di correlazione	,700	1,000
		Sig. (a due code)	,188	.
		N	5	5

Figure S4.4. Correlation analysis

In *Sarcophyton sp.* ∑PAEs and the ecological volume resulted to be correlated but not significantly (Spearman coefficient, ρ = 0.700, p = 0.188).

Correlazioni

			ecologicalvol	DBP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	-,154
		Sig. (a due code)	.	,805
		N	5	5
	DBP	Coefficiente di correlazione	-,154	1,000
		Sig. (a due code)	,805	.
		N	5	5

Figure S4.5. Correlation analysis

In *Coelogorgia palmosa* no correlation among DBP and the ecological volume (Spearman coefficient, ρ = - 0.154, p = 0.805).

Correlazioni

			ecologicalvol	DEHP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,671
		Sig. (a due code)	.	,215
		N	5	5
	DEHP	Coefficiente di correlazione	,671	1,000
		Sig. (a due code)	,215	.
		N	5	5

Figure S4.6. Correlation analysis

In *Coelogorgia palmosa* DEHP and the ecological volume resulted to be correlated but not significantly (Spearman coefficient, ρ = 0.671, p = 0.215).

Correlazioni

			DMP	ecologicalvol
Rho di Spearman	DMP	Coefficiente di correlazione	1,000	,205
		Sig. (a due code)	.	,741
		N	5	5
	ecologicalvol	Coefficiente di correlazione	,205	1,000
		Sig. (a due code)	,741	.
		N	5	5

Figure S4.7. Correlation analysis

In *Coelogorgia palmosa* no correlation among DMP and the ecological volume (Spearman coefficient, ρ = - 0.205, p = 0.741).

Correlazioni

			ecologicalvol	TOTFTALATI
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,200
		Sig. (a due code)	.	,747
		N	5	5
	TOTFTALATI	Coefficiente di correlazione	,200	1,000
		Sig. (a due code)	,747	.
		N	5	5

Figure S4.8. Correlation analysis

In *Coelogorgia palmosa* no correlation among ∑PAEs and the ecological volume (Spearman coefficient, ρ = - 0.200, p = 0.747).

Correlazioni

			ecologicalvol	DBP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	-,154
		Sig. (a due code)	.	,805
		N	5	5
	DBP	Coefficiente di correlazione	-,154	1,000
		Sig. (a due code)	,805	.
		N	5	5

Figure S4.9. Correlation analysis

In *Lobophytum* sp. no correlation among DBP and the ecological volume (Spearman coefficient, ρ = - 0.154, p = 0.805).

Correlazioni

			ecologicalvol	DEHP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	-,224
		Sig. (a due code)	.	,718
		N	5	5
	DEHP	Coefficiente di correlazione	-,224	1,000
		Sig. (a due code)	,718	.
		N	5	5

Figure S4.10. Correlation analysis

In *Lobophytum sp*. no correlation among DEHP and the ecological volume (Spearman coefficient, ρ = - 0.224, p = 0.718).

Correlazioni

			ecologicalvol	DMP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,289
		Sig. (a due code)	.	,638
		N	5	5
	DMP	Coefficiente di correlazione	,289	1,000
		Sig. (a due code)	,638	.
		N	5	5

Figure S4.11. Correlation analysis

In *Sinularia* sp. no correlation among DMP and the ecological volume (Spearman coefficient, ρ = 0.289, p = 0.638).

Correlazioni

			ecologicalvol	DBP
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	,500
		Sig. (a due code)	.	,391
		N	5	5
	DBP	Coefficiente di correlazione	,500	1,000
		Sig. (a due code)	,391	.
		N	5	5

Figure S4.12. Correlation analysis

In *Sinularia* sp. no correlation among DBP and the ecological volume (Spearman coefficient, ρ = 0.500, p = 0.391).

Correlazioni

			ecologicalvol	TOTFTALATI
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	-,200
		Sig. (a due code)	.	,747
		N	5	5
	TOTFTALATI	Coefficiente di correlazione	-,200	1,000
		Sig. (a due code)	,747	.
		N	5	5

Figure S4.13. Correlation analysis

no correlation among ∑PAEs and the ecological volume (Spearman coefficient, ρ = -0.200, p = 0.747).

Correlazioni

			ecologicalvol	TOTFTALATI
Rho di Spearman	ecologicalvol	Coefficiente di correlazione	1,000	-,300
		Sig. (a due code)	.	,624
		N	5	5
	TOTFTALATI	Coefficiente di correlazione	-,300	1,000
		Sig. (a due code)	,624	.
		N	5	5

Figure S4.14. Correlation analysis

In *Sinularia* sp. no correlation among ∑PAEs and the ecological volume (Spearman coefficient, ρ = - 0.300, p = 0.624).

Correlazioni

			EcoloV	DEHP
Rho di Spearman	EcoloV	Coefficiente di correlazione	1,000	-,900*
		Sig. (a due code)	.	,037
		N	5	5
	DEHP	Coefficiente di correlazione	-,900*	1,000
		Sig. (a due code)	,037	.
		N	5	5

*. La correlazione è significativa a livello 0,05 (a due code).

Figure S4.15. Correlation analysis

Significant correlation among the ecological volume and the encountered DEHP concentration (Spearman coefficient, ρ = 0.900, p = 0.037).

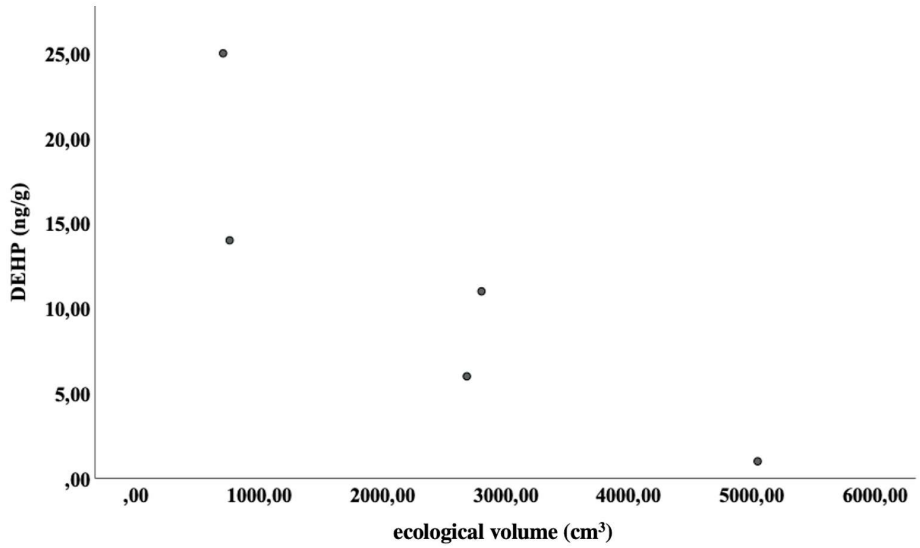

Figure S4.16. Correlation analysis among the ecological volume and the encountered DEHP concentration.

Correlazioni

			ecologicalV	DMP (ng/g)
Rho di Spearman	ecologicalV	Coefficiente di correlazione	1,000	-,002
		Sig. (a due code)	.	,992
		N	20	20
	DMP (ng/g)	Coefficiente di correlazione	-,002	1,000
		Sig. (a due code)	,992	.
		N	20	20

Figure S4.17. Correlation analysis

No correlation among DMP and the ecological volume (Spearman coefficient, ρ = -0.002, p = 0.992).

Correlazioni

			ecologicalV	DEHP (ng/g)
Rho di Spearman	ecologicalV	Coefficiente di correlazione	1,000	,013
		Sig. (a due code)	.	,956
		N	20	20
	DEHP (ng/g)	Coefficiente di correlazione	,013	1,000
		Sig. (a due code)	,956	.
		N	20	20

Figure S4.18. Correlation analysis

No correlation among DEHP and the ecological volume (Spearman coefficient, ρ = 0.013, p = 0.956).

Correlazioni

			ecologicalV	DBP (ng/g)
Rho di Spearman	ecologicalV	Coefficiente di correlazione	1,000	,195
		Sig. (a due code)	.	,410
		N	20	20
	DBP (ng/g)	Coefficiente di correlazione	,195	1,000
		Sig. (a due code)	,410	.
		N	20	20

Figure S4.19. Correlation analysis

No correlation among DBP and the ecological volume (Spearman coefficient, ρ = 0.195, p = 0.410).

Correlazioni

			ecologicalV	∑PAEs (ng/g)
Rho di Spearman	ecologicalV	Coefficiente di correlazione	1,000	-,111
		Sig. (a due code)	.	,642
		N	20	20
	∑PAEs (ng/g)	Coefficiente di correlazione	-,111	1,000
		Sig. (a due code)	,642	.
		N	20	20

Figure S4.20. Correlation analysis

No correlation among ∑PAEs and the ecological volume (Spearman coefficient, ρ = -0.111, p = 0.642).

Correlazioni

			DMP (ng/g)	DEP (ng/g)	DBP (ng/g)	BBzP (ng/g)	MBP (ng/g)	MBzP (ng/g)	MEHP (ng/g)	DEHP (ng/g)
Rho di Spearman	DMP (ng/g)	Coefficiente di correlazione	1,000	-,383	,005	-,377	-,196	,183	,129	-,319
		Sig. (a due code)	.	,095	,982	,102	,407	,439	,587	,170
		N	20	20	20	20	20	20	20	20
	DEP (ng/g)	Coefficiente di correlazione	-,383	1,000	-,078	,069	,369	-,241	-,240	-,334
		Sig. (a due code)	,095	.	,744	,774	,109	,306	,308	,150
		N	20	20	20	20	20	20	20	20
	DBP (ng/g)	Coefficiente di correlazione	,005	-,078	1,000	-,086	,171	-,232	-,095	,100
		Sig. (a due code)	,982	,744	.	,720	,472	,326	,691	,676
		N	20	20	20	20	20	20	20	20
	BBzP (ng/g)	Coefficiente di correlazione	-,377	,069	-,086	1,000	,072	,043	-,300	,080
		Sig. (a due code)	,102	,774	,720	.	,764	,857	,199	,737
		N	20	20	20	20	20	20	20	20
	MBP (ng/g)	Coefficiente di correlazione	-,196	,369	,171	,072	1,000	-,176	-,176	-,113
		Sig. (a due code)	,407	,109	,472	,764	.	,457	,458	,635
		N	20	20	20	20	20	20	20	20
	MBzP (ng/g)	Coefficiente di correlazione	,183	-,241	-,232	,043	-,176	1,000	-,176	-,452*
		Sig. (a due code)	,439	,306	,326	,857	,457	.	,458	,045
		N	20	20	20	20	20	20	20	20
	MEHP (ng/g)	Coefficiente di correlazione	,129	-,240	-,095	-,300	-,176	-,176	1,000	,215
		Sig. (a due code)	,587	,308	,691	,199	,458	,458	.	,363
		N	20	20	20	20	20	20	20	20
	DEHP (ng/g)	Coefficiente di correlazione	-,319	-,334	,100	,080	-,113	-,452*	,215	1,000
		Sig. (a due code)	,170	,150	,676	,737	,635	,045	,363	.
		N	20	20	20	20	20	20	20	20

*. La correlazione è significativa a livello 0,05 (a due code).

Figure S4.21. Correlation matrix

DEHP significantly correlated with MBzP (Spearman coefficient, ρ = - 0.452, p = 0.045).

Table S3.1. Correlation matrix of the whole dataset (ρ = Spearman correlation coefficient).

		DBP	DEP	DMP	BBP	**DEHP**	MBP	**MBzP**	MEHP
DBP	ρ	1.000	- 0.078	0.005	- 0.086	0.100	0.171	- 0.232	- 0.095
	p	.	0.744	0.982	0.720	0.676	0.472	0.362	0.691
DEP	ρ	- 0.078	1.000	- 0.383	0.069	- 0.334	0.369	- 0.241	- 0.240
	p	0.774	.	0.095	0.774	0.150	0.109	0.306	0.308
DMP	ρ	0.005	- 0.383	1.000	- 0.377	- 0.319	- 0.196	0.183	0.129
	p	0.982	0.095	.	0.102	0.170	0.407	0.439	0.587
BBP	ρ	- 0.086	0.069	- 0.377	1.000	0.080	0.072	0.043	- 0.300
	p	0.720	0.774	0.102	.	0.737	0.764	0.857	0.199
DEHP	P	0.100	- 0.334	- 0.319	0.080	1.000	- 0.113	**- 0.452**	0.215
	p	0.676	0.150	0.170	0.737	.	0.635	**0.045**	0.363
MBP	ρ	0.171	0.369	- 0.196	0.072	- 0.113	1.000	- 0.176	- 0.176
	p	0.472	0.109	0.407	0.764	0.635	.	0.457	0.458
MBzP	ρ	- 0.232	- 0.241	0.183	0.043	**- 0.452**	- 0.176	1.000	- 0.176
	p	0.326	0.306	0.439	0.857	**0.045**	0.457	.	0.458
MEHP	P	0.691	- 0.240	0.129	- 0.300	0.215	- 0.176	- 0.176	1.000
	p	0.170	- 0.308	0.587	0.199	0.363	0.458	0.458	.

Correlazioni

			DBP	DEP	DMP	BBP	DEHP	MBP	MBzP	MEHP
Rho di Spearman	DBP	Coefficiente di correlazione	1,000	-,289	,000	,000	-,100	,354	.	-,354
		Sig. (a due code)	.	,638	1,000	1,000	,873	,559	.	,559
		N	5	5	5	5	5	5	5	5
	DEP	Coefficiente di correlazione	-,289	1,000	,167	-,304	-,866	,612	.	-,408
		Sig. (a due code)	,638	.	,789	,619	,058	,272	.	,495
		N	5	5	5	5	5	5	5	5
	DMP	Coefficiente di correlazione	,000	,167	1,000	-,913*	-,289	,612	.	,612
		Sig. (a due code)	1,000	,789	.	,030	,638	,272	.	,272
		N	5	5	5	5	5	5	5	5
	BBP	Coefficiente di correlazione	,000	-,304	-,913*	1,000	,527	-,559	.	-,559
		Sig. (a due code)	1,000	,619	,030	.	,361	,327	.	,327
		N	5	5	5	5	5	5	5	5
	DEHP	Coefficiente di correlazione	-,100	-,866	-,289	,527	1,000	-,707	.	,354
		Sig. (a due code)	,873	,058	,638	,361	.	,182	.	,559
		N	5	5	5	5	5	5	5	5
	MBP	Coefficiente di correlazione	,354	,612	,612	-,559	-,707	1,000	.	-,250
		Sig. (a due code)	,559	,272	,272	,327	,182	.	.	,685
		N	5	5	5	5	5	5	5	5
	MBzP	Coefficiente di correlazione	.	.	.	.	.	.	.	.
		Sig. (a due code)	.	.	.	.	.	.	.	.
		N	5	5	5	5	5	5	5	5
	MEHP	Coefficiente di correlazione	-,354	-,408	,612	-,559	,354	-,250	.	1,000
		Sig. (a due code)	,559	,495	,272	,327	,559	,685	.	.
		N	5	5	5	5	5	5	5	5

*. La correlazione è significativa a livello 0,05 (a due code).

Figure S4.22. *Sinularia*: correlation matrix for DMP e BBP (Rho =- 0.913, p =0.030).

Table S3.1 3. Correlation matrix of *Sinularia* sp. (ρ = Spearman correlation coefficient).

		DBP	DEP	**DMP**	**BBP**	DEHP	MBP	MBzP	MEHP
DBP	**ρ**	1.000	- 0.289	0.000	0.000	- 0.100	0.354	.	- 0.354
	p	.	0.638	1.000	1.000	0.873	0.559	.	0.559
DEP	**ρ**	- 0.289	1.000	0.167	- 0.304	- 0.866	0.612	.	- 0.408
	p	0.638	.	0.789	0.619	0.058	0.272	.	0.495
DMP	**ρ**	0.000	0.167	1.000	**- 0.913**	- 0.289	0.612	.	0.612
	p	1.000	0.789	.	**0.030**	0.638	0.272	.	0.272
BBP	**ρ**	0.000	- 0.304	**- 0.913**	1.000	0.527	- 0.559	.	- 0.559
	p	1.000	0.619	**0.030**	.	0.361	0.327	.	0.327
DEHP	**P**	- 0.100	- 0.866	- 0.289	0.527	1.000	- 0.707	.	0.354
	p	0.873	0.058	0.638	0.361	.	0.182	.	0.559
MBP	**ρ**	0.354	0.612	0.612	- 0.559	- 0.707	1.000	.	- 0.250
	p	0.559	0.272	0.272	0.327	0.182	.	.	0.685
MBzP	**ρ**	.	.	.	.	.	.	.	.
	p	.	.	.	.	.	.	.	.
MEHP	**P**	- 0.354	- 0.408	0.612	- 0.559	0.354	- 0.250	.	1.000
	p	0.559	0.495	0.272	0.327	0.559	0.685	.	.

DMP e BBP (Rho =- 0.913, p =0.030)

CHAPTER 5

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

Sara Vencato et al.

In preparation

Abstract

During ageing or particular processes, such as digestion, plastic debris can leach associated additives in the marine environment. This study investigates simultaneously the occurrence of microplastics (MPs) and phthalate esters (PAEs), ubiquitous plasticizers, in wild *Actinia equina* and *Anemonia viridis*, two common and edible sea anemones species, widely present in Sardinia (Western Mediterranean Sea, Italy). MPs were found with a 100% occurrence in both species, dominated by microfibers (94%) and followed by fragments (6%), with an average concentration of 0.79 ± 0.12 particles/g in *A. equina* and 0.79 ± 0.13 particles/g in *A. viridis*. PAEs were detected in the 70% of the sampled specimen, with concentration ranging from 0 to 150.06 ng/g in *A. equina* and between 0 and 144.29 ng/g for *A. viridis*. The occurrence of both MPs and PAEs contamination in sea anemone tissues seems to mirror the seawater plastic pollution conditions of the study area. Given the rapid degradation of PAEs, the concentrations and changing levels of such plastic additives and their metabolites may be employed as an indicator of short term interaction between sessile organism and plastic debris.

5.1. Introduction

Nowadays, over 80% of marine litter is made up of plastic debris (Barnes et al., 2009; Bellas et al., 2016), a worldwide source of pollution due to plastics increasing request and production, high durability and improper disposal (Eriksen et al., 2014). Plastic can persist in the environment for decades (Barnes et al., 2009), thus, it is exposed to all the mechanical, chemical, and biological degradation that lead to the formation of microplastics (MPs), plastic particles smaller than 5 mm in size (Arthur et al., 2009). Currently, MPs are a matter of great environmental concern, mainly because of their ubiquity and their aptitude to interact with the aquatic life both directly, i.e. through physical interactions, like ingestion (Wright et al., 2013), and indirectly, for example as vectors of alien species, diseases and contaminants (Browne et al., 2011; Koelmans et al., 2016; Lamb et al., 2018; Teuten et al., 2009). Since during their production various additives such as plasticizers, flame retardants, stabilizers and pigments are added to plastic polymers, MPs are not only pollutants carriers, but may themselves be a source of pollutants. Among plasticizers, Phthalic Acid Esters (PAEs) or phthalates are chemicals widely employed as softeners in most plastics (Paluselli et al., 2019), mainly polyvinyl chloride (PVC) (Guerranti et al., 2013) but even in other plastic polymers, like polyethylene (PE) (Paluselli et al., 2019), polypropylene (PP) and polyethylene terephthalate (PET) (Rani et al., 2015). Consequently, PAEs can be found in a wide variety of products, like packaging, medical devices, cosmetics, clothing, children's toys, piping, and other building materials (Guerranti et al., 2013; Krauskopf & Godwin, 2005; Oehlmann et al., 2009). PAEs have recently turned into a serious problem owing to their ubiquitous presence in the environments (Net et al., 2015; Xie et al., 2005), their potential to accumulate in aquatic environments (Paluselli et al., 2019; Zhang et al., 2018), their endocrine-disrupting nature and carcinogenic properties, even at low concentrations (Oehlmann et al., 2009; Ye et al., 2014). Not chemically but only physically bound to the polymeric matrix, PAEs can easily be released into the

environment or inside an animal stomach (Andrady et al., 2011), where, thanks to their lipophilic nature, they became easily available for bio-concentration in marine organism tissues (Jaeger & Rubin, 1973; Mathieu-Denoncourt et al., 2016; Net et al., 2015). Indeed, phthalates were found in different organisms, ranging from plankton (Fossi et al., 2012) to fishes (Guerranti et al., 2016), but no bio magnification was observed through the food web (Mackintosh et al., 2006; Hu et al., 2016). Therefore, PAEs occurrence is linked with the diet, living habits and trophic levels of the organisms (Hu et al., 2016) and may depend on the interaction between phthalates and single organism over time. Thus, MPs interaction (e.g. ingestion) may constitute a route of transfer of PAEs in marine organisms. Indeed, a possible correlation between MPs exposure and the presence of PAEs concentrated in tissues was highlighted in diverse marine species (Baini et al., 2017; Fossi et al., 2012, 2014; Vered et al., 2019). Consequently, PAEs detection in marine organisms' tissues has been proposed as a marker of plastics exposure in the marine environment. Still, in the literature the potential role of MPs interactions to the accumulation of PAEs in sea organism tissues due to leaching from plastic debris has been scarcely examined (Saliu et al., 2020a; Vered et al., 2019) and studies on PAEs in aquatic wildlife are rare (Hu et al., 2016). Anthozoans, including sea anemones, stony corals and soft corals, are a class of marine invertebrates still underrepresented in the microplastics literature (Savage et al., 2022). Nevertheless, due to their proximity to the coastlines (Andrady, 2011), their benthic and sedentary nature and non-selective feeding mechanisms (Shick 2012), they are highly exposed to MPs litter (Corona et al., 2020; Soares et al., 2020) and, likely, interface with plastic additives. Currently, most of the studies on the interaction mechanisms and impacts between microplastics (MPs) and anthozoans have been conducted on scleractinian corals, the main builders and major occupiers of reef frameworks. However, other shallow tropical marine environments, many temperate coasts and deep-water marine habitats, are dominated by non-scleractinian anthozoans (Fautin 1989). Sea anemones are non-coral anthozoans worldwide distributed, with over one thousand different species reported

(Thangaraj et al., 2019). Thanks to characteristics like their semisessile nature and heterotrophic, opportunistic and non-selective suspension feeding strategy (Gili & Coma, 1998; Shick, 2012), sea anemones have recently been investigated for macrolitter (Weideman et al., 2020) and microlitter ingestion (Savage et al., 2022) and proposed as potential bioindicators of microplastic contamination (Fang et al., 2021; Morais et al., 2020). Moreover, with the increasing consumption of novel foods, several species of sea anemones have been reported for human consumption (Silva et al., 2017). Anemonia viridis (Forsskål, 1775) and Actinia equina (Linnaeus, 1758) are two wide known sea anemone species, that, due to their widespread distribution and edible nature (González et al., 2001; Silva et al., 2017), are consumed in some Mediterranean regions like Sardinia (Italy) and across the Andalusian coast (Spain). The snakelocks anemone (Anemonia viridis) is a useful anthozoan model organisms, ease of maintenance in aquarium facilities and commonly found in the North East Atlantic, on the western shores of the UK and in the Mediterranean Sea (GBIF Secretariat, 2021; Savage et al., 2022). It is principally planktonivorous and carnivorous (crustaceans, mollusks) and actively search for foods in the surrounding waters through its long tentacles. The beadlet sea anemone Actinia equina (Figure 1) is a common intertidal anthozoan in the Mediterranean and in the Black Sea, diffused a little everywhere from the Atlantic to the Indo-Pacific (Chomsky et al., 2004). It is less active respect to the snakelocks anemone, acting as a sit-and-wait predator that feed on whatever falls onto the tentacles and oral disc (Shick 2012). Generally, they are carnivore (insects, crustaceans, mollusks) and detritivore (organic detritus) (Chintiroglou & Koukouras, 1992). These two species are easily found in the surface portion of the water column, where, they are likely exposed to high concentrations of both MPs (Galgani et al., 2015) and plastic-associated chemicals (Koelmans 2015). With this background, purpose of this work is to investigate the potential use of PAEs as an assessment index of organisms' exposure to plastic microlitter in free ranging marine wildlife. Particularly, this study aims to assess simultaneously the occurrence of MPs and PAEs in wild A. equina and A. viridis

specimens, collected from a small area (scale of around 100 km2) in the west coast of Sardinia (Italy), selected for its high hydrodynamics and wind forces and characterized by a medium-low plastic pollution grade, still comparable to the levels detected in other areas of the Mediterranean Sea (0.15 items/m3) (de Lucia et al., 2014). By exploring the levels and composition of both the contaminants in the target sea anemones, this work aims to explore the application of PAEs levels detection in common and sessile marine organisms as a proxy to track interactions with MPs on a short-term temporal scale.

5.2. Materials and methods

5.2.1. Study area

The study was carried out along the coasts of the Sinis Peninsula (Sardinia), in the middle of the Western Mediterranean Sea sub-region (Marine Strategy Framework Directive, MSFD), which lies between the bay of Is Arenas to the north and the Gulf of Oristano to the south (Figure 1). Off to the Sinis peninsula, there are the island of Mal di Ventre and the Catalano outcrop. The typical wind patterns are the Mistral from north-west (NW), the Libeccio from south-west (SW) and the Sirocco from south-east (SE). The Mistral can be considered the main wind force acting in the area. The long-scale offshore circulation presents strongly different dynamics, mainly characterised by anticyclonic gyres generated by the Algerian Current system (Olita et al., 2013). At surface the mean circulation of the area is characterized by a southward current flowing close to the western Sardinia shore (Olita et al., 2013), characterized by upwelling phenomena on some spots of the west Sardinia coast (Olita et al., 2013).

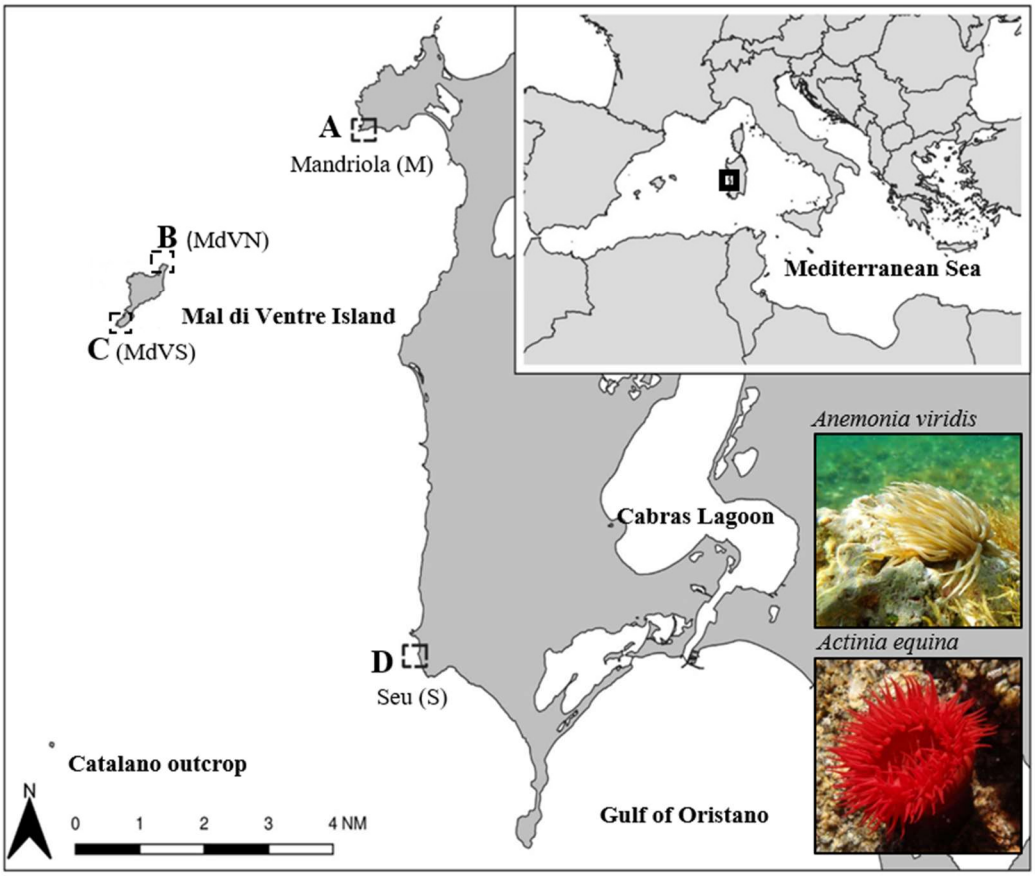

Figure 1. Map of the study area showing the 4 sampled sites around the Sinis Peninsula (Sardinia); A) Mandriola, B) Mal di Ventre Island North, C) Mal di Ventre Island South, D) Seu. On the right, a picture of the target sea anemone species *Anemonia viridis* (picture above) and *Actinia equina* (picture below).

5.2.2. Sample collection

The snakelocks anemone *Anemonia viridis* (Forsskål and Niebuhr, 1775) (Figure 1) is a temperate sea anemone in the class Anthozoa. This anemone species is a useful anthozoan model organisms ease of maintenance in aquarium facilities and commonly found in the North East Atlantic, on the western shores of the UK and in the Mediterranean Sea (GBIF Secretariat, 2021; Savage et al., 2022). Long tentacles surround the mouth, sweeping the ocean water, actively searching for foods, while the column and oral disk are less active than in *Actinia equina* (Shick, 2012). It is principally

planktonivorous and carnivorous (crustaceans, mollusks). The beadlet sea anemone *Actinia equina* (L.) (Figure 1) is a common intertidal actinian anthozoan in the Mediterranean and in the Black Sea, but actually diffused a little everywhere from the Atlantic to the Indo-Pacific (Chomsky et al., 2004). Technically, beadlet anemones are sedentary, since they are capable of very slow movement, but in terms of feeding they are regarded as sessile sit-and-wait predators that appear to feed on whatever falls onto the tentacles and oral disc (Shick 2012). Generally, they are carnivore (insects, crustaceans, mollusks) and detritivore (organic detritus) (Chintiroglou & Koukouras, 1992).

5 specimens of each sea anemone species were randomly collected in each one of the 4 sites, for a total of 40 specimens of sea anemones, 20 *Actinia equina* and 20 *Anemonia viridis*. The choice of the species was based on an initial evaluation of the most common and easily available sea anemone species in the area and on which we had enough information related to their ecology and biology. Samplings were conducted in 4 coastal sites: Mandriola (M) (Figure 1A), Mal di Ventre North (MdVN) (Figure 1B), Mal di Ventre South (MdVS) (Figure 1C) and Seu (S) (Figure 1D). A pre-survey along the Sinis coastline has been done in order to identify the sites where both the species could be found simultaneously. Moreover, in order to favour spatial comparisons and avoid intrinsic variability to local environmental conditions, field expeditions were concentrated in only one month (May 2022). The individuals were collected by two operators by snorkelling, depth range 0 – 2.5m. The entire body of each individual was collected from the field by gently detaching the pedal disc from the substrate with the aid of a metal spatula. Then, the specimens were stored in individual glass jars filled with seawater from the collection site. Moreover, 6 aliquots of 20 mL in triplicates were collected in glass vials from each site in order to evaluate the average concentration levels of phthalates in the seawater.

5.2.3. Sample processing

In the laboratory, the sea anemones were immediately weighed (wet weight, second decimal point; g) and thoroughly rinsed with pre-filtered saltwater in order to collect potentially adhered microplastics onto a 50 µm sieve (Giuliani steel sieves). Any material regurgitated due to contraction of the central column or attached on tentacles or external tissues was collected by filtering the seawater of each glass container on the same steel sieve (Chintiroglou & Koukouras, 1991; Morais et al., 2020). 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 glass vials at – 80 °C for future phthalates assessment analyses. Water sample aliquots from each sample site were stored at – 80 °C as well. Specimens were then placed into individual glass beakers (200 mL) and 15% H_2O_2 1:20 (w/v) was added in order to digest the organic matter (Nuelle et al., 2014), keeping them at room temperature (~25-30 °C) for 20 days. Once the organic material had been digested, the solution was filtered onto 50 µm sieve (Giuliani steel sieves) and positioned in single glass Petri dish in order 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). An item was considered to be a microplastic particle if no cellular or organic structure was visible and it was homogeneously coloured (Hidalgo-Ruz et al., 2012; Primpke et al., 2020). Fine-tipped tweezers were used to position the detected microplastic particles onto individual Petri dishes. Potential MPs items were photographed, counted, and the maximum length was measured by means of image analysis. In the case of fibres that presented bendings, the length was estimated when possible. The items were classified by colours (transparent white, blue, light blue, black, yellow, red, orange, pink, purple, green) and shapes (circular, angular, spherical, flat, irregular and cylindrical). Then, MPs were subdivided into different typologies according to Hidalgo-Ruz et al. (2012):

fragment, film, sphere, rope/filament, sponge/foam and fibre. Natural food items were also counted.

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

Common practices to minimize contamination were adopted during collection and processing of the samples. To reduce phthalate background contamination during sample collection and manipulation no plastic items were used. All glassware was baked at 300 °C and precleaned with acetone before use. The number of people in circulation at the laboratory was reduced. Before and after being used, all laboratory and field equipment, glassware and tools were cleaned with 90% alcohol diluted in distilled water. Other precautions were also taken during manipulation, extraction, sorting and visual identification such as: wearing a cotton laboratory coat, cleaning all surfaces with alcohol, covering the samples at all times during analysis and positioning clean filters while analysing samples to collect any atmospheric microplastics created during laboratory procedures. Airborne contamination has been recognised as an important parameter to monitor while performing any study involving synthetic microlitters. Therefore, laboratory atmospheric deposition was monitored to obtain an estimate of the level of potential airborne contamination.

5.2.5. Phthalates analysis

In both sea anemone samples and seawater samples, the presence and quantification of the 5 target phthalates congeners (butyl benzyl 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 mono benzyl phthalate or MBP (monoester of BBP) were assessed by employing the solid-phase micro-extraction in vivo (BioSPME) coupled with liquid chromatography and mass spectrometry (LC–MS) analyses, following the method described in Saliu et al. (2020a) adapted to the sea

anemone samples. The SPME is a technique of extraction based on the equilibration of analytes between the sample matrix (gaseous, aqueous or solid) and an organic polymeric phase, through a coated fused-silica fiber. Before the use, the fibers were activated in 1 mL of methanol for 15 min. The fiber extraction step was performed in direct immersion mode for 40 min at room temperature (25 °C). The SPME fibers (C18 purchased from Supelco) were inserted directly in the sea anemones tissues to enable the extraction of the analytes. Then the fibers were removed from the sample and placed in a glass vial containing 1 ml of ultrapure water for the washing step, then placed in 80 µL of pure methanol for performing the desorption step for 30 minutes. Then, the fiber was taken out from the vial and the final extract was submitted to LC/MS analysis. LC/MS analyses were carried out by using a ThermoScientific TSQ quantum access max instrument, following the instrumental set up and applying, for PAEs detection, the selected reaction monitoring (SRM) of the mass transitions described in Saliu et al. (2021). Calibration of the system was obtained by using standard calibration mixture and labelled internal standards as described in Saliu et al. (2021).

5.2.6. Statistical analysis

Two-way permutational analysis of variance (Permanova) was used to check for any significant difference in the sea anemone weight and any significant difference in the abundance of fibres and fragments (and their sum) as well as in the PAEs concentrations according to species (fixed factor with two levels*: Actinia equina*, *Anemonia viridis*) and sites (random factor with 4 levels: S, M, MdVN, MdVS). All these univariate tests were run with the PRIMER6 statistical software (Plymouth Marine Laboratory, UK) complete with PERMANOVA+ package on square root transformed data and based on Euclidean distance; each term was analysed using 9999 random permutations and associated with a Monte Carlo test (Anderson et al., 2008). Pearson correlation tests were performed to investigate the linear relationship between the total plastic items, fibres and fragments and the sum of the 8 phthalates (Σ_8PAEs), the sum of the 5 phthalate congeners (Σ_5PAEs),

the sum of the phthale monoesters (Σ_3MPEs) and single PAEs (DBP, BBP, DEP, DEHP, DMP, MBP, MBzP and MEHP).

5.3. Results

5.3.1. Sea anemones morphometric parameter

The mean wet weight was 15.73 ±1.3 g for *Actinia equina* samples, ranging from 7.81 to 35.19 g. For *Anemonia viridis*, the mean wet weight was 20.54 ±2.10 g, ranging from 8.39 to 43.04 g. Actinia equina specimens showed an average wet weight of 20.85 ± 3.63 in Seu, 11.78 ± 0.86 in Mandriola, 14.17 ± 2.54 in Mal di Ventre North and 18.93 ± 3.77 in Mal di Ventre South. Anemonia viridis specimen showed an average wet weight equal to 20.60 ± 3.73 in Seu, 21.29 ± 6.3 in Mandriola, 21.36 ± 3.88 in Mal di Ventre North and 18.93 ± 3.78 in Mal di Ventre South. The weight of sea anemone specimens did not differ significantly between species ($p = 0.1426$) nor between sampled sites ($p = 0.4107$).

5.3.2. Microplastics assessment

The sample analysis under the stereoscope revealed 100% of MPs occurrence for both species. A total of 513 microplastic items in the size range 330µm – 5mm were found (*Actinia equina* N = 247; *Anemonia viridis* N = 266) with an average concentration of 12.35 ± 2.06 particles/ ind. and 0.79 ± 0.12 particles/g in *A. equina* and 13.30 ± 1.68 particles/ind. and 0.79 ± 0.13 particles/g in *A. viridis* (Figure 2). In both the species, 94% of the microplastic items assessed were microfibres and 6% fragments. The most abundant colours were transparent and blue (36% and 35% respectively), followed by black (20%), green (4%), light blue (2%), purple (2%) and red (1%). Specifically, with regard to *Actinia equina*, a minimum number of 1 plastic item (found in Mal di Ventre South) and a maximum of 35 plastic items (found in Seu) were assessed. Seu displayed the highest level of MPs contamination (17.8 ± 5.15 items/ind), followed by Mal di Ventre North (12.8 ± 2.52 items/ind), Mal di Ventre South (9.6 ± 5.23 items/ind) and

Mandriola (9.2 ± 2.94 items/ind). *A. viridis* specimens showed a minimum number of 2 plastic items (found in Mal di Ventre South) and a maximum number of 33 plastic items (Seu). Mandriola was the site with *A. viridis* most contaminated (16.6 ± 2.48 items/ind), followed by Seu (15.6 ± 4.95 items/ind) and the two sites in Mal di Ventre island (12.2 ± 1.98 items/ind North; 8.4 ± 4.06) items/ind South). Overall, Mal di Ventre South site was the less impacted by MPs presence (N = 87), followed by Mandriola (N=129), Mal di Ventre North (N=130) and Seu (N=167). In all the sites, fibres were the major component of the plastic items found for both the sea anemone species (Figure 2A, 2B). Remarkably, *A. equina* sampled in Seu and *A. viridis* specimens sampled in Mal di Ventre South, respectively, presented no plastic fragments (Figure 2C), so the MPs items found in such specimens were only fibres. Plastic fragments were a minor component in all the sea anemone specimens sampled in all the sites. Both for *A. equina* and *A. viridis*, Mandriola resulted the site with the major number of fragments (*A. equina*: 7, 1.4 ± 0.93 frags/ind; *A. viridis*: 13, 2.6 ± 1.43 frags/ind). For *A. equina*, followed Mal di Ventre South (N = 6, all found in a single sample), Mal di Ventre North (N = 3, all found in a single sample) and Seu, with no fragments found. For *A. viridis*, Seu was the second site in terms of fragments found (N = 3, in three different samples), Mal di Ventre North (N = 1) and finally Mal di Ventre South with zero plastic fragments. The analysis of variance on total MPs abundance, fibres abundance and fragments abundance among samples did not show any significant differences between species (MPs: p = 0.5262; Fibres: p = 0.503; Fragments: p = 0.7837), nor between the sites (MPs: p = 0.1389; Fibres: p = 0.1236; Fragments: p = 0.0949). In 13 specimens (5 *A. viridis* and 8 *A. equina*) small limpet shells, sea-urchin spikes and crustacean claws were found, all expelled in the glass container after field collection and in the solution after H_2O_2 digestion.

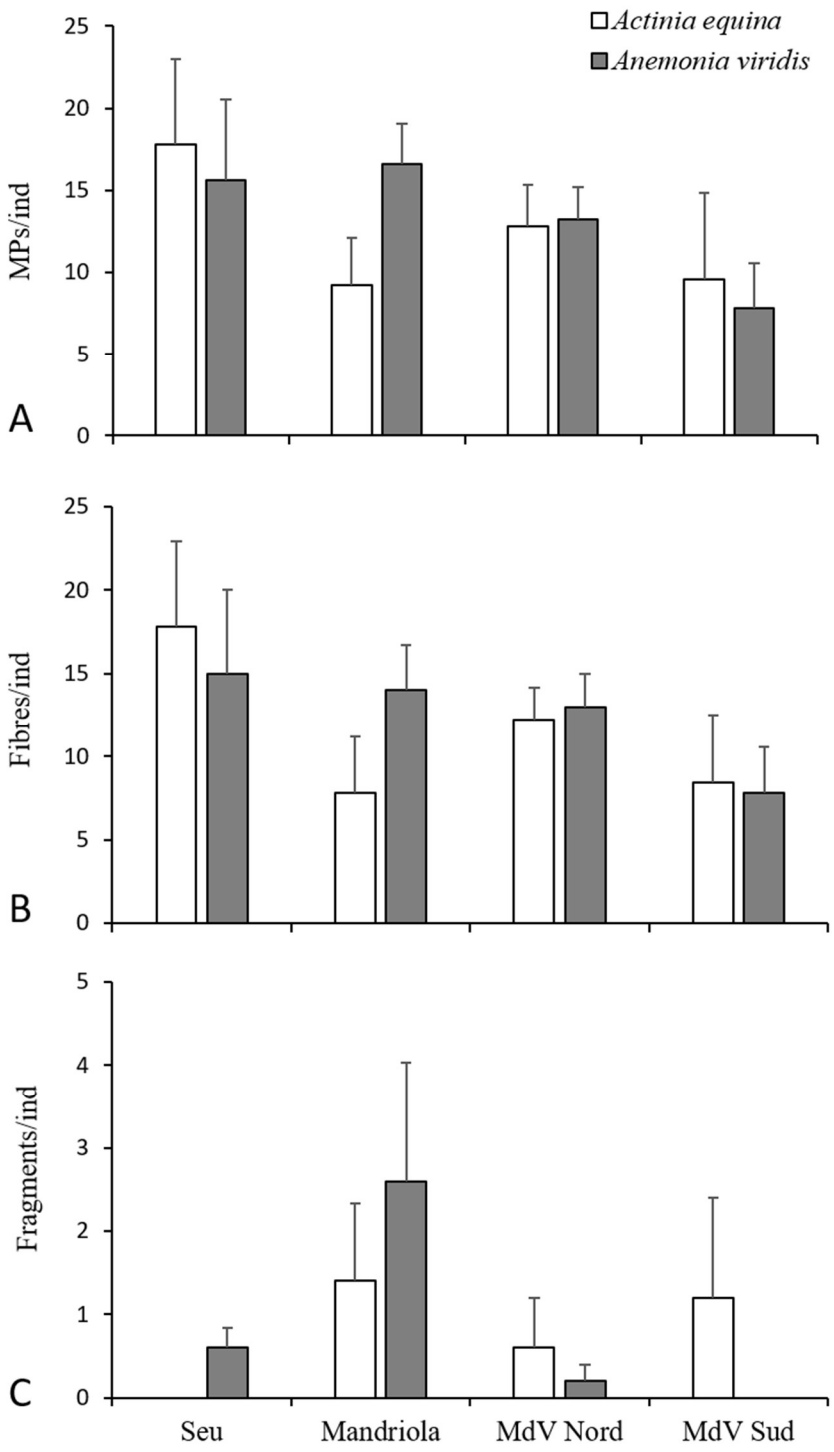

Figure 2. Average concentrations of A) all the MPs items, B) only fibres and C) only fragments detected in *Actinia equina* (white) and *Anemonia viridis* (grey) sea anemone specimens according to the various sites considered. All the concentrations are reported with SE.

5.3.3. PAEs assessment by BioSPME-LC/MS

The mean values of PAEs concentration, obtained through SPME-LC/MS and assessed per species and sites are reported in figure 3A. Phthalates were found in all the sampled sites and in the 70% of all the sea anemone samples (13 *A. equina* and 15 *A. viridis*), with an average concentration of 67.74 ± 13.30 ng/g and 61.80 ± 11.67 ng/g for *A. equina* and *A. viridis* respectively. In *A. equina* specimens, the maximum concentration value for the total sum of PAEs was 150.06 ng/g, with 90.35 ng/g as the highest value for the Σ_5PAEs congeners and 119.92 ng/g as the maximum concentration for the sum of the monoester phthalates MBP, MEHP and MBzP (Σ_3MPEs). In general, Σ_3MPEs was more represented in relation to Σ_5PAEs (Figure 3B). In particular, Seu showed an average concentration of Σ_5PAEs of 21.57 ± 17.61 ng/g and 46.45 ± 28.44 ng/g for the metabolites concentration; Mandriola showed an average concentration of the 5 phthalate congeners Σ_5PAEs=13.12 ng/g and average concentration of metabolites = 46.46 ± 28.45 ng/g; MdVN showed an average concentration of 11.60 ± 7.11 for Σ_5PAEs and of 58.91 ± 26.48 ng/g for the metabolites; MdVS did not show PAEs congeners but only metabolites, for a concentration of 69.84 ± 28.51 ng/g. For *A. viridis* specimens, the maximum concentration value for the total sum of PAEs was 144.29 ng/g, with 116.74 ng/g as the highest value for the Σ_5PAEs congeners and 144.29 ng/g as the maximum concentration for the sum of the metabolites MBP, MEHP and MBzP. Specifically, Seu

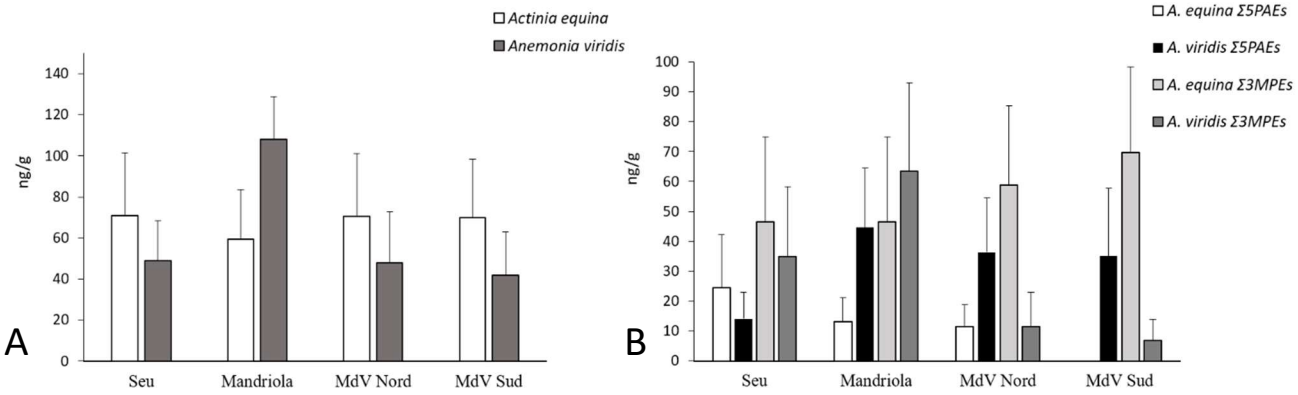

Figure 3 A) average concentrations of the sum of the 8 PAEs and B) average concentrations divided by 5 PAEs congeners (Σ_5PAEs) and 3 metabolites (Σ_3MPEs) detected in *Actinia equina* and *Anemonia viridis* samples in the different sites surveyed. Error bars indicate Standard Errors.

showed an average Σ_5PAEs concentration of 14.17 ± 8.74 ng/g and 34.88 ± 23.30 ng/g for the Σ_3MPEs concentration; Mandriola showed average Σ_5PAEs concentration of 44.64 ± 19.90 ng/g and average concentration of metabolites = 63.56 ± 29.49 ng/g; MdVN showed an average concentration of 36.43 ± 18.06 for Σ_5PAEs and of 11.51 ± 11.51 ng/g for the metabolites; MdVS showed average Σ_5PAEs concentration of 35.06 ± 22.76 and a concentration of 6.93 ± 6.93 ng/g for metabolites (Figure 3B). The most represented between the considered 8PAEs is the short chain phthalate MBP, with a total average of 40.81 ± 8.41 (55.41± 13.03 for *A. equina*; 26.21 ± 9.92 for *A. viridis*), followed by DEP, with a total average of 11.17 ± 3.47 (7.8 ± 3.11 for *A. equina*; 14.54 ± 6.20 for *A. viridis*) and DBP, with a total average of 6.73 ± 3.78 (4.52 ± 4.52 for *A. equina*; 8.94 ± 6.15 for *A. viridis*) (Figure 4). Between the target PAEs and MPEs considered, BBP was the only phthalate ester not detected in any of the samples (Figure 4). Permanova analyses did not show any significant variation for the distribution of the Σ_5PAEs congeners and for Σ_3MPEs among the 2 target species (Σ_5PAEs: p =0.1183; Σ_3MPEs: p = 0.2786) nor between the sites (Σ_5PAEs: p =0.6102; Σ_3MPEs: p = 0.9042) considered. No

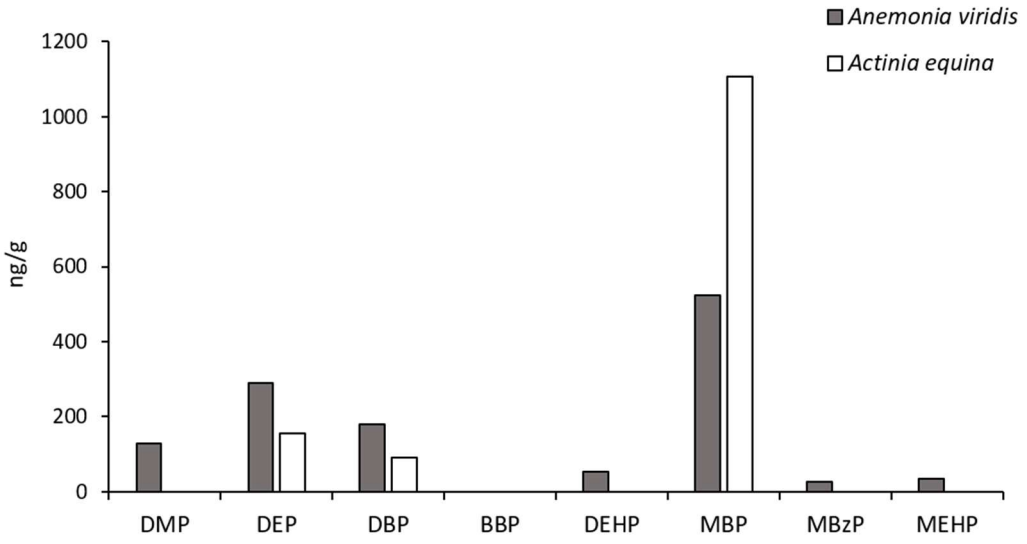

Figure 4. Single PAE congeners (DMP, DEP, DBP, BBP and DEHP) and target metabolites (MBP, MBzP and MEHP) concentrations detected in *Actinia equina* and *Anemonia viridis* samples. Concentrations are reported in ng/g of sea anemone specimen.

significant differences were highlighted among the concentrations of each phthalate congener and each metabolite between the two sea anemone species (p = 0.3406) nor between the different sampling sites (p = 0.8316) (Figure 5A, 5B). Seawater samples showed an average total concentration of 13.99 ±3.77, with a higher concentration of Σ_5PAEs with respect to Σ_3MPEs in each sampled site (Table 1).

Table 1. Average concentration of phthalate ester congeners (Σ5PAEs), monoester phthalates (Σ3MPEs) and the sum of all the phthalates (Σ8PAEs) found in seawater collected in each sampled site (mean ± ES). All values are expressed as ng/g.

	Σ_5PAEs	Σ_3MPEs	Σ_8PAEs
Seu	1.8 ± 1.8	0.4 ± 0.3	2.2 ± 2.1
Mandriola	15.9 ± 12.5	5.5 ± 2.5	21.4 ± 11.5
Mal di Ventre North	10.7 ± 4.2	5.6 ± 2.7	16.2 ± 4.5
Mal di Ventre South	9.1 ± 5.8	7.0 ± 3.0	16.1 ± 8.0

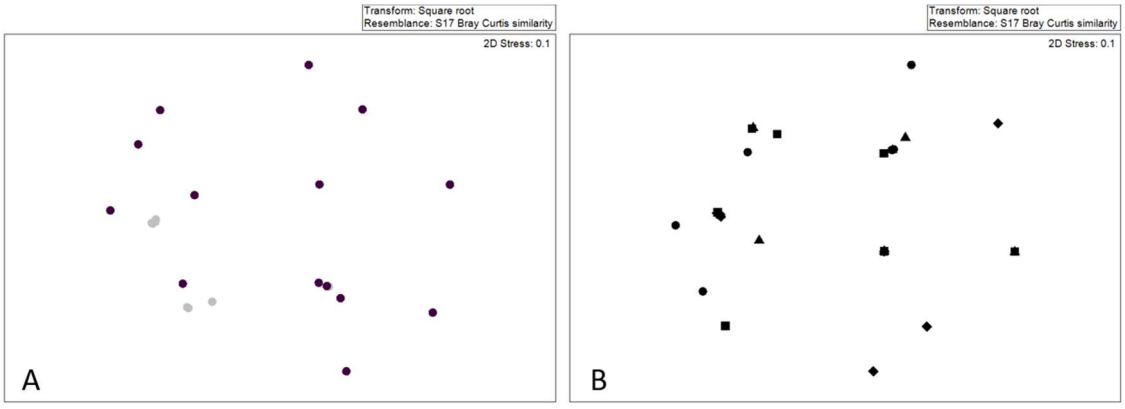

Figure 5 A) multidimensional scaling plot (MDS) showing the distribution of the phthalate esters according to the different sea anemone species considered. The grey circles represent *Actinia equina* and dark circles represent *Anemonia viridis* and B) multidimensional scaling plot (MDS) showing the distribution of the phthalate esters according to the different sites considered (Seu black triangle; Mandriola black circle; Mal di Ventre N. black square; Mal di Ventre S. black rhomus).

5.3.4. Statistical analyses on MPs and PAEs dataset

Pearson's product-moment correlation tests were run to assess the relationship between the total plastic items, fibres and fragments with the Σ_8PAEs, Σ_5PAEs, Σ_3MPEs and each PAEs. There was a statistically significant, moderate positive correlation between the total number of fibres and the Σ_8PAEs, (ρ (38) = .31, $p < 0.05$), with a statistically significant, moderate positive correlation between the number of fibres and Σ_8PAEs found in *Anemonia viridis*, (ρ (18) = .48, $p < 0,05$). Moreover, an increase in Σ_5PAEs was moderately correlated with an increase in the total number of MPs items for the sum of sea anemone specimens, (ρ (38) = .32, $p < 0,05$), with a statistically strong positive correlation between the Σ_5PAEs and the MPs items in *Actinia equina*, (ρ (18)= .61, $p < 0,01$).

5.4. Discussion

The current study explores the occurrence and distribution of both microplastics and phthalate esters in *Actinia equina* and *Anemonia viridis* investigating at the same time the potential use of PAEs levels detection in marine organism's tissues as proxy of their interaction with plastic litter. To this aim, samples were collected in an area expressly chosen for its its high hydrodynamics and wind forces, in order to detect any potential different pattern in the interactions of both MPs and PAEs contaminants with the target anemones. In this study plastic microlitter is always present either at spatial scale and in the sea anemones collected, while PAEs are detected in all the sampled sites and in the 70% of the collected organisms. Remarkably, PAEs levels seem to follow the behaviour shown by MPs pollution patterns: indeed, both the pollutants are characterized by a uniform occurrence and distribution between *A. viridis* and *A. equina*. Such uniformity is highlighted even at spatial scale, with a homogenous distribution of plastic microlitter and phthalates in the 4 sampled sites. Particularly, the results highlight the interaction of wild sea anemones with MPs. Indeed, the 100% frequency of plastic occurrence

detected in our samples confirm that even *A. equina* and *A. viridis* sea anemones, besides the species already mentioned in the literature, widely interact with MPs particles, supporting the potential role anemones can play as environmental plastic bioindicators (Morais et al., 2020; Savage et al., 2022). Noteworthy, the contribution of fragments compared to the total MPs found in our samples is negligible, since 94% of the MPs particles that interacted with both of the sea anemone specimens are fibres. Savage et al. (2022) describes a similar situation, with fibres representing the majority of particles (91%) taken up by *A. viridis* specimens, followed by fragments (9%) both in experimental and field studies. A comparable pattern is described even in Morais et al. (2020), where plastic fibres recovered from the sea anemone *Bunodosoma cangicum* comprise about 84% of the ingested plastics, followed by fragments (~12%). These abundances of detected plastic fibres may be related to the particular environmental habitat occupied by the anemones investigated. Indeed, our anemones were all collected in the surface compartment of the water column, where, due to their slower vertical advection velocity (Ballent et al., 2012; Reisser et al., 2013), fibres reside for longer time, being potentially more available for interactions with sea organisms respect to other MPs shapes. Laboratory exposure experiments underline for *A. viridis* a limited uptake selectivity for different particle shapes and sizes (Savage et al., 2022), and that sea anemones feeding behaviour is regularly mediated by chemical cues from natural food items (Romanó de Orte et al., 2019). Knowing that they feed using a variety of mechanisms, for example active chemoreception, passive suspension feeding or incidental ingestion (Gili and Coma, 1998; Kamio & Derby, 2017), the observed condition boosts the idea that the MPs content found in sea anemones may not be explained by a particular feeding preference of the organism, but by the MPs contamination conditions of the surrounding environment, that is consequently mirrored in the anemone diet. Our results corroborate such hypothesis: in our anemone samples, MPs contamination results homogeneous, with no significant differences in the plastic occurrence and distribution at spatial scale nor between the two sea anemone species.

Therefore, these last outcomes suggest that the exposition to plastic litter and interactions of our target species with MPs are similar, reflecting the environmental component despite the ecological niche of the two different anemone species. The spatial homogeneity in MPs contamination found reflects the seawater plastic pollution condition of the Sinis region. Indeed, the waters in internal and adjacent areas to the Gulf of Oristano are described as a "plastic soup" (Suaria et al., 2016), characterized by a ubiquitous presence of plastic microlitter in the entire area (de Lucia et al., 2014), with a high average presence of microplastics (0.15 items/m^3). The homogeneity in MPs and PAEs levels and distribution found in this study reflects such conditions at a smaller geographical scale, mirroring the homogeneous situation that characterized the entire area. Consequently, in order to notice potential differences in the uptake of such contaminants and their distribution patterns, it should be necessary to consider a broader study area, characterized by defined differences in terms of plastic pollution and see if the already detected differences in the presence of plastic contamination, are reflected by differences in phthalates pollution, both in seawater and biota samples. As previously mentioned, phthalate esters presence and distribution detected in this study follow the pattern shown by MPs occurrences found in the sea anemone specimens and in the Sinis region. Indeed, Σ_8PAEs (BBP, DBP, DEHP, DEP, DMP, MEHP, MBP and MBzP) has been detected in all the sampled area, with uniform concentrations between species and sites. Among the phthalate esters and metabolites considered in this study, MBP, DEP and DBP were the PAEs most represented in our biota samples. Particularly, DEP was the phthalate congener mostly found and, even if no statistical significance was detected, it was present a little more in *A. viridis* with respect to *A. equina*, while it was homogenously present in all the sampled sites. It is used in the manufacture of perfumes, plastics, mosquito repellents and cleaning products (United States Environmental Protection Agency, 2023) and finds some use as a specialist plasticiser in PVC. It easily penetrates soil, contaminates groundwater and, through nearby waterways, it reaches the sea (Dionisio et al., 2018). Such phthalate ester is denser than

water and insoluble in water, therefore DEP tends to sink (CAMEO Chemicals, 2022) towards the benthic environment, where sea anemone specimens lie. DBP is principally used as plasticizers for PVC different items as well as for fibre and fabric manufacturing in textiles industries (Haz-Map, 2022). The wide occurrence of such phthalate and especially of its principal metabolite (MBP), can be linked to the high presence of fibres found in this study. Indeed, microfibres are usually released during washing processes of synthetic clothes, with numbers that range from 124 to 308 mg for kg of washed fabric depending from the type of washed garment, that corresponds to a number of microfibres ranging from 640,000 to 1,500,000 (De Falco et al., 2018). Conversely to our results, DEHP is generally one of the predominant PAEs found in seawaters and marine biota (Fossi et al, 2012). However, there are studies that showed that DBP, instead of DEHP, was the predominant PAEs compound in fish (Lin et al., 2003) and indicate that the distribution of PAEs in the biota is source-specific (Hu et al., 2016). This result could be related to the fact that DEHP consumption has gradually been replaced by diisononyl phthalate (DiNP e Pubchem ID: 590836), diisodecyl phthalate (DiDP e Pubchem ID: 33599) and di(2-Propyl Heptyl) phthalate (DPHP e Pubchem ID: 92344), which represented 57% of plasticizer consumption in Europe in 2015 (Koch et al., 2007). Also, such PAEs pattern could be related to different degradation rate at environmental conditions. For example, Paluselli et al., (2019) show in an experimental study that DEP exhibits a major half-live (t1/2 = 53 days) compared to DEHP half-life (t1/2 = 26 days) in seawater at dark biotic condition, suggesting a faster biodegradation of DEHP respect to DEP at environmental conditions. Moreover, despite DEHP is usually used as the most common plasticizer to soften plastic (mainly in PVC), multiple phthalates were detected in different plastic marine debris (Rani et al., 2015). Thus, as suggested by Li et al. (2016), to identify the compound or its degradation products that could interact with the marine organisms, chemical identification should be undertaken during leaching experiments with a focus on plastic additives. PAEs undergo trophic dilution in the marine food web, which is likely to be the combined results of low assimilation efficiencies and efficient

metabolic transformation at higher trophic levels (Mackintosh et al., 2006). Indeed, phthalates do not bioaccumulate in living organisms, but are quickly metabolized within a few hours into their corresponding monoesters (Frederiksen et al., 2007; Liang et al., 2008), through mechanisms of bio-concentration and metabolism that in wild aquatic species remain mostly unclear. Generally, higher detection frequencies of phthale monoesters (MPEs) are observed than those of parent PAEs (Hu et al., 2016). Such condition is present in this study too, since our target sea anemone specimens present an almost double Σ_3MPEs concentration (1692,68 ng/g) with respect to the Σ_5PAEs concentration (897,97 ng/g). In particular, there is no sea anemone specimen where a phthalate congener and its principal metabolite are simultaneously present and only in 6 specimens out of 40, Phthalate congeners and MPEs were found at the same time. These outcomes seem to boost the idea to use the primary metabolites of PAEs, as a PAEs presence indicator (Hu et al., 2016), with MPEs that could qualitatively reflect contamination by PAEs and low molecular-weight MPEs (e.g. MBP) can quantitatively reflect PAEs contamination in wild marine organisms and be used as marker for PAEs exposure (Hu et al., 2016). As described in section 3, Σ_8PAEs concentrations in our biota samples resulted higher than Σ_8PAEs in our seawater samples, especially referring to the Σ_3MPEs. Indeed, in every sampled site, the Σ_3MPEs detected in seawater resulted lower than the sum of the 5 phthalate congeners, describing an opposite pattern compared to *A. equina* and *A. viridis*, where in every targeted site the sum of metabolites resulted always greater than the sum of the 5 PAEs congeners. The fact that in sea anemones PAEs were detected at higher levels with respect to the seawater samples, should be a proof of the ability of sea anemones to bio-concentrate PAEs with respect to the surrounding environment. Moreover, the higher levels of monoalkyl phthalates in the tissues of the examined sea anemones respect to the 5 PAEs congeners detected should be considered a proof of a possible metabolic pathway, since monoalkyl phthalates are obtained from hydrolyzation as the first step of metabolic pathways of phthalates in many organisms (Blair et al., 2009; Silva et al., 2007) before excretion. However, such

considerations must be carefully examined in future lab feeding experiments and confirmed by additional on-field assessment. Indeed, physiological aspects, such as the residence time of MPs in the sea anemone coelenteron and kinetic rates in the metabolization of PAEs in MPEs are important variables that can influence the MPs and PAEs profiles. In this context also the choice of the analytical procedure for the extraction of PAEs is highly relevant. In fact, by SPME only the "free and unbounded" fraction of PAEs is extracted (Isa et al., 2022), thus the detected PAEs are those that accumulated in the tissue due to the uptake from seawater or by leaching after ingestion or interaction with plastic debris. Since PAEs are not persistent and are biodegraded in the environment (Net et al., 2015), they are ubiquitous in the environment because of the continuous dispersion of plastic and thus living organisms can be continuously exposed to PAEs (Baini et al., 2017; Mackintosh et al., 2006; Net et al., 2015). A significant linear correlation between the MPs presence and PAEs contamination was found in both the two sea anemone species. Specifically, in *A. viridis* there was a statistically significant moderate positive relationship between the number of fibres and Σ_8PAEs, while for *A. equina* an increase in Σ_5PAEs was moderately correlated with an increase in the total number of MPs items. This can be explained as the result of a complex interaction between direct bio-concentration and metabolism in the biota, which should be species-dependent, but highlight that with increasing of the microplastic litter levels (fibres or total MPs items), a moderate increase of PAEs levels is found in both *A. equina* and *A. viridis*. These results support the concept of PAEs (or especially MPEs) as MPs pollution tracers. Particularly, given the rapid degradation of PAEs congeners into their corresponding MPEs, the concentrations and changing levels of both PAEs congeners and MPEs should be employed as an indicator of short-term interaction with plastic, where, for example, constant levels of PAEs could be related to continuous dispersion of plastic, higher PAEs congeners levels could be index of a recent approach with plastic debris and growing levels of MPEs could indicate a past interaction, according to the metabolic pathways of the considered organism indicator.

This could be particularly relevant during monitoring activities of microplastic presence in aquatic environments, allowing, for example, to appreciate the effectiveness of mitigation measures in the short term or seasonal changes in the source of such pollutants. However, our data did not show a clear correlation pattern between the total concentration of MPs and the levels of PAEs, and thus did not totally confirm the contribution of MPs by leaching to the contamination of sea anemones tissues, which should be further investigated. In the last few decades, several efforts have been devoted to the development of reliable methods to evaluate MPs concentrations in the marine environments and different methodologies are used to measure microplastic contamination (Hidalgo-Ruz et al., 2012). Microlitter ingestion is currently being assessed in various organisms ranging from invertebrates to vertebrates (Wright et al., 2013). However, in order to investigate the presence of plastic items in biota, the extraction of microplastics from biological matrices required usually lethal approaches, with the necessity to suppress the target organisms. This point should be carefully taken into account for conducting research programs in vulnerable or protected marine environments, particularly if involving endangered species. SPME is a not exhaustive extraction technique with a good sensitivity in the analyses of marine invertebrates (Saliu et al., 2020a). Such methodology involves the use of a fiber in a needle probe format directly immerged in the investigated matrices. The introduction of biocompatible coatings (BioSPME) allows direct extraction of analytes from biological matrices (Kennedy et al., 2010) and even *in vivo* (Saliu et al., 2020b). From this point of view, sea anemones may be very promising organisms: indeed, it is potentially possible to detach them from the sediment and, after the PAEs absorption through the BioSPME fiber applied *in vivo* (e.g. in Saliu et al., 2020b), easily put them back without causing too many damage to the specimens and without suppress them. However, further investigations are needed to prove that the insertion of the C18 fibers into the tissue of such organisms do not cause any change in the health status of sea anemones. Sea anemones have great culinary potential and possess excellent nutritional properties

(Silva et al., 2017), and are particularly popular as a delicacy along the coasts of Sardinia. Together with gastronomic, economic, and ecological considerations, also marine pollution is becoming a new issue to be included in the overall evaluation of seafoods (Vital et al., 2021). Of course, the consumption of sea anemones must be considered occasional and depending on the traditional habits. However, the 100% frequency of MPs detection found in this work, show how through the consumption of both *A. viridis* and *A. equina*, humans are exposed to MPs ingestion. Since phthalates are hepatotoxic, teratogenic, and carcinogenic by nature (Liang et al., 2008) and given evidence regarding human exposure to microplastics via seafood with potential effects on human health (Smith et al., 2018), our results on the occurrence of such pollutants in these organisms justify further studies to assess the safety of sea anemones for human consumption. However, it is necessary to consider that sea anemones are fried before the consumption and that phthalate concentrations in food usually decline after cooking (Fierens et al., 2012), even if no data are available to confirm such information for sea anemones too.

5.5. Conclusions

This study documents microplastics and phthalate esters uptake by the snakelocks anemone *Anemonia viridis* and the beadlet anemone *Actinia equina* in the marine environment. Both the pollutants were detected in the target sea anemone species, highlighting the ability of such organisms to bio-concentrate at once both MPs and PAEs. Our results support the use of sea anemones as bioindicator for microplastic contamination in marine environments (Fang et al., 2021; Morais et al., 2020; Savage et al., 2022). Moreover, since the MPs interaction patterns and PAEs characteristics found for *A. equina* and *A. viridis* bodies appear to reflect the plastic conditions of the area where the target sea anemones were collected, this work suggest to apply the detection of phthalate ester levels in sea anemone tissues as a proxy of their interaction with

environmental found MPs. So, further studies in new areas characterized by different plastic pollution conditions is necessary to collect valuable information to better investigate the potential role of PAEs levels as marker of MPs contamination using sea anemones as PAEs indicators.

5.6. References

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CHAPTER 6

6.1. Conclusions

The ocean is of eminent importance to mankind. Twenty-three per cent of the world's population (~1.2 billion people) live within 100 km of the coast (Small and Nicholls 2003), a figure, which is likely to rise up to 50 % by 2030 (Adger et al., 2005). Although human welfare is intricately linked with the sea and its natural resources, people have substantially altered the face of the ocean within only a few centuries. As a result, marine environmental protection and management have become integral political and societal issues in many countries worldwide (Bergmann et al., 2015). However, effective environmental management requires a proper understanding of the ecological implications of human activities. The pollution of the seas caused by the dispersion of anthropogenic litter has been recognized as one of the most serious environmental emergencies worldwide. Various studies show that the majority of marine litter items in the sea consists primarily of plastics (Barnes et al., 2009; Bellas et al., 2016), mainly due to their continuously increasing global production and the fact that they are virtually immune to environmental degradation. Plastic debris exists worldwide and research on such topic and particularly on microplastic pollution has gradually included ocean, freshwater and terrestrial systems (Bergmann et al., 2015).

Coral reefs are large ecological reservoirs of marine biodiversity, providing indispensable habitats for marine life, and one of the most productive and valuable ecosystems on earth (D'Angelo & Wiedenmann, 2014; Han et al., 2020). Prolonged warming, ocean acidification, deoxygenation, weakening of benthic-pelagic coupling, high intensity and frequency of heat waves, and marine pollution have caused mass mortality of reef-building corals in the ongoing Anthropocene (Altieri et al., 2017; Eyre et al., 2018; Rossi et al., 2019; Hughes et al., 2020). Pollutant release into marine environments poses one of the biggest threats to coral reefs health and survival. However, only recently the abundance and distribution characteristics of microplastics (MPs) in coral reef systems

have received scientific attention (Soares et al., 2020; Huang et al., 2021). Indeed, anthozoans, including sea anemones, stony corals and soft corals, are a class of marine invertebrates still underrepresented in the microplastic literature (Lusher 2015). Recent research suggests that microplastics may cause a plethora of impacts on corals in shallow, mesophotic, and deep-sea zones at different latitudes, with a variety of species-specific influences from cellular-physiological to ecological scales (Soares et al., 2020; Huang et al., 2021). However, most of these studies focus on scleractinian (stony) corals, neglecting the effects of microplastics on other benthic reef dwellers, such as soft corals and sea anemones. Indeed, scleractinian corals are the main builders and, commonly, the major occupiers of reef frameworks (Fautin 1989) and in studies of habitat-forming species, those that are not spatially dominant are often considered “non-primary” and may be overlooked (Steinberg et al., 2020). However, in other shallow, tropical marine environments, or in the same habitats under different conditions, non-scleractinian anthozoans - typically zoanthids and octocorals - occupy comparable expanses of substratum. Likewise, some temperate and deep-water marine communities are dominated by anthozoans, generally actinians. Although these animals do not structure their communities physically, they are, in many respects, functionally comparable to reef-building corals (Fautin 1989). Nevertheless, it is fundamental to have a broader overall environmental picture and, so, to assess the interaction and the potential impacts of microplastics on different and multiple organisms.

In order to gain a better understanding regarding the microplastic impacts, most studies have focused on quantifying MPs abundance and on the assessment of their distribution and composition in various habitats. Different sampling methodologies have been employed to document microplastics presence in marine environments. Among them, there is the use of biological indicators, which measure litter levels in their environments in a way that is impossible to replicate by direct physical measurements (Fossi et al., 2018; Palazzo et al., 2021). The search for indicators for microplastic interaction in diverse environment is still ongoing and various efforts have been made to cover

different ecological and biological aspects (Galimany et al., 2009; Vandermeersch et al., 2015). For example, levels of accumulated plastic additives in the environment or within organism tissues have often been considered as a proxy indicator of plastic exposure in the oceans, as a consequence of the release of such additives from dispersed plastic debris (Hermabessiere et al., 2017). Between them, phthalate esters (PAEs) have been proposed as a tracer to evaluate organisms MPs exposition in marine environments (Fossi et al., 2012), due to their ubiquity as contaminants introduced in nature via anthropogenic activities (Chen et al., 2019; Huang et al., 2021; Jafarabadi et al 2021), their chemical characteristics and their wide use as plasticizers (Zheng et al., 2014). However, at the present time, the contribution of microplastic interactions to the accumulation of PAEs in sea organism tissues due to leaching (the possible role of MPs as "Trojan Horse" for PAEs) has still scarcely been considered in the literature. The BioSPME-LC/MS technique (Saliu et al., 2020a, Saliu et al., 2020b) gives the possibility to further investigate the use of PAEs as a proxy of MPs interaction with marine organisms, overcoming some analytical issues and proposing at the same time a methodology that could be easily used *in vivo* and potentially *in situ* (Saliu et al., 2020b).

With such background, this PhD thesis set out to address some of the previously described knowledge gaps, by examining microplastics and phthalate esters occurrences and their interactions with soft benthic overlooked anthozoan organisms, specifically soft corals and sea anemones. To achieve this and further investigate the possible use of PAEs as a proxy to evaluate microplastics exposition in marine environments, this research suggests the application of BioSPME-LC/MS methodology to detect PAEs levels in the soft benthic anthozoan tissues, exploring its validity and sensibility under very different conditions (i.e. in the field and in laboratory), and with different anthozoan species.

Therefore, first of all, in chapter two the capacity of a soft coral species (*Coelogorgia palmosa*) to interact with MPs is tested, analysing the effects of MPs interaction with

such organism through feeding and adhesion tests performed in expressly created microcosms with different microplastic experimental concentrations. At the end of the treatments, *C. palmosa* fragments which interacted with microplastic beads showed evidence of stress by an abnormal mucus production and the shrinkage of polyp tentacles. The results highlighted the microplastic ingestion by an octococal species and the adhesion of MPs particles on its "soft" surface, describing that such MPs were mostly trapped by the produced abnormal mucus. As already proposed for scleractinian corals (Martin et al., 2019; Corona et al., 2020), adhesion resulted the main form of MPs-coral interaction for this soft coral, suggesting that the adhesion driven by the abnormal mucus excreted by corals in stressed status resulted the main mechanism of microplastic trap. In different anthozoan species, mucus has been recorded to trap vary exogenous materials upon release into the seawater environment (Bythell & Wild, 2011). Since anthozoans produce abnormal mucus when subjected to stress (Brown & Bythell 2005), factors that induce stress conditions in the organism may enhance the adhesion of random plastic present in the water column, enhancing the stress status of the organism. So, the occurrence and the intensity of the coral responses to the presence of plastic debris (e.g. abnormal mucus production, adhesion and ingestion) might depend more on the time of interaction with microplastic debris respect to the concentration of MPs in the environment. This potentially makes impacts caused by microplastic presence a time-dependent disorder, suggesting that chronical interaction, even at minor MPs concentrations, might be more impacting for the organism health status, due to the ubiquity of plastic pollution in the marine environment.

Soft corals, as well as sea anemones, are benthic organisms which lack the physical defense of a mineralized skeleton (Silva et al., 2017). Thus, they must rely solely on cellular and molecular mechanisms as a first line of defence against abiotic or biotic stressors (Kültz 2005; Mydlarz et al., 2010). Given the ability of microplastic to impair different cellular processes and possibly generate oxidative stress and damage, through

the production of reactive oxygen species (ROS), soft corals can be particularly affected by this source of contamination (Wright et al., 2013; RochaGalloway et al., 2017). Chapter three analyses and describes the effects of a short term MPs exposure on *Coelogorgia palmos*a cellular physiology. Therefore, the cellular oxidative status and the cellular oxidative damage were investigated through the analysis of three antioxidant enzymes involved in ROS detoxification (Glutathione reductase, Catalase, Superoxide dismutase) and through the analysis of cellular lipid peroxidation respectively. In addition, the impact of microplastics on the cellular protein homeostasis was also analysed, through the analysis of Heat shock proteins Hsp60 expression. Overall, the results highlight that microplastic contamination in soft corals can generate oxidative stress, cellular damage and possibly suppress protein homeostasis defensive mechanisms. Indeed, an increase of antioxidant enzymatic activity and malondialdehyde (MDA) levels (signs of lipid peroxidation) were recorded with exposure to increasing microplastic experimental concentrations. However, the observed decrease of Hsp60 level with the increase of microplastic concentrations represents a surprise, since Hsp expression is usually upregulated when organisms face conditions that may affect their cellular protein structure. This could indicate that microplastic impaired the expression of the Hsp60 protein and, consequently, a suppression of antioxidant defensive mechanisms in soft corals. So, in addition to the physical damage showed in chapter 2, chapter 3 provides the first evidence of impacts at cellular level of short-term microplastic exposure on a soft coral species. *C. palmosa* was found to be a good model during stress experiments in tanks and for laboratory analysis, that might be used for further investigation. Moreover, the results suggest that the length of microplastic exposure may play a role in the levels of antioxidant defences in benthic organisms and, as detected even in the previous chapter, even low concentrations of microplastic can become stressful for the organism physiology following chronic exposure. Consequently, the effect of exposure time to low concentrations of microplastic should also be further investigated when studying cellular oxidative stress of marine benthic organisms, with

the necessity to better elucidate the tolerance threshold for marine organisms to these contaminants (Rocha et al., 2020).

Afterward, in chapter four, the phthalates occurrence and distribution were investigated in soft corals, to explore their capacity to interact and concentrate such plasticizers. The presence of 5 different PAEs congeners (DMP, DEP, DBP, BBP, DEHP) and three phthalate monoesters or MPEs (MBP, MBzP, MEHP), was assessed in soft coral samples of the species *Coelogorgia palmosa, Sinularia sp., Sarcophyton glaucum*, and *Lobophytum sp*. collected from colonies raised in the Acquario di Genova tanks, an environment considered as a microcosm defined by characteristic PAEs concentration levels. Moreover, the bioconcentration factors of the target phthalate esters were measured through the BioSPME-LC/MS procedure, to evaluate the inclination of soft corals to accumulate PAEs from their surrounding environment (Jafarabadi et al., 2021). PAEs were detected in all the collected soft coral samples and the results indicated that the short chain phthalates (DMP and DEP) display higher levels of accumulation in the soft coral tissue than theoretically expected, while the larger phthalates (BBP and DEHP) display levels of accumulation lower than expected. Such observation was already reported in previous lab and field studies involving other aquatic organisms and is generally considered a proof of metabolic transformation. This indicate a possible metabolic pathway that in soft corals transform long chain/high molecular weight phthalates into shorter chain/monoalkyl phthalates. Such hypothesis is supported even by the fact that we detected short chain phthalates in the tissues of the examined soft corals and not in the water. The findings in this chapter provide important new insight, demonstrating that PAEs do interact and concentrate in soft corals, suggesting that the longer chain phthalates are degraded into shorter chain phthalates by a species-specific metabolic pathway before the excretion. Moreover, SPME fiber application coupled with LC-MS methodology has proven to be efficient in determining PAEs contamination, even in traces, in soft corals samples.

Currently, there is no literature data regarding the rates for the direct transfer of PAEs into cnidarian tissues based on microplastics exposure. Thus, understanding whether the level of PAEs found in organism's tissues is related to the direct interaction with plastic particles, like the ingestion, more so than other mechanism is a very important topic. The assessment that soft coral species can interact both with MPs (chapter 2 and 3) and PAEs (chapter 4) done in the previous works was carried out under controlled parameters and by employing artificial conditions (i.e. experimental medium-high concentrations of commercial plastic particles within a microcosm, where coral colonies were exposed to an average similar concentration of phthalates in the water). However, in nature there are different MPs concentrations, while PAEs levels are usually extremely variable in terms of space, time and plastic conditions. So, in order to evaluate the role of MPs in transferring PAEs into marine organisms at "natural" concentrations of such contaminants, it is necessary to collect *in situ* evidences of such possible interactions at the present MPs and PAEs environmental concentrations.

To this end, chapter 5 investigates simultaneously the occurrence of both MPs and PAEs contamination into wild sea anemones sampled in the Western Mediterranean Sea (Sardinia, Italy), in a study area specifically choose for the particular MPs environmental conditions and hydrodynamics (Olita et al., 2013; de Lucia et al., 2014). Hence, we assessed the presence and distribution of both PAEs and MPs in *Actinia equina* (Linnaeus, 1758) and *Anemonia viridis* (Forsskål, 1775), exploring simultaneously the pattern of MPs and PAEs levels in two common and wide distributed sea anemone species to detect any potential difference in the occurrence of such contaminants at environmental and spatial scale. Overall, proof of the interactions of sea anemones of both the species with MPs and PAEs at environmental concentrations were highlighted: *A. equina* and *A.viridis* widely interacted through ingestion and adhesion with plastic microlitter, particularly with microfibers. Remarkably, every single sea anemone specimen was found with microplastics in its tissues, for a 100% frequency of MPs

occurrence. Phthalates were detected in the 70% of the collected animals, with higher levels of phthalate monoesters (MPEs) in the tissues of the examined sea anemones respect to the PAEs congeners. This fact could be considered a proof of a metabolic pathway, as previously noted for soft corals (chapter 4) and in other sea organisms (Blair et al., 2009). Detected PAEs occurrence seem to follow the pattern shown by MPs contamination: indeed, both the pollutants are characterized by a uniform occurrence and distribution between *A. viridis* and *A. equina* and at spatial scale, mirroring the seawater plastic pollution conditions of the study region. Indeed, the waters inside and around the study area are described as a "plastic soup" (Suaria et al., 2016), characterized by a homogeneous and ubiquitous presence of plastic microlitter in the entire area (de Lucia et al., 2014), with a medium-high average presence of microplastics. A general moderate positive relationship between the MPs presence and PAEs contamination has been highlighted in both the two sea anemone species, in *A. viridis* between the number of fibres and Σ_8PAEs, while for *A. equina* between the total number of MPs items and Σ_5PAEs. This can be explained as the result of a complex interaction between direct bioaccumulation and metabolism in the biota, which should be species-dependent, but highlight that with increasing of the microplastic litter levels (fibres or total MPs items), a moderate increase of PAEs levels is found in both *A. equina* and *A. viridis*.

In summary, this thesis contributes to the field of microplastic research by exploring MPs-biota interactions and the relationship with the common plastic additives phthalate esters, with a particular focus on overlooked soft-benthic anthozoan organisms. The chapters show the interaction of both soft corals and sea anemones with microplastic particles and phthalate esters at environmental and experimental conditions. Particularly, the adhesion and ingestion of microplastic items were for the first time assessed for soft corals and confirmed for sea anemones, alongside their capacity to interact with and concentrate plasticizers. At present, studies highlight diversified

possible interactions between scleractinian corals and microplastics, with different coral species that respond differently to diverse MPs exposures. Such observations are extended to soft corals and sea anemones too, suggesting that microplastics and plasticizers must be considered a novel source of anthopogenic contamination in different reef dwellers, particularly at environmental conditions, which are usually characterized by chronic plastics presence, even in cases of low microplastic concentrations. These findings trigger new questions and, hopefully, serve as foundations for future researches. For example**,** further investigations are needed to improve the knowledge on the here presented microplastic impacts on different anthozoans. Moreover, additional studies at environmental microplastic concentrations and long-term exposure are required to obtain a clearer picture of the effects of microplastics on the here considered organisms, both at physical and physiological levels.

The European Marine Strategy Framework Directive (2008/56/EC) highlighted concerns for the environmental implications of marine litter and underlined the urgent need for member countries to "Determine trends in the amount, distribution and composition of micro-particles (mainly microplastics) in European waters and to establish baseline quantities, properties and potential impacts (Galgani et al., 2010). The importance of this issue has also been acknowledged by the G7 world leaders, who committed to a global action plan to combat marine litter (G7-Alliance on Resource Efficiency, 2015). The detection of plastic associated chemical in organism tissues have been widely employed to assess levels of plastic exposure and phthalate esters are the most common plasticizers used in plastic items and found in the environment (Rani et al., 2015; Paluselli et al., 2019). With such considerations and given the rapid degradation of PAEs into MPEs, the outputs here found suggest that the concentrations and changing levels of both PAEs and MPEs should be employed as a proxy of short term interaction with plastic, where, for example, constant levels of PAEs could be related to continuous dispersion of plastic, higher PAEs congeners levels could be index of a recent approach

with plastic debris and growing levels of MPEs could indicate a past interaction, according to the metabolic pathways of the considered organism. This could be particularly relevant during monitoring activities of microplastic presence in aquatic environments, allowing, for example, to appreciate the effectiveness of applied mitigation measures in the short term or seasonal changes in the source of such pollutants.

This thesis provides further information on MPs presence and interactions alongside PAEs detection in different soft coral and sea anemone species, expanding such evaluation even beyond coral reef environment, thanks to the worldwide distribution of some organisms (e.g. sea anemones). Indeed, an overall picture on the interaction and the potential impact of microplastics is needed, not only on charismatic and ecosystem-builder species, but on multiple organisms, in order to understand how reefs and other ecosystems will be affected and respond to microplastic pollution and who the ecological winners or losers will be in an increasingly polluted marine environment.

6.2. References

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APPENDIX

Abstracts of articles produced during the PhD programme, which are not related to the topic of the PhD thesis are listed below.

I. First observation of cushion seastar *Culcita sp*. spawning simultaneously with other echinoderms species in central Indian Ocean

Enrico Montalbetti, Sara Vencato, Luca Saponari, Davide Seveso

Galaxea, Journal of Coral Reef Studies, 22(1), 51–52. https://doi.org/10.3755/galaxea.22.1_51

Culcita spp. are facultative corallivores occurring throughout the Indo-Pacific Ocean. In the Maldives, *C. schmideliana* (Bruzelius, 1805) was reported as one of the main contributors to a delay in coral recovery after the 2016 bleaching event and the resulting coral mortality, due to the large densities of specimens recorded and their preferential predation on coral recruits (Bruckner and Coward 2019). To date, little information is available on the timing and controlling factors of the reproductive cycle of the seastar, with only few reports of spawning in the wild (Otha et al. 2011). On the 7th of March 2020, spawning by Culcita sp. and several other echinoderms was observed on the shallow reef (< 8 m) adjacent the island of Thudufushi, South Ari Atoll, Republic of Maldives. The spawning took place at 1730 hrs following the peak of low tide (1715 hrs), two days before the full moon. Fifteen individuals of *Culcita sp*., 3 *Linckia multifora* (Lamarck, 1816), 2 *Fromia indica* (Perrier, 1869) and 1 *Pearsonothuria graeffei* (Semper, 1868) (Echinodermata: Holothuroidea) were observed showing spawning behaviour such as an arched body shape or releasing sperm from the gonopores within an estimated area of 200 m^2. The event took place in the same tide and lunar cycle conditions of previous unreported events recorded in Faafu Atoll in April 2019 (Personal observation), suggesting that the period of March- April could coincide with a spawning season of *Culcita sp*. Interestingly, the butterfly fish *Chaetodon falcula* (Bloch, 1975) was observed feeding on recently released *Culcita sp*. sperm. *Culcita spp*. are known to feed on coral recruits (Montalbetti et al. 2019) and together with *Drupella spp*. and *Acanthaster planci* (Linnaeus, 1758) are recognized as a potential threat for Maldivian coral reefs (Saponari et al. 2018). This observation represents the first record of natural spawning for this genus in the Republic of Maldives as well as in Central Indian Ocean, and it may contribute to an increased understanding of the reproductive cycle for *Culcita spp.* in this area.

II. Characterization and Assessment of Micro and Macroscopic Litter in Sardinian Beaches (Western Mediterranean Sea)

Andrea Camedda, Stefania Coppa, Luca Palazzo, Stefano Marra, Giorgio Massaro, Fabrizio Serrentino, Sara Vencato, Roberto Brundu, Giuseppe Andrea de Lucia

Water, Air, & Soil Pollution, 232(2), 1-14. https://doi.org/10.1007/s11270-021-04993-9

The presence of beach litter along the coast is due to the indirect input by waves, wind, rivers and currents and to the direct deposition by beach users. This study, conducted in Sardinia (Western Mediterranean Sea), aims to quantify and characterize beach litter all around the island and to suggest the main sources of impact. Five monitoring campaigns (autumn 2013; spring 2014; autumn 2015; spring 2016; autumn 2016) were conducted considering 3 "exposed" and 4 "sheltered" beaches by means of 33.3-m linear transects in which all "macroscopic" items (> 5 mm) were collected. "Micro" litter (< 5 mm) sampling was performed in 6 beaches through 10-m linear transects. For both sampling designs, abundance and litter typologies were assessed following the protocols of the Marine Strategy Framework Directive (MSFD) and BASEMAN project. Repeated measures permutational analysis of variance was performed to detect any difference in abundance and composition of marine litter according to season, wind exposure and site. Exposure was the factor that better explains the distribution of litter: higher values were found on the "exposed" (E) sites with respect to the "sheltered" (S) ones, both for macroscopic (E: 1696.56 ± 219.25 items/100 m; S: 420.5 ± 74.73 items/100 m) and micro litter (E: 7990.67 ± 2319.44 items/10 m; S: 111.78 ± 25.91 items/10 m). All the 8 typologies were recorded, and litter composition significantly varied according to exposure and site over time. This work provides key information about litter presence and sources, useful to suggest possible mitigation measures.

III. What is hidden in the luggage? First assessments of illegal seashells gathering in Sardinia (Italy)

Stefania Coppa, Andrea Camedda, Giorgio Massaro, Sara Vencato, Franco Murru, Maria Tiziana Pinna, Davide Urrai, Antonio Casula, Maurizio Riccitelli, Giuseppe Andrea de Lucia

Submitted to *Conservation Science and Practice* on 05/08/2022

Natural souvenirs collection has been identified as a driving force in biodiversity and habitat degradation of tropical marine ecosystems. This work considers this phenomenon in the Mediterranean region taking Sardinia (Italy), one of the most renowned tourism destinations, as a case study. The biological material seized at Cagliari-Elmas Airport (years 2019-2020: 138 kg) was analysed: 199 taxa were identified, gastropods (112 species, 7,866 pieces) and bivalves (63 species, 34,218 pieces) resulted the most represented classes. Twenty-two protected species were found in the tourists' luggage including Patella ferruginea and Pinna nobilis, the invertebrates most threatened with extinction in the Mediterranean Sea. This study demonstrates that the illegal collection of natural mementos is common in Sardinia, thus its relevance is not limited to tropical regions. Regulation, enforcement and compliance shortcomings emerged, highlighting the importance of strengthening stakeholders' collaboration for a deeper insight on this phenomenon and implementing effective conservation strategies.

IV. Biodegradable Zein-based biocomposite films for underwater delivery of Curcumin reduce thermal stress effects in corals

Marco Contardi, Marta Fadda, Valerio Isa, Yohan Louis, Andrea Madaschi, Sara Vencato, Enrico Montalbetti, Laura Bertolacci, Luca Ceseracciu, Davide Seveso, Silvia Lavorano, Paolo Galli, Athanassia Athanassiou, Simone Montano

Submitted to *ACS Applied Materials & Interfaces Manuscript* on 25/01/2023

Massive bleaching episodes are one of the first causes of coral death worldwide. The overproduction of reactive oxygen species (ROS) is considered the principal cause of splitting between polyps and algae within coral tissue during bleaching events. Here, we propose Zein/Polyvinylpyrrolidone(PVP)-based biocomposite films laden with curcumin as advanced mitigation tools. By varying the Zein/PVP weight ratio, we can easily tune the biocomposites' mechanical, swelling, and release properties. The curcumin release profile can also be regulated by temperature showing an on-demand modality activated in case of heat-induced bleaching episodes. Strips of the biodegradable films were applied to Stylophora pistillata. After immersion in seawater, the biocomposites become soft hydrogels, resulting in biocompatible and not affecting coral viability. Finally, in bleaching simulation tests at 29 and 33°C, the presence of the antioxidant significantly ameliorated the coral condition. The treated corals did not undergo the release of algae and had an overall healthier condition with respect to untreated corals. Hence, this study provides insights into new strategies for mitigating coral bleaching by using natural antioxidants and biocomposites to deliver them into corals.

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