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Exploring Microplastics Contamination in Mediterranean Octocorals

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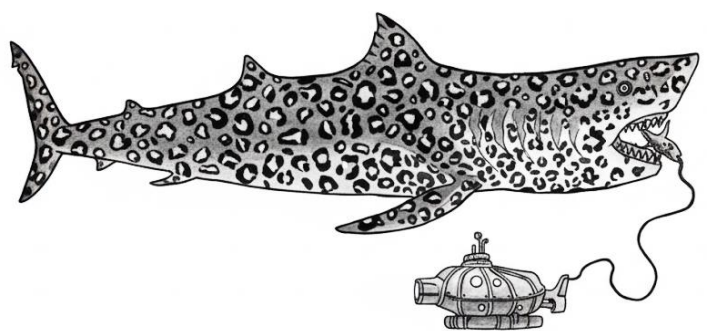
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Exploring Microplastics Contamination in Mediterranean Octocorals

Antonio Giova

Messina, Italy, 2026



Festival Director: “That's an endangered species at most. What would be the scientific purpose of killing it?”

Steve Zissou: “Revenge.”

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Summary

Marine litter (ML), and particularly microplastics (MPs), has emerged as a pervasive form of pollution affecting marine ecosystems from coastal areas to the deep sea, with increasing evidence of their occurrence across environmental compartments and taxonomic groups. Despite growing attention, deep-sea ecosystems and habitat-forming invertebrates remain poorly explored, especially in the Mediterranean Sea. Octocorals are among the most emblematic and ecologically relevant components of Mediterranean benthic communities, from shallow circalittoral assemblages to deep-sea coral forests, where they act as ecosystem engineers by increasing habitat complexity, supporting associated biodiversity, and contributing to benthic–pelagic coupling. Their functional role, longevity, and capacity to interact with particulate matter make them suitable indicators of environmental contamination; however, information on MP occurrence and biological effects in Mediterranean octocorals is still extremely limited.

This PhD thesis aimed to characterize key aspects of MP pollution in Mediterranean octocorals through an integrated approach combining methodological development, field-based assessment, and experimental exposure studies.

The first objective was to develop and validate a reliable digestion protocol for the isolation and identification of MPs from octocoral tissues, addressing the lack of standardized methodologies for anthozoans. Using the deep-sea bamboo coral *Isidella elongata* as a model species, a wide range of chemical digestion approaches was systematically tested in order to identify a protocol that ensured efficient organic matter removal while preserving MP integrity. This methodological framework provided the baseline for all subsequent investigations presented in this thesis.

Following protocol validation, MP occurrence was assessed under natural conditions in *I. elongata* collected from a poorly explored deep-sea area of the Ionian Sea (Western Mediterranean). MPs were quantified in coral colonies and in deep-sea fish species living in close association with coral forests, using these organisms as biological indicators of

contamination. In addition, seafloor ML deposited around coral collection sites was characterized to provide environmental context. By integrating biological and environmental compartments, this study represents one of the first ecosystem-based assessments of ML pollution in Mediterranean deep-sea coral forest systems. In addition, the first evidence of MP occurrence in Mediterranean octocorals was reported, with concerning implications for cold-water coral habitats.

To further investigate biological interactions with MP, experimental exposure studies were conducted under controlled laboratory conditions. Due to practical constraints associated with the rarity and maintenance of *I. elongata*, experiments were performed on two Mediterranean mesophotic gorgonians with established laboratory protocols, *Paramuricea clavata* and *Eunicella singularis*, which share key functional traits with deep-sea octocorals, including suspension feeding and habitat-forming roles. Two complementary experiments were carried out, targeting different life history stages. The first focused on adult colonies of *P. clavata*, assessing MP ingestion and egestion dynamics during exposure. The second addressed early life history stages of *E. singularis*, evaluating larval survival, settlement, and MP uptake. By covering both adult and early developmental phases, this work provides insight into potential MP impacts across multiple biological levels.

Overall, this thesis contributes novel methodological tools, field-based evidence, and experimental data to the understanding of MP contamination in Mediterranean octocorals. By integrating different spatial scales, ecological compartments, and life stages, it highlights the relevance of habitat-forming corals as indicators of ML pollution and underscores the need for further research on MP impacts in mesophotic and deep-sea ecosystems.

General Background

From Plastic to Microplastic: Global Threats to Marine Ecosystems

Plastics are synthetic organic polymers with thermo-plastic or thermo-set properties (synthesized from hydrocarbon or biomass raw materials), elastomers (e.g. butyl rubber), material fibers, monofilament lines and coatings (GESAMP 2019). Plastics are often produced as a mixture of different polymers and various plasticizers, colorants, stabilizers and other additives (UNEP, 2021). Most plastics are classified into two main categories: thermoplastics, sensitive to deformation by heating, such as polyethylene (PE), polypropylene (PP), and polystyrene (PS), and thermosetting plastics, which are harder and non-deformable even under high temperatures, e.g. polyurethane (PUR), paints, and epoxy resins (Issac and Kandasubramanian, 2021; Kibria et al., 2023; Lithner et al., 2011).

Thanks to their durability, resistance to degradation, excellent mechanical properties, thermal and electrical insulation, bio-inertness and relatively low cost, plastics have rapidly expanded across industries and daily life (Barnes et al., 2009; Lebreton and Andrady, 2019; Napper and Thompson, 2020; Ryan, 2015). Their versatility allows for the production of a wide range of items, from flexible films and foams to rigid containers, adhesives, and synthetic fibers (Napper and Thompson, 2020; Ryan, 2015).

Since the 1950s, when large-scale production of plastics began (Geyer et al., 2017; Harris et al., 2023; Lebreton and Andrady, 2019), plastic production reached approximately 430.9 million tons in 2024 (Figure 1) (PlasticsEurope, 2025). Among the most produced polymers, PE accounts for 26% (combining low-, middle- and high density), followed by PP (19.0%) polyvinyl-chloride (PVC; 12.8%), polyethylene terephthalate (PET; 6.2%), PUR (5.3%) and PS (5.1%) (PlasticsEurope, 2025). A large part

of plastic production (89.8%) comes from the extraction of fossil fuels, namely crude oil petroleum hydrocarbons, natural gas and coal, carrying with it a substantial environmental burden from the very beginning of the lifecycle, from the resource extraction to energy-intensive refining and manufacturing processes, and finally with waste management and disposal (Kibria et al., 2023; Lebreton and Andrady, 2019; Macheca et al., 2024; MacLeod et al., 2021).

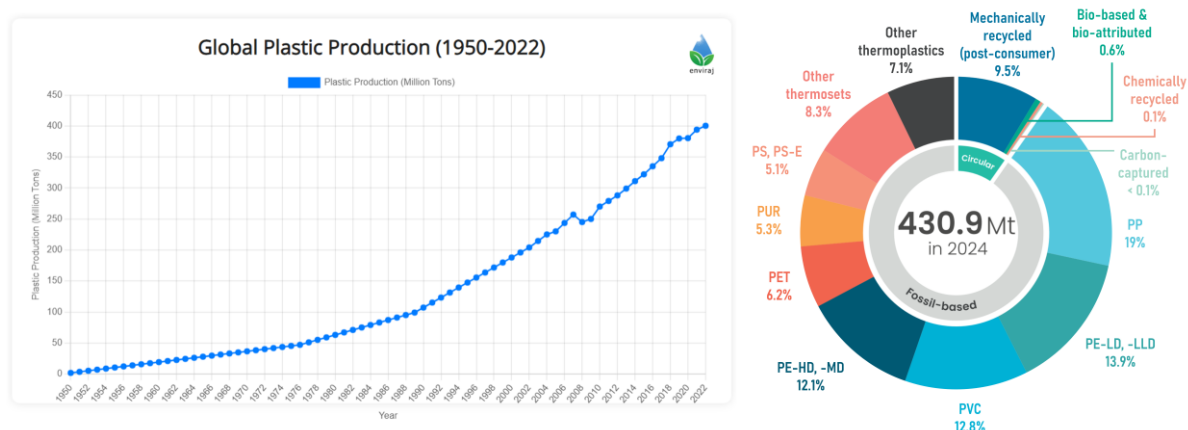


Figure 1. (Left) trend in global plastic production from 1951 to 2022. It excludes PET-, PA-, and polyacryl-fibers from 1951 to 2018. Starting from 2018, it also excludes polymers not used in making plastic parts and products (i.e. quantities used for adhesives, sealants, coatings, paints, varnishes, textiles, waterproofing, or within the production of cosmetics, medicines or chemical processes). Credits: Enviraj consulting. (Right) Global plastics production in 2024 by polymer. PP: Polypropylene, PE: Polyethylene, PE-HD: High-Density Polyethylene, PE-MD: Medium-Density Polyethylene, PE-LD: Low-Density Polyethylene, PE-LLD: Linear Low-Density Polyethylene, PVC: Polyvinyl Chloride, PET: Polyethylene Terephthalate, PS: Polystyrene, PS-E: Expanded Polystyrene, PUR: Polyurethane, PA: Polyamide. Credits: PlasticsEurope.

Plastic pollution is found globally from deserts to farms, from mountaintops to the deep ocean, in tropical landfills and in Arctic snow (MacLeod et al., 2021). Plastic waste generation exceeded 367 million tons in 2020 (Macheca et al., 2024). This proliferation has been driven by a consumerist culture, fueled by the capitalistic economic model, leading to a sharp rise in single-use plastic consumption and the expansion of a 'throw-away' culture (Lau et al., 2020; Lebreton and Andrady, 2019). Unfortunately, global waste

management systems lack the capacity to safely dispose of or recycle such volumes, and even a significant increase in this capacity would still fall short of keeping up with the projected growth in plastic waste (Borrelle et al., 2020; Chen, 2015; Lau et al., 2020). Furthermore, the same properties that make plastics so versatile in innumerable applications, namely durability and resistance to degradation, make these materials difficult or impossible for nature to assimilate (Andrady, 2015; Geyer et al., 2017; Worm et al., 2017). As a result, these persistent materials have accumulated across ecosystems, with the marine environment emerging as the primary sink for plastic waste, leading the United Nations Environment Programme (UNEP) to recognize plastics as one of the most problematic pollutants affecting oceans worldwide (Macheca et al., 2024). It has been estimated that plastic litter represents at least 85% of marine litter (ML) found in surveys, as a result of both land-based and sea-based human activities (GESAMP, 2019; UNEP, 2021).

ML is defined as any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment (UNEP, 2009). It is commonly classified by size, as it strongly influences transport pathways, environmental fate, degradation processes, and type and severity of biological interactions with marine organisms. Based on this criterion, ML is divided into macrolitter (>25 mm), mesolitter (5–25 mm), and microlitter (<5 mm), with the latter further categorized into large microlitter (5–1 mm) and small microlitter (1–0.1 mm) (Hanke et al., 2013).

Waste from land-based sources may be transported to the sea by wind, rivers (Moss et al., 2021; Rech et al., 2014), road runoff, storm water runoff, sewage discharges or drains (Galgani et al., 2015). Likewise, marine activities such as commercial shipping, ferries and liners, ships, both commercial and recreational fishing vessels, offshore installations and aquaculture sites can be direct sources of ML (Deville et al., 2023; Galgani et al., 2015; Horsman, 1982). The different transport patterns and modification processes at sea, along with the intrinsic characteristics of debris (type of material, size and density), determine the fate of ML. Floating ML can be spread on the ocean surface or stranded on the shorelines by currents, wind, waves and tides (Declerck et al., 2019; Maclean et al., 2021; Ourmieres et al., 2018). Indeed, ocean circulation patterns play a

key role in determining the distribution of floating ML, with convergence zones where surface debris tends to accumulate, commonly referred to as “garbage patches”, especially located within the subtropical oceanic gyres (Kubota, 1994; Lebreton, 2022; Lebreton et al., 2018; Moore et al., 2001). While floating litter represents the most visible fraction of marine pollution, a large proportion of plastic debris is subjected to sinking through the water column to the seabed (Fazey and Ryan, 2016). Increasing evidence from ROV surveys and sediment cores reported litter concentrations at the seafloor several orders of magnitude higher than those recorded at the surface (Galgani et al., 1996; Hanke et al., 2025; Martin et al., 2022; Peng et al., 2020). A combination of physical and biological processes can affect the vertical transport of plastic debris from the sea surface to the seabed. Even though most polymers are positive buoyant, they can be forced to deeper layers by hydrodynamic, such as waves and turbulence, or progressively increase the effective density due to biofouling (Amaral-Zettler et al., 2021; De Haan et al., 2019; Fazey and Ryan, 2016; Kowalski et al., 2016; Woodall et al., 2014). Another sinking mechanism for small-sized plastic is through magnification, with planktonic and mesopelagic organisms that can ingest particles near the surface and subsequently transfer them to depth in faecal pellets or marine snow aggregates (Cole et al., 2014; Long et al., 2015; Lusher et al., 2016; MacLeod et al., 2021). Many plastics eventually become neutrally buoyant or sink slowly through the water column, relocating in the entire water column and accumulating at stratification layers (Zhao et al., 2025). In deeper marine layers, additional mechanisms such as turbidity currents and dense shelf water cascading contribute to the transfer and final deposition of plastics on the deep seafloor (Chen et al., 2025), turning deep basins, especially canyons and trenches, into major long-term sinks for ML (Galgani et al., 1996; Hanke et al., 2025; Harris et al., 2023; Martin et al., 2022; Peng et al., 2020; Pierdomenico et al., 2023; Woodall et al., 2014).

It is well established that ML, and in particular plastics, poses a large scale and serious threat to the welfare of marine organisms and habitats (Napper and Thompson, 2020; Werner et al., 2016), causing collateral aesthetic, social and economic implications (Galgani et al., 2000; Werner et al., 2016). Focusing on plastic pollution, accounting for 60 to 80% of total ML items (Galgani et al., 2015; Pedà et al., 2025), it has been reported that more than 900 marine species have interacted with plastic debris (Gall and

Thompson, 2015; Kühn et al., 2015; MacLeod et al., 2021; Napper and Thompson, 2020; Worm et al., 2017), resulting in severe physical harm or mortality, as well as more subtle impacts on behavior and ecological functions (e.g., predator evasion or migration). The most visible effect of plastic pollution on marine organisms is the entanglement of organisms in marine debris, often in discarded or lost fishing gear and ropes, compromising their ability to move, feed, and breathe (Anastasopoulou and Fortibuoni, 2022a; Kühn et al., 2015; Napper and Thompson, 2020; Worm et al., 2017; Zhang et al., 2025). In addition, many marine organisms can unintentionally ingest plastic, mistaking litter for food, or indirectly swallowing during feeding activities (Derraik, 2002; Kühn et al., 2015; Laist, 1997; Werner et al., 2016; Worm et al., 2017). Indigestible debris such as plastics may accumulate in the stomach and impair individual fitness, with repercussions for reproduction and survival, even without directly leading to mortality (Bjørndal et al., 1994; McCauley and Bjørndal, 1999; Van Franeker, 1985). Secondary ingestion also occurs when a predator consumes prey that has previously ingested plastic particles (Pedà et al., 2020). The ingestion phenomenon is particularly relevant due to its potential implications for bioaccumulation and biomagnification, as it may facilitate the trophic transfer of plastics and associated contaminants across different levels of the trophic web. While marine birds, turtles, and mammals have historically received most of the attention, the impacts of entanglement and ingestion on other groups, such as fish and invertebrates, are becoming increasingly evident (Consoli et al., 2019; Derraik, 2002; Laist, 1997; Pedà et al., 2025; Schirinzi et al., 2020; Sciutteri et al., 2023; Worm et al., 2017).

Although most plastic polymers are generally considered biochemically inert due to their large molecular weight and their insolubility in water (Lithner et al., 2011; Rodrigues et al., 2019; Teuten et al., 2009), their production involves the use solvents that may be used as initiators and catalysts, and the integration of chemical additives (Rochman, 2015; Rodrigues et al., 2019). Plastics are manufactured from monomers, some of which (e.g., styrene and vinyl chloride) are carcinogenic or mutagenic (Lithner et al., 2011; Papaleo et al., 2011), and which polymerization reaction can remain incomplete, leaving residual monomers, oligomers and solvents embedded within the polymer matrix (Araújo et al., 2002; Crompton, 2007). In addition, plastics typically

contain substantial quantities of chemical additives, including plasticizers (phthalates, Bisphenol A), flame retardants (PBDEs), stabilizers, pigments and UV filters, introduced to enhance flexibility, durability, color or microbial resistance (Deanin, 1975; Lithner et al., 2011; Rodrigues et al., 2019). Additives can migrate from plastic products to air, water, sediment, to other contact media (e.g. food) or, upon ingestion, to tissues, releasing compounds known to be toxic or endocrine-disrupting, such as phthalates and bisphenol A (Andrady, 2011; Crompton, 2007; Darbre, 2020; Hermabessiere et al., 2017; Wilkins and Richmond, 2025). Moreover, the hydrophobic surface of plastic particles facilitate the adsorption and concentration of metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, organochlorine pesticides as well as pharmaceutical and personal care products present in seawater (Ashton et al., 2010; Browne et al., 2013; Prunier et al., 2019; Teuten et al., 2009). Such toxic substances accumulate on plastics, often at higher concentrations on smaller particles (Cormier et al., 2022; Wang et al., 2015), and can subsequently be taken up after ingestion, increasing the toxicological hazard on marine organisms (Bakir et al., 2012; Rochman et al., 2013).

ML has been increasingly recognized as a global environmental problem and a major threat to marine biodiversity, leading to its inclusion in several international and regional policy frameworks (Galgani et al., 2000; Gall and Thompson, 2015). At a global scale, ML is addressed within agreements such as 11th Conference of the Parties to the Convention on Biological Diversity (CBD COP 11 Decision XI/18), the 10th Conference of the Parties to the Convention on the Conservation of Migratory Species of Wild Animals (CMS Resolution 10.4), the International Convention for the Prevention of Pollution From Ships (MARPOL) Annex V (Gall and Thompson, 2015; Sciutteri et al., 2023). In Europe, the Marine Strategy Framework Directive (MSFD, 2008/56/EC) and the four Regional Sea Conventions (RSCs), OSPAR Commission for the Northeast Atlantic region, HELCOM (Helsinki Commission – Baltic Marine Environment Protection Commission), Black Sea Commission (BSC) and UNEP/MAP Barcelona Convention for the Mediterranean Sea are providing frameworks for monitoring and large-scale actions to assess and mitigate the impacts of ML (Galgani et al., 2024).

Monitoring programs play a central role in supporting policy implementation, providing the evidence needed to assess trends, identify impacts and evaluate the effectiveness of mitigation measures (Galgani et al., 2024; Nielsen et al., 2023). The MSFD established the main tool for addressing the litter issue in the marine environment at the European level, which main goal is the assessment of trends toward the Good Environmental Status (GES) and the identification of impacts of ML on marine ecosystems (GESAMP, 2019; Werner et al., 2016). Over time, monitoring has expanded from beach and floating litter surveys to include seafloor litter and interactions with marine biota, particularly ingestion and entanglement (Consoli et al., 2019; Galgani et al., 2024; Pedà et al., 2025). ML is addressed under Descriptor 10 through criteria targeting both environmental presence and biological impacts. Primary criteria (D10C1–D10C2) assess the composition, amount and spatial distribution of litter and microlitter across marine compartments, while secondary criteria (D10C3–D10C4) focus on litter ingestion and entanglement impacts on marine organisms (Galgani et al., 2023).

A large and growing subset of ML is composed of microplastics (MPs), defined as plastic particles smaller than 5 mm in size (Galgani et al., 2023; GESAMP, 2019). According to the MSFD, this size class is further categorized into large MPs (from 5 mm to 1 mm) and small MPs (from 1 mm to 0.1 mm) (GESAMP, 2019). MPs originate either directly from the runoff of various sized industrially produced microbeads commonly found in consumer and personal care items (primary MPs), or through the breakdown of larger plastics affected by abiotic and biotic factors (secondary MPs) (Andrady, 2017, 2011; Cole et al., 2011). Primary MPs are mostly small pellets or granules produced as precursors for other plastics, or used directly in the cosmetic industry (Thompson, 2015), as powders in stamping (Kershaw and Rochman, 2015), in abrasives (Gregory, 1996), in the clothing industry (Martellini et al., 2018) and as drug carriers in the medical field (Ibrahim et al., 2022). Secondary MPs can result from the plastic fragmentation into smaller pieces due to a combination of abiotic (i.e., photodegradation and mechanical degradation) and biotic (biodegradation) factors (Anastasopoulou and Fortibuoni, 2022b; Andrady and Koongolla, 2022; Derraik, 2002).

The transport and fate of MPs largely mirror those of larger ML but occur at smaller scale. MPs are transported through the marine environment by the same physical and biological mechanisms that govern the distribution of larger debris, including currents, wind, waves and vertical transfer (Chen et al., 2025; Chubarenko et al., 2016; De Haan et al., 2019; Hidalgo-Ruz et al., 2012; Kane and Clare, 2019). However, due to their small size and large surface area to volume ratios (Fazey and Ryan, 2016), they are even more susceptible to displacement, redistribution and re-suspension, as well as to accidental ingestion and trophic transfer through biomagnification (Cole et al., 2014; Kowalski et al., 2016; Long et al., 2015; Lusher et al., 2016; MacLeod et al., 2021). As a consequence, MPs have been detected in all marine compartments, from surface waters to the water column and the seafloor (Chen et al., 2025; Cole et al., 2011; De Haan et al., 2019; Isobe et al., 2019; Karlsson et al., 2017; Kowalski et al., 2016; Martin et al., 2022), and in organisms across virtually all taxonomic groups (Bottari et al., 2026; Janssens and Garcia-Vazquez, 2021; Karlsson et al., 2017; Lusher et al., 2017; Pedà et al., 2025, 2022a, 2020; Saliu et al., 2022; Scherer et al., 2017; Schirinzi et al., 2020), with the number of affected *taxa* continuously increasing.

The significant persistence and availability of MPs in the marine environment may lead to both direct and indirect impacts on marine organisms' health, mainly through the ingestion, transport of chemical additives and associated plastisphere (Andrady, 2011; Cole et al., 2011; De Sá et al., 2018; Deudero and Alomar, 2015; Issac and Kandasubramanian, 2021; Pedà et al., 2022b, 2016). Understanding the extent of MPs accumulation by biota at different trophic levels and its impact on health using harmonized protocols is essential to monitor plastic pollution and implement measures to address this issue. This is required at the Mediterranean level for monitoring ML under Descriptor 10 of the EU Marine Strategy Framework Directive (2008/56/EC) for the achievement of Good Environmental Status (GES) (Galgani et al., 2024; Lusher et al., 2017).

Ecology and Vulnerability of Mediterranean Octocorals

Octocorallia, along with Hexacorallia, is a class of anthozoans (Phylum Cnidaria: Subphylum Anthozoa) that host more than 3,500 nominal species of mainly non-stony corals distributed across more than 75 families (McFadden et al., 2022). They are exclusively marine organisms, occurring worldwide from shallow coastal waters to the deep sea and across all marine ecoregions (Gómez-Gras et al., 2025; Pérez et al., 2016). The class includes corals such as those under the common names of sea fans, sea pens, soft corals, blue corals, precious red corals and bamboo corals. Octocorals exhibit a remarkable diversity of growth forms, ranging from encrusting and stolonal webs to erect, bushy and complex arborescent structures (Bayer, 1973; Enrichetti et al., 2025; Pérez et al., 2016; Williams and Alderslade, 2011). This morphological variability allows them to colonize a wide range of environments and substrates, including rocky outcrops, biogenic structures and soft sediments (Bryce and Radford, 2018; Ruppert et al., 1994; Zapata-Guardiola and López-González, 2012).

Octocorals are colonial coelenterates characterized by polyps bearing eight pinnate tentacles arising from a basal common tissue called coenenchyma (Bayer, 1973; Breedy, 2009; Sánchez et al., 2019). Small calcareous skeletal elements in form of calcite—the sclerites—are embedded in the coenenchyma. The sclerites stiffen parts of the polyp body, fill the coenenchyma, and provide support for the octocoral colony (Grasshoff and Bargibant, 2001). While all sharing these features, octocorals display highly variable skeletal architectures. Some, such as the so-called soft corals, lack a compact axial structure and rely on flexible tissues only supported by sclerites (Breedy, 2009). In contrast, other octocorals possess a well-developed axial skeleton, whose composition and structure vary markedly among groups. In gorgonians (sea fans), the axis is mainly constituted by sclerotized collagen-like protein (gorgonin) associated with calcitic deposits, providing both flexibility and mechanical support (Sánchez et al., 2019). Similarly, sea pens (Pennatuloidae spp.) share a gorgonin-based axial structure that is only weakly calcified or entirely non-calcified (Ledger and Franc, 1978). Red corals (*Corallium* spp.), instead, are characterized by a fully calcified axis made out of calcite (Le Roy et al., 2021), whereas bamboo corals (Isididae spp. and Keratoisididae spp.)

skeleton is articulated in calcitic internodes separated by proteinaceous nodes (Saucier et al., 2021). An exception within Octocorallia is represented by blue corals (*Heliopora* spp.), which are the only hermatypic corals in the class, producing a massive aragonitic skeleton functionally analogous to that of stony corals (Class Hexacorallia: Order Scleractinia) (Richards et al., 2018).

Although octocorals play an important role in shallow-water ecosystems, particularly on tropical reefs where they can be abundant and even dominant space occupiers (Fabricius, 2011; Lasker et al., 2020; Riegl, 1999), their highest diversity is found in deep-sea environments (Enrichetti et al., 2025). Indeed, approximately three-quarters of the species occur below 50 m depth, with diversity peaks associated with continental shelves, slopes and seamounts (Cairns, 2007; McFadden et al., 2022; Pérez et al., 2016; Yesson et al., 2012).

The distribution of benthic communities, including anthozoans, mainly depends on physical oceanographic features including light availability, water temperature and salinity. Other constraints come from water chemistry, as the case of hydrothermal vents, and biological interactions, namely substrate competition and co-occurrence (Borcard et al., 1992). In the Mediterranean Sea, benthic communities are traditionally described along a vertical zonation based on light availability, as originally formalized by Pérès and Picard (1964) in the famous "*Nouveau manuel de bionomie benthique de la mer Méditerranée*". The two authors identified two macrosystems based on light availability. The phytal (or coastal) system, where autotrophic processes occur, and the aphytal system, where the absence of light does not allow the photosynthetic processes to take place (Figure 2). The phytal system includes the supralittoral plane, located above sea level and generally reached by sea spray, where organisms experience high humidity without being submerged; the mesolittoral plane, bounded by the highest high tide and the lowest low tide levels, characterized by difficult living conditions due to alternation between immersion and emersion but higher species richness than the supralittoral; the infralittoral plane, extending below the lowest low tide level to the lower limit of seagrass meadows (about 25–40 m depending on water transparency), representing the area with the highest species diversity; and the circalittoral plane,

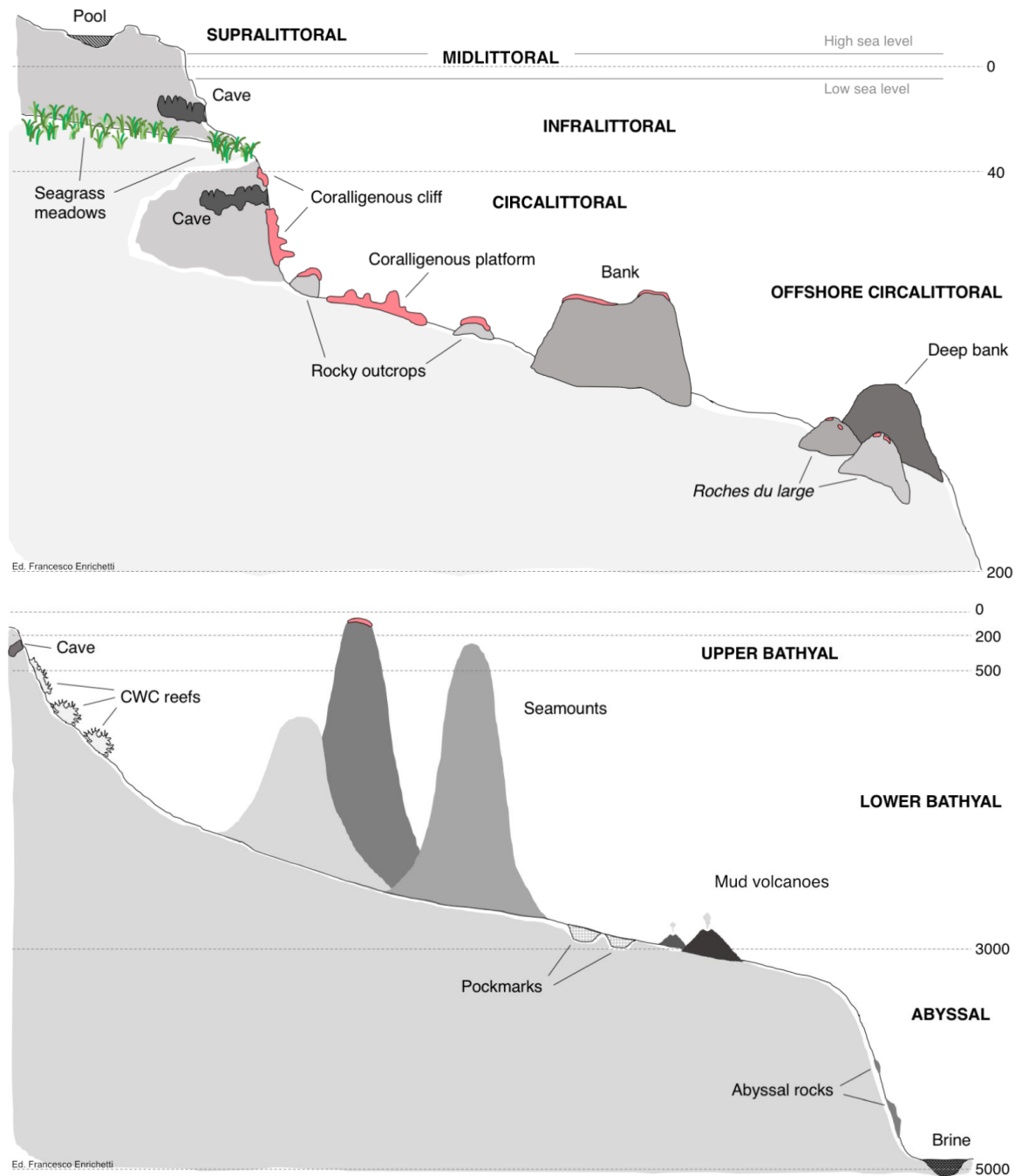


Figure 2. Zonation of the benthic domain proposed by Pérès e Picard. The image above represents the horizons of the phytal system, while the image below reports the horizons of the aphytal system.

Credits: F. Enrichetti in SPA/RAC (in press).

extending to the lower limit of algal growth, where light becomes a limiting factor and sciaphilous (organisms living at low-light conditions) algae dominate, often considered

the initial plane of the mesophotic or twilight zone, marking a transition from algal- to animal-dominated biocoenoses. The aphytal system includes the bathyal plane, extending from the continental shelf to about 3000 m depth; the abyssal plane, reaching 6000–7000 m, whose presence and fauna in the Mediterranean are debated; and the adal plain, corresponding to oceanic trenches below 7000 m, which is not present in the Mediterranean Sea.

At mesophotic and deep-sea depths, gorgonians, soft corals and pennatulaceans frequently form mono- or multi-specific assemblages that generate structurally complex habitats commonly referred to as coral meadows, coral gardens or coral forests (Bo et al., 2015, 2014b; Buhl-Mortensen and Mortensen, 2004; Rossi et al., 2017; Stone, 2006). These aggregations represent a key component of marine animal forests (*sensu* Rossi et al., 2017), communities comparable in complexity, biodiversity, and structuring role to the terrestrial forests, but composed of suspension-feeding organisms, e.g. sponges, anthozoans, bryozoans and bivalves (Bo et al., 2015, 2009; Buhl-Mortensen et al., 2010; Cerrano et al., 2010; Rossi et al., 2017). The three-dimensionality of marine animal forests provides secondary substrates and niches, forming a spawning, nursery and shelter ground for a rich associated fauna (Bo et al., 2014a; Cau et al., 2017; Cerrano et al., 2010; D'Onghia et al., 2017; Rueda et al., 2019). Within these systems, octocorals have been demonstrated to function as foundation species, enhancing habitat complexity and promoting high levels of associated biodiversity by providing attachment surfaces, refugia and feeding grounds for a wide range of invertebrates and fishes (Buhl-Mortensen et al., 2010; De Vincenzis et al., 2026; Gomes-Pereira et al., 2017; Gómez-Gras et al., 2025). Their complex morphologies can locally modify near-bottom hydrodynamics, particle retention and light regimes, potentially favoring the settlement of sessile organisms and contributing to the stability and persistence of benthic assemblages (Ponti et al., 2018, 2014). As suspension feeders, octocorals play an important role in benthic-pelagic coupling thus the transfer of energy, biomass and nutrients between the pelagic and the benthic ecosystems – by recycling particulate organic matter sinking from upper photosynthetic layers, directly influencing nutrient cycling and trophic pathways (Bo et al., 2014a; Corry et al., 2018; Fabricius, 2011; Gili and Coma, 1998). Although they are not classical reef builders, octocorals can also contribute

to carbon cycling through the production of calcium carbonate sclerites and, in some species, photosynthetic activity mediated by symbiotic zooxanthellae (Atrigenio et al., 2020; Matsumoto, 2010; Shoham et al., 2019).

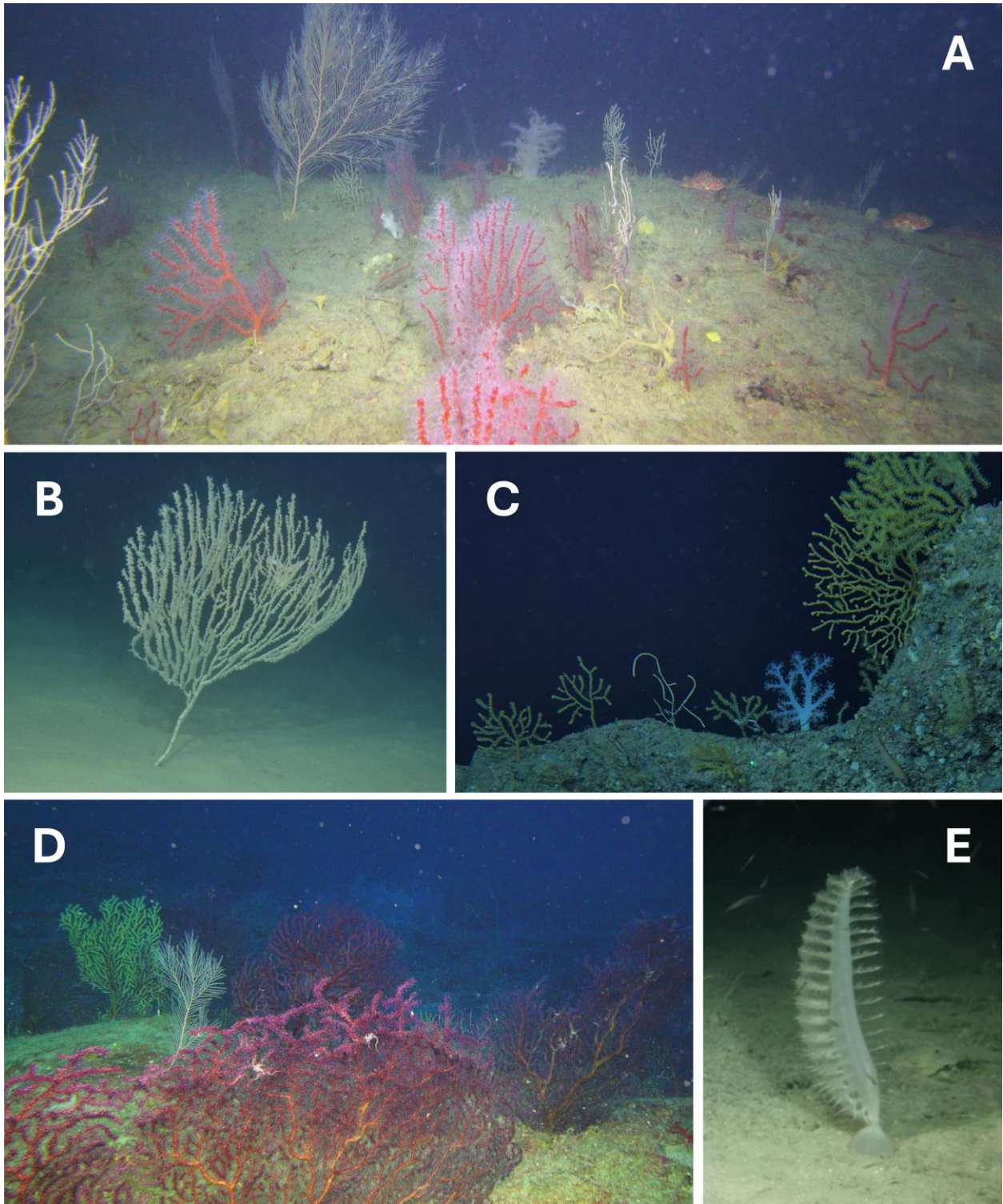


Figure 3. Mediterranean octocorals in their environment from ROV surveys. (A) Multispecific assemblage of the precious red coral *Corallium rubrum*, *Callogorgia verticillata*, *Eunicella verrucosa* and an unidentified soft coral at mesophotic depths. (B) The bamboo coral *Isidella elongata* hosting the spider crab *Anamathia rissoana* on bathyal muddy bottoms. (C) Population of the gorgonian *Acanthogorgia* sp. and a single colony of the soft coral *Chironephthya mediterranea* on bathyal rocky bottoms. (D) Coralligenous assemblage with the red gorgonian *Paramuricea clavata* and a *C. verticillata* colony at mesophotic depths. (E) The sea pen *Pteroides griseum* on bathyal muddy planes. Credits: Stazione Zoologica Anton Dohrn.

Habitat-forming octocorals are particularly vulnerable to anthropogenic disturbances, especially in deep-sea environments. Demersal fishing practices, particularly bottom trawling, represent one of the main threats, as octocorals are frequently impacted by direct mechanical damage, bycatch and entanglement in lost or discarded fishing gear (Aguilar et al., 2017; Consoli et al., 2019; Freiwald et al., 2004; Hall–Spencer et al., 2002; Stone, 2006). In some regions, historical harvesting for the jewellery trade has also contributed to population declines, especially for species with slow growth and high market value (Santangelo and Bramanti, 2010). Given their slow growth rates, longevity and limited recruitment, many species exhibit low resilience, with recovery times often spanning decades, if recovery occurs at all (Althaus et al., 2009; Freiwald et al., 2004). In recent decades, increasing attention has been given to the role of octocorals in ecosystem reconfiguration under global change scenarios. In several tropical regions, particularly in the Caribbean Sea, long-term observations suggest persistent shifts from stony coral- to octocoral-dominated communities, likely reflecting differences in life history traits, skeletal composition and physiological tolerance (Edmunds and Lasker, 2016; Lasker et al., 2020; Lenz et al., 2015; Nelson and Bramanti, 2020). Unlike scleractinians, most of octocorals’ skeletons are made of sclerotized collagen (Sánchez et al., 2019) with calcium carbonate confined to sclerites, a feature that has been proposed as one of the mechanisms underlying their relative resistance to ocean acidification and other climate-related stressors (Ateweberhan et al., 2013; Gabay et al., 2014; Gómez et al., 2015). Several studies indicate that many octocoral species show higher tolerance to bleaching, nutrient enrichment and certain biological stressors compared to stony corals, although extreme thermal anomalies can still induce tissue necrosis and mass mortality events (Cerrano et al., 2000; Garrabou et al., 2021; Prada et

al., 2010). In the Mediterranean Sea, recurrent marine heatwaves, which are discrete prolonged anomalously warm water events, have further exacerbated population declines of several habitat-forming octocoral species, highlighting their sensitivity to cumulative stressors and the need for targeted conservation and management strategies (Garrabou et al., 2021). However, the effects of environmental stressors are not spatially uniform, and increasing evidence suggests that certain areas may function as temporary or long-term climate refuges, partially sheltered from warming disturbances (Beauvieux et al., 2024; Bongaerts and Smith, 2019; Bramanti et al., 2023; Georges et al., 2024; Glynn, 1996). In this context, the “depth refuge hypothesis”, proposed by Glynn (1996) for shallow-water corals, but further applied to other environments, suggests that mesophotic depths (approximately 30–150 m) may provide cooler and more stable thermal conditions, potentially offering refuge for shallow-water populations from surface-driven thermal stress.

Considering the key ecological role, the biological traits (i.e., slow growth, recovery rates, recruitment, and long lifespan), and the high sensitivity to anthropogenic stressors, most octocorals are recognized under several national and international conservation frameworks. Nine octocorals are classified as threatened species in the IUCN Red List of Mediterranean Anthozoans (Critically endangered: *Isidella elongata*; Endangered: *Corallium rubrum*, *Crassophyllum thessalonicae*; Vulnerable: *Ellisella paraplexauroides*, *Funiculina quadrangularis*, *Pennatula rubra*, *Pennatula phosphorea*, *Pteroeides spinosum*, *Paramuricea clavata*), three are listed in Annexes II and III of the SPA/BD protocol of the Barcelona Convention (Annex II: *C. verticillata*, *E. paraplexauroides*, *I. elongata*; Annex III: *C. rubrum*), and one species (*C. rubrum*) belongs to Annex III of Bern Convention and Annex V of Habitats Directive Framework. In addition, the habitat created by octocorals are considered Vulnerable Marine Ecosystems in relation to several human activities (FAO, 2009).

State of the Art of Microplastic Contamination in Octocorals

Although some large-scale impacts of human activities on marine animal forests are evident and extensively documented, including the desolation caused by bottom trawling (Bo et al., 2014a; Clark et al., 2016), the volume of debris impacting benthic ecosystems (Angiolillo and Fortibuoni, 2020; Consoli et al., 2019; Hanke et al., 2025; Pham et al., 2014) and mass mortality events due to marine heatwaves (Garrahou et al., 2022; Gómez-Gras et al., 2021; Viladrich et al., 2022), MP pollution represents an invisible threat, whose ecological consequences are only beginning to emerge and remain challenging to anticipate.

Anthozoans interact with MPs either passively, by adhesion to their surfaces or actively, by ingestion (Figure 4) (Huang et al., 2021; Pereira et al., 2024). The adhesion of MPs to the coenosarc can cause stress by interfering with the coral cleaning mechanisms, causing mucus overproduction and/or increasing particle expulsion mechanisms (Corona et al., 2020; Martin et al., 2019; Reichert et al., 2022). The particles can also cause tissue necrosis with particles migrating into the tissue and being overgrown by tissue material, eventually becoming embedded in the skeleton following the radial growth, as reported for some scleractinians (Ding et al., 2019; Hierl et al., 2021). Moreover, MPs fall within the size range of their natural prey and can be ingested by corals (Hall et al., 2015). Corals, gorgonians included, are typically described as non-selective suspension feeders that depend mainly on seston suspended in the water column, with zooplankton constituting one of the principal components of their diet (Gili and Coma, 1998; Hamann and Blanke, 2022). However, species-specific feeding behavior affects the size and shape of ingested particles (Pantos, 2022; Savage et al., 2022). Currently, the ability of coral polyps to discriminate between MPs and food items has still not been clarified. Reichert et al. (2024) tested the feeding response of 11 reef-building corals (including one octocoral species) to food, sand and MPs. Corals appeared able to discriminate between food and non-food particles, rejecting sand and preferentially ingesting natural food, while MPs elicited feeding responses similarly to natural prey and were not immediately recognized as non-food items. However, at later

stages of the feeding process, corals increasingly discriminate against MPs, indicating that MP particles are mainly confused with natural food in the initial phase of the feeding response, where cnidocytes are fired and prey is captured. In addition, MPs colonized by a bacterial biofilm (a complex formed by bacteria cells, generally referred to as plastisphere, and a self-produced matrix of extracellular polymeric substances) caused higher reaction and ingestion rates than virgin MPs, suggesting that biofilm-associated compounds may act as phagostimulants, as already observed for other filter-feeders (Fabra et al., 2021). Conversely, Allen et al. (2017) reported a higher ingestion of virgin MPs compared to bio-fouled MPs in a stony coral species, hypothesizing that the phagostimulants might be intrinsic to the plastic itself. The authors proposed three possible explanations: exposure to seawater may alter plastic surface chemistry by removing phagostimulants, biofilm formation may obscure phagostimulant, or microbial films may introduce feeding deterrents. Another possible pathway for MP uptake is through biomagnification, through which MPs can be transferred across trophic levels along the food web (Allen et al., 2017; Corinaldesi et al., 2021; Hall et al., 2015; Morais et al., 2020; Rotjan et al., 2019). However, laboratory studies have demonstrated coral ability to recognize and egest MP particles during feeding activities (Allen et al., 2017; Hankins et al., 2018; Pereira et al., 2024; Syakti et al., 2019).

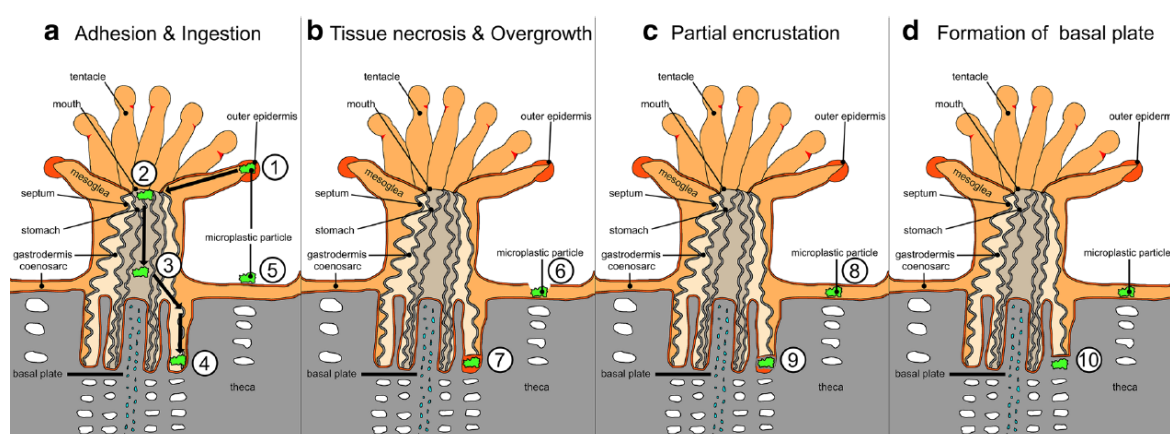


Figure 4. Possible microplastic (MP) incorporation scenario. (A) (1) MP particle becomes adhesive to the outer epidermis area of the coral tentacle. (2) Food response is triggered and the particle is transported to the mouth of the polyp. (3) Ingestion begins and the particle is transported into the gastrovascular cavity. (4) The MP particle becomes stuck inside the gastral area. (5) Adhesion to the coenosarc as a possible mechanism of attachment. (B) (6) Tissue necrosis occurs and particles migrate

into the tissue. (7) Tissue healing process starts and the particle is overgrown by tissue material. (C) (8) Tissue healing process is completed. The particle is fully surrounded by coral tissue. (9) Partial overgrowth by aragonite is induced. Aragonite crystals form a layer surrounding the foreign body, later connected to the theca. (D) (10) The formation of a new basal plate surrounding the MP particle by encrustation is complete. Credits: Hierl et al., 2021

Most of the knowledge about the effects of MP exposure to anthozoans comes from studies targeting species from the class Hexacorallia. These experimental studies have documented a broad spectrum of biological responses, affecting mechanical damage, polyp behavior and physiological regulation.

One of the most consistently reported impacts concerns feeding behavior and trophic interactions. MPs can interfere with prey capture causing reduced feeding performance on natural food, with increasing energetic costs associated with particle rejection (Chapron et al., 2018; Mouchi et al., 2019; Rades et al., 2022; Rotjan et al., 2019). Adhesion of MP particles to coral surface also activate cleaning processes, such as egestion and mucus production, that are likely energetically costly (Allen et al., 2017; Hankins et al., 2018; Reichert et al., 2018; Savage et al., 2022). Direct contact of MPs with coral surfaces has also been associated with abrasion and tissue necrosis (Reichert et al., 2018; Syakti et al., 2019). Furthermore, several studies have observed the incorporation of MPs particles into coral skeletal structures (Ding et al., 2019; Hierl et al., 2021; Krishnakumar et al., 2021; Reichert et al., 2018), while others reported a reduction of calcification and growth rates (Chapron et al., 2018; Mouchi et al., 2019; Reichert et al., 2019), raising concerns about long-term impacts on skeletal integrity. In symbiotic corals, MPs have also been shown to affect host-symbiont interactions leading to bleaching in some cases including changes in photosynthetic efficiency, symbiont density, and chlorophyll content, with potential implications for health and resilience of coral reefs under multiple stress scenarios (Axworthy and Padilla-Gamiño, 2019; Okubo et al., 2020; Reichert et al., 2024, 2021, 2019, 2018; Rocha et al., 2020; Syakti et al., 2019; Tang et al., 2018; Xiao et al., 2021). In addition, the potential effects of MPs on coral reproduction and early life history stages are raising concern, although evidence from laboratory studies is scarce and controversial (Berry et al., 2019; Wilkins et al., 2024; Wilkins and Richmond, 2025). At

the physiological level, MP exposure has been linked to a range of sublethal stress responses involving metabolism, oxidative balance, and immune-related processes. Several studies report metabolic alterations and shifts in energy allocation, often accompanied by increased oxidative stress and modulation of antioxidant defenses (Grillo et al., 2021; Lanctôt et al., 2020; Rocha et al., 2020; Tang et al., 2018; Xiao et al., 2021). A study conducted by Tang et al. (2021) demonstrated directly *in situ* the correlation between MP pollution in the surrounding environment and symbiotic density in the tropical scleractinians *Pocillopora damicornis* and *Galaxea fascicularis*, while reporting no signs of oxidative stress. In addition, an increased caspase-3 activity, indicative of apoptotic processes and cellular stress, was observed only in *P. damicornis*. Another ecotoxicological impact comes from the plastsphere as a potential vector of pathogens, as well as organic pollutants and heavy metals absorbed from the surrounding environment (Wang et al., 2022; Zettler et al., 2013). Rotjan et al. (2019) observed the transfer of *Escherichia coli* microbes marked with GFP (green fluorescent protein) from the plastsphere to surfaces and tissues of the northern star coral *Astrangia poculata*, causing higher mortality than controls. Nevertheless, a recent study reported a shift in coral microbiome after MP exposure but observing very few specific bacteria directly transferred from plastsphere, suggesting that shift in the microbiome was due to general dysbiosis from stress (Chapron et al., 2025).

Coral responses to MP exposure appear to be highly species-specific, with some species showing pronounced stress responses while others appear more tolerant under similar exposure conditions (Mouchi et al., 2019; Reichert et al., 2019; Tang et al., 2021).

The severity and consistency of these effects strongly depend on experimental design and exposure conditions. Marked negative responses are most frequently observed in studies employing MP concentrations that exceed those currently reported in natural marine environments. In contrast, experiments conducted under environmentally relevant concentrations often report weak, variable, or negligible effects on coral physiology and performance, especially when compared to other major stressors such as ocean warming or acidification (Berry et al., 2019; Grillo et al., 2021; Reichert et al., 2021).

To date, experimental evidence on octocoral exposure to MPs remains limited and fragmented, with only a small number of laboratory-based studies available, covering a restricted set of taxa and experimental conditions.

The first experimental study specifically addressing MP exposure in Octocorallia was conducted by Reichert et al. (2019), who compared the response of the blue coral *Heliopora coerulea* to that of several hexacorallian species under long-term exposure (six months) to PE MPs (200 particles L⁻¹). While *H. coerulea* exhibited a slight reduction in calcification rates (-3%), photosynthetic performance increased and no signs of bleaching, tissue necrosis or parasitic scars were observed, in contrast to the hexacorals included in the experiment.

A subsequent study by the same research group extended the exposure to 15 months under identical MP conditions (Rades et al., 2022), revealing reduced feeding rates in *H. coerulea* without alterations in its ability to discriminate between MPs and zooplankton. Notably, no stress-related responses such as mucus release were detected, whereas co-exposed hexacorals showed clear stress signals.

In contrast, short-term exposure experiments on gorgonians have generally reported pronounced stress responses. Two studies on *Coleogorgia palmosa* exposed to increasing concentrations of PE (10–70 mg L⁻¹) for 48 h documented enhanced mucus production, elevated antioxidant enzyme activity and lipid peroxidation damage relative to controls (Montalbetti et al., 2022; Vencato et al., 2021).

Similarly, the Indo-Pacific gorgonian *Pinnigorgia flava* exhibited bleaching, tissue swelling and increased mucus production following 72 h exposure to polypropylene (PP) nanofibers derived from surgical masks, together with marked oxidative stress responses at the cellular level (Isa et al., 2025a, 2023). Exposure of the same species to LDPE and PP microfragments, including both virgin and beach-stranded plastics and their leachates, induced polyp retraction and physiological stress, although no mortality or tissue necrosis was observed (Isa et al., 2025b).

Evidence from deep-sea octocorals remains extremely scarce. Pereira et al. (2024) exposed the sea pen *Viminella flagellum* from the northeastern Atlantic to PE microspheres (1500 particles L⁻¹), observing particle adhesion and ingestion during a 24 h exposure phase, followed by complete rejection during a subsequent depuration period.

Additional studies have reported molecular and physiological effects of MP exposure in other octocoral taxa. Gao et al. (2025) demonstrated that exposure of *Junceella squamata* to polystyrene MPs (1–1000 mg L⁻¹) induced transcriptional responses associated with microbiome alteration and impaired nutrient digestion and absorption. Prieto et al. (2025) investigated the shallow-water octocoral *Xenia elongata* under repeated exposure to PE and PP MPs (200 particles L⁻¹), both individually and in combination, reporting polymer-specific effects, with mixed treatments leading to severe tissue necrosis and complete mortality, alongside altered respiration and growth.

To date, the only experimental study addressing MP exposure in a Mediterranean octocoral is that by Corinaldesi et al. (2021), who investigated the response of the red coral *Corallium rubrum* to a mixture of polymers (PE, PP, PS, PET and PVC). High and intermediate MP concentrations (1000 and 500 particles L⁻¹) induced impaired feeding activity, oxidative DNA damage, altered gene expression, increased mucus production and physical injuries. In contrast, exposure to low MP concentrations (100 particles L⁻¹) did not result in significant molecular alterations, although transient increases in feeding activity and physical damage were observed during the early phase of the experiment.

Most of the studies focused on MP in anthozoans have investigated the effects of MP exposure under laboratory conditions, while information on MP abundance in corals directly collected from the field remain scarce. Once again, the research effort has been mainly focused on the class Hexacorallia, including both reef-building corals and sea anemones. These studies consistently report the presence of MPs on coral surfaces and within tissues or skeletons across a wide range of environments, from shallow tropical reefs to polar and deep-sea settings (Bian et al., 2024; Ding et al., 2019; Janssens and

Garcia-Vazquez, 2021; Krishnakumar et al., 2021; Lei et al., 2021; Lim et al., 2022; Morais et al., 2020; Raguso et al., 2022; Rotjan et al., 2019; Savage et al., 2022; Savinelli et al., 2020; Sfriso et al., 2020; Sutthacheep et al., 2025; Tang et al., 2021).

Taylor et al. (2016) provided one of the earliest field observations of MP contamination in deep-sea organisms by visually inspecting external surfaces and dissecting internal cavities. Among the analyzed taxa, single specimens of a soft coral (*Anthomastus* sp.) and an unidentified sea pen collected in the southwest Indian Ocean were examined, revealing the presence of four microfibers within sea pen tentacles and the upper mesentery.

Ding et al. (2019) investigated MP pollution in seawater, fish and corals from the atolls surrounding the Xisha Islands (South China Sea). In a single specimen of the soft coral *Sinularia* sp., 37 particles (0.99 items g⁻¹) were isolated from the coral surface, while 7 particles (0.27 items g⁻¹) were detected within the skeleton after chemical digestion, representing the highest contamination levels among the 22 analyzed coral species, most of which were scleractinians. The same genus was later examined by Bian et al. (2024) in a nearby coastal area, where significantly lower MP concentrations were reported (0.16 items g⁻¹), corresponding to the lowest values among the investigated reef-building corals.

More recently, Helmberger et al. (2025) analyzed 19 cold-water octocoral species, including soft corals and sea fans, collected around the Phoenix Archipelago (Central Pacific Ocean) using chemical digestion protocols. Only microfibers were recovered, and their abundance did not exceed that detected in procedural blanks, leading the authors to conclude that there was no evidence of MP accumulation in the analyzed specimens.

Research Objectives

This project aims to contribute to the expanding research on MP contamination in octocorals. To address this, the project follows a structured approach, starting with the development and validation of standardized analytical methodologies for MP extraction from octocoral tissues. Establishing a reliable and replicable digestion protocol is a crucial first step in ensuring accurate assessments of MP contamination. Building on this methodological foundation, the second objective is to investigate the occurrence and abundance of MPs in Mediterranean cold-water octocoral samples collected directly from their natural environment, providing the first field-based insights into their exposure levels. Finally, the project will move towards an experimental phase, in which studies in laboratory conditions will be conducted to assess the biological effects of MP exposure on habitat-forming mesophotic octocorals. This will allow for the evaluation of physiological and behavioral responses, shedding light on potential long-term consequences for these fragile deep-sea organisms. By integrating these three key aspects method development, field assessment, and experimental exposure the project aims to provide a comprehensive understanding of MP pollution in mesophotic and cold-water octocorals, laying the groundwork for future monitoring and conservation efforts.

Chapter 1

Finding a Digestion Protocol for Microplastic Extraction

Rationale for the study

Microplastic (MP) pollution in marine ecosystems is an issue of growing concern, with increasing evidence of MP occurrence from coastal waters to deep-sea environments and across all taxonomic levels. As the first objective of this PhD project aimed at characterizing key aspects of MP pollution in Mediterranean octocorals, the assessment of MP contamination in deep-sea octocorals collected *in situ* was planned.

The decision to focus on the deep-sea octocoral *Isidella elongata* (Esper, 1788) was driven by both ecological and scientific considerations. Octocorals are recognized as ecosystem engineers by creating and maintaining complex three-dimensional habitats that support high levels of associated biodiversity and providing for substantial ecosystem services (Bo et al., 2014a; Cerrano et al., 2010; D'Onghia et al., 2017; Rueda et al., 2019). *I. elongata* is among the most important habitat former on deep-sea muddy bottoms, acting as spawning and nursery ground for several cephalopods, crustaceans and fishes (Angiolillo et al., 2024; Carbonara et al., 2020; Gerovasileiou et al., 2019; Mastrototaro et al., 2017). Despite their importance, deep-sea ecosystems still remain among the least explored regions of the planet, and knowledge of their interaction with MPs is still extremely limited.

During the preparation of the MP assessment experiment, it became evident that no established or validated protocols existed for the isolation, identification, and quantification of MPs in octocorals, and in anthozoans in general. The few studies

addressing this topic (see Santana et al., 2022; Lim et al., 2022; Reichert et al., 2022; Savage et al., 2022) have used methodologies often characterized by weaknesses in validation and reproducibility. These methodological limitations include the use of restricted particle diversity, unrealistic particle sizes, lack of combined chemical testing, and insufficient quantitative reporting, consequently limiting cross-study comparability.

To address these limitations, the first stage of the doctoral research focused on the critical selection, testing, and validation of digestion methods applicable to octocoral samples. The goal was to identify a protocol that achieved an optimal balance between efficient organic matter removal and the complete preservation of MP integrity. In this phase, a wide range of experimental conditions was systematically explored to determine the most suitable approach for *I. elongata*. This study was conducted at the facilities of the Sicily Marine Centre of Stazione Zoologica Anton Dohrn (Messina, Italy).

Multiple chemical agents commonly used in MP workflows were assessed, including alkaline solutions (KOH), oxidizing agents (NaClO and H₂O₂), and mineral acids (HNO₃ and HCl), applied either to individual matrices (polyps, coenenchyma, sclerites) or to whole-colony samples. These reagents were tested at different concentrations, temperatures, exposure times, and in several orders of application to determine their digestion efficiency and their compatibility with subsequent MP recovery. Across the trials, the main limitations encountered included incomplete tissue degradation under milder alkaline or oxidative conditions, persistent undigested organic materials, and excessively violent reactions under stronger acidic treatments, which risked compromising sample and plastic integrity. Tests highlighted the need to treat organic tissues and carbonate structures with separate reagents, leading to the selection of a two-step digestion strategy based on an initial mild organic digestion followed by a targeted carbonate removal step. Finally, the two pipelines that satisfied our goals were compared for being subjected to validation.

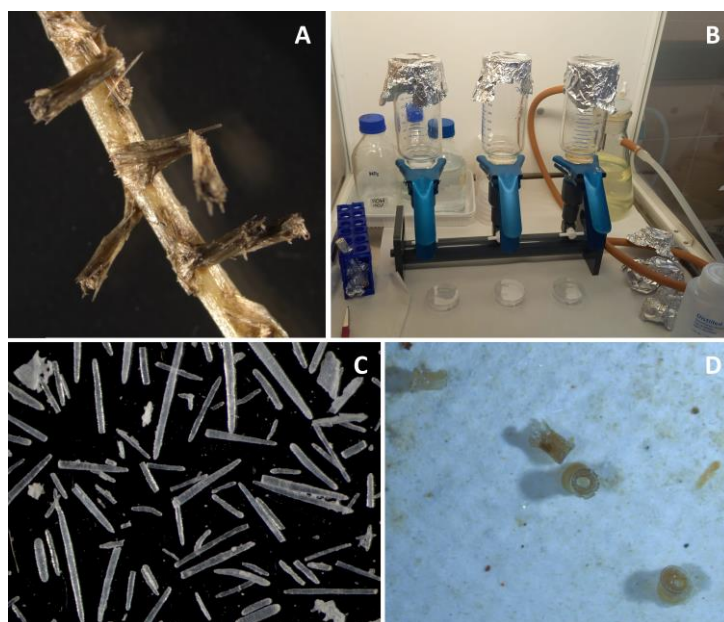


Figure 1.1. (A) Close up on a branch of *Isidella elongata* showing the carbonate skeleton covered by the coenenchyma and the polyps. (B) The vacuum filtration apparatus under the fume hood which was used for the filtration of digested material. (C) The carbonate sclerites resulting from the digestion of organic matter during the first step. (D) Sclerotized organic nodes partially persisting after both digestion steps.

The plastic polymers subjected to validation were polyethylene (PE), polypropylene (PP) and polystyrene (PS), recognized as the most abundant polymers found in marine environment and biota (Andrady, 2017; De Sá et al., 2018; González-Ortegón et al., 2024; Palmieri et al., 2024; Rodrigues et al., 2019; Suaria et al., 2016). The polymer size range was chosen to reflect what is expected to occur in our samples, in accordance with the size range of the prey typically ingested by the species.

The methodological framework established within this chapter represented a baseline for all subsequent field- and laboratory-based investigations discussed in the next chapters.

The following section presents the article published in the *Journal of Hazardous Materials: Plastics* (Elsevier) reporting the validation of the digestion protocol applied to the deep-sea octocoral *I. elongata*.

Identifying a Suitable Digestion Protocol for Microplastic Extraction from *Isidella elongata* (Esper, 1788): New Insights for Cold-Water Corals

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Graphical abstract

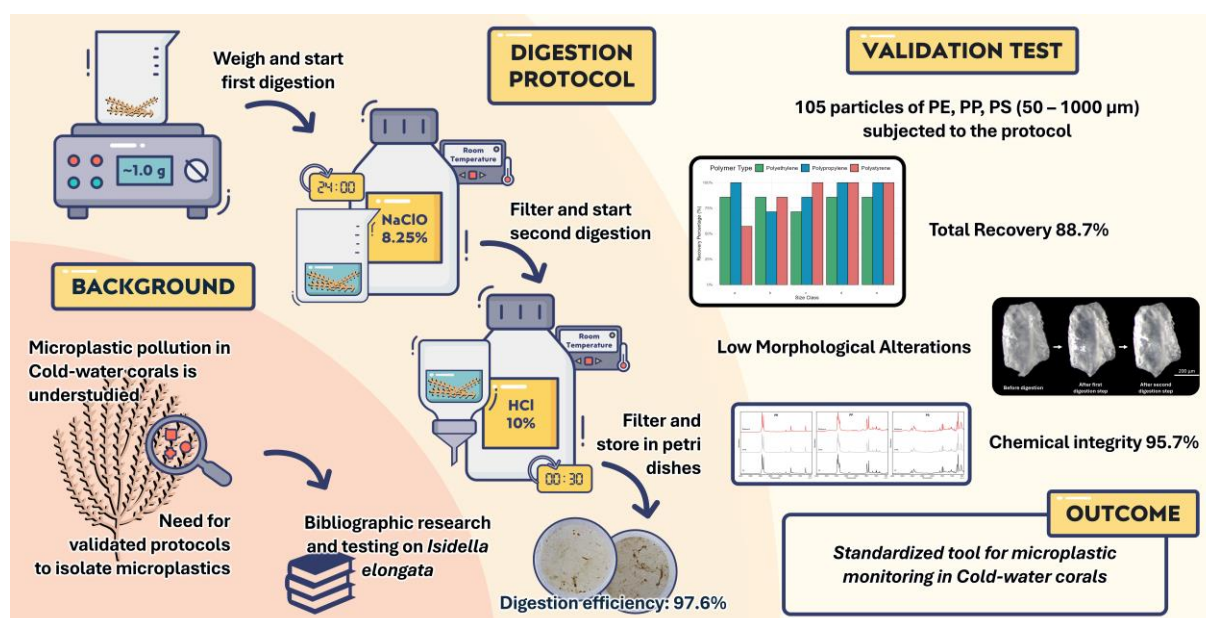


Figure 1.2. Graphical abstract resuming the need for the study, the digestion protocol pipeline, the main results of validation test and the outcome of the study.

Abstract

The persistence and widespread distribution of microplastics (MPs) in marine ecosystems raise concerns about their ecotoxicological impact on biota. Monitoring MP pollution requires standardized methodologies, particularly for smaller particles and those targeting invertebrates. Most studies on MP impacts in anthozoans are based on mesocosm experiments, while field data are scarce and rely on unvalidated protocols. This study aims to identify and document a suitable digestion protocol for small-sized MP isolation from Anthozoa and to validate the method according to standardized protocols using the bamboo coral *Isidella elongata*, a key Mediterranean deep-sea species. Two protocols were adapted and compared in terms of digestion efficiency, with the most effective combining sodium hypochlorite and hydrochloric acid. Its effects were investigated on 105 particles of three polymers (polyethylene, PE, polypropylene, PP, and polystyrene, PS) ranging from 50 to 1000 μm , with and without coral tissue. Spiked MPs were analyzed before and after digestion steps to assess recovery rate and potential alterations in size, morphology, and polymer composition. After chemical digestion, 87.6% of particles were recovered, most of which preserved their integrity. Only minor deviations in average size were reported, while no discoloration and minimal or absent degradation and deformation occurred. In contrast, fragmentation was a common alteration in PS given the inherent properties of the polymer. According to FT-IR spectral analysis, polymer composition was well preserved after digestion. These results suggest that the proposed approach represents a reliable methodology for future MP monitoring in cold-water corals, paving the way for standardized biomonitoring approaches for assessing MP pollution in deep-sea benthic fauna.

Keywords: Plastic extraction, Chemical digestion, Validation test, Marine pollution, Deep-sea, Octocorals

Highlights

- A chemical digestion protocol was validated for MP extraction from *Isidella elongata*.
- 105 PE, PP, and PS particles (50–1000 μm , five size classes) were tested.

- The method ensured high digestion efficiency while preserving particle morphology and chemistry.
- This is the first validated protocol for Mediterranean cold-water coral and aids MP monitoring standardization in deep-sea fauna

Introduction

Microplastics (MPs, plastic particles <5 mm) pollution is one of the major global concern in the 21st century due to the continued massive production of plastic, reaching approximately 413.8 million tons in 2023, with polyethylene (PE) accounting for 26.2% (combining low-, middle- and high density), followed by polypropylene (PP; 19.0%) and polyvinyl-chloride (PVC; 12.8%), polyethylene terephthalate (PET; 6.2%), polyurethane (PUR; 5.3%) and polystyrene (PS; 5.2%) (PlasticsEurope, 2024). This global production reflects the composition of polymers found in the marine environment and organisms, where indeed the most observed MP types are PE, PP, and PS (Andrady, 2017; De Sá et al., 2018; González-Ortegón et al., 2024; Palmieri et al., 2024; Rodrigues et al., 2019; Suaria et al., 2016).

MPs enter marine ecosystems either directly by running off industrially manufactured microbeads of different sizes, widely used in consumer and personal care products (primary MPs) or from the degradation of larger plastic exposed to abiotic and biotic drivers (secondary MPs) (Andrady, 2017, 2011). Depending on the composition and chemical/physical properties of the polymer, the item's size and the abiotic (e.g., currents and water circulation) and biotic (biofouling) factors, plastics may distribute along the water column, from the sea surface to the deep sea, affecting different habitats and ecosystems (Andrady, 2017). In particular, the high persistence and availability of MPs in the marine environment may lead to both direct and indirect impacts on marine organisms' health, mainly through the ingestion, transport of chemical additives and associated plastisphere (Andrady, 2011; Cole et al., 2011; De Sá et al., 2018; Deudero and Alomar, 2015; Inobeme et al., 2024; Issac and Kandasubramanian, 2021; Kumar et al., 2025; Megha et al., 2025). Understanding the extent of MPs accumulation by biota at different trophic levels and its impact on health using harmonized protocols is essential to monitor plastic pollution and implement measures to address this issue. This is required at the Mediterranean level for monitoring marine litter under Descriptor 10 of the EU Marine Strategy Framework Directive (2008/56/EC) for the achievement of Good Environmental Status (GES) (Galgani et al., 2024; Lusher et al., 2017).

To date, several chemical digestion protocols for extracting MPs from biological material have been developed (e.g., Lusher et al., 2017; Schirinzi et al., 2020; Tsangaris et

al., 2021), using mainly acid (*e.g.*, Hongsawat et al., 2024; Van Cauwenberghe and Janssen, 2014; Vandermeersch et al., 2015; Yozukmaz, 2021) and alkaline agents (*e.g.*, Avio et al., 2015; Digka et al., 2024; Foekema et al., 2013; Fraissinet et al., 2024; Giani et al., 2019; Karami et al., 2017; Li et al., 2018; Thiele et al., 2019) with oxidizing and/or hydrolyzing properties, as well as proteolytic enzymes, varying agents' concentrations, temperatures and time exposure depending on the species-specific composition of tissues and the complexity of the biological matrix (Von Friesen et al., 2019). Although these chemical digestion protocols are generally validated for the chemical resistance of polymers, some of these methodologies, particularly those targeting smaller particle size classes, still lack validation (Aliani et al., 2023; Galgani et al., 2024). The validation is an essential step to establish reliable and reproducible pipelines that allow researchers to isolate, identify, and quantify MP debris accumulated in biota (Lusher et al., 2017). This is especially needed in sediments and invertebrates, where monitoring protocols for MPs are less established than in other marine matrices (De Sá et al., 2018; Galgani et al., 2024).

To the best of our knowledge, most of the studies documenting MP ingestion rates and impacts in anthozoans (Cnidaria: Anthozoa) have been conducted under laboratory conditions, while field-based data remain scarce. Although comprehensive reviews have summarized MP occurrence across marine organisms intended for human consumption (seafood) (*e.g.*, WHO, 2022), targeted information on coral species remains limited. Experimental evidence has shown that these organisms can directly ingest MPs, as they fall within the size range of their natural prey and can be covered by organic matter (Hall et al., 2015; Rotjan et al., 2019), or indirectly through biomagnification along the trophic web (Corinaldesi et al., 2021; Morais et al., 2020; Okubo et al., 2020). Exposure to MPs has been linked to several biological responses, including mechanical damage, physiological stress and behavioral alterations (Chapron et al., 2018; Corinaldesi et al., 2021; Lartaud et al., 2020; Reichert et al., 2021; Tang et al., 2018; Xiao et al., 2021). Therefore, developing and applying appropriate methods for MP extraction is essential to further investigate the effects of plastic ingestion and accumulation in these organisms.

Most of the studies investigating MP occurrence in anthozoan tissues have applied chemical digestion protocols that lack formal validation (Allen et al., 2017; Corinaldesi et al., 2021; Ding et al., 2019; Helmberger et al., 2025; Lei et al., 2021; Mateos-Cárdenas et

al., 2024; Morais et al., 2020; Rotjan et al., 2019; Sfriso et al., 2020; Tang et al., 2021). Although some recent efforts have been made toward validating the methods (Santana et al., 2022; Lim et al., 2022; Reichert et al., 2022; Savage et al., 2022), most of these tests still exhibit methodological limitations. For instance, Lim et al. (2022) applied 35% H₂O₂ and 37% HCl on five scleractinians (Anthozoa: Hexacorallia) belonging to three genera (*Acropora*, *Galaxea*, *Pocillopora*). Both reagents may chemically affect several polymer types (PA, PET, and PC for HCl; PA and PET for H₂O₂; Lusher et al., 2017). Moreover, the study employed relatively large MP particles (2.5–4.5 mm), far exceeding the size range expected to be ingested by coral polyps, and the results were not quantitatively reported. Reichert et al. (2022) tested NaClO (7%) and HCl (5.5%) on scleractinian corals (*Acropora muricata*, *Pocillopora verrucosa*, *Porites lutea*, and *Heliopora coerulea*). While these concentrations generally preserve most polymer types, except for PA under acid exposure, the test was limited to only 20 PE particles per reagent and did not account for possible synergistic effects between sequential chemical treatments. Santana et al. (2022) carried out a rigorous validation on *Acropora millepora*, comparing 1 M KOH, 10% KOH, and 70% HNO₃ using PE, PS, PET, PVC, and rayon under various exposure times. Although the results were reported with methodological detail, both KOH treatments proved inefficient, while HNO₃ achieved complete digestion with acceptable recovery and limited morphological alteration, these tests were mostly performed on millimeter-sized particles (2.7 ± 0.6 mm). Moreover, recovery was assessed for smaller particles (<0.5 mm) without specifying their exact size range, and the chemical stability of polymers under 70% nitric acid contradicts previous findings (Lusher et al., 2017). Finally, Savage et al. (2022) applied a Fenton's reagent-based method (1:1 H₂O₂ 30% and FeSO₄· 7H₂O) to *Anemonia viridis*, following a protocol previously validated by Hurley et al. (2018) on several polymers (PP, LDPE, HDPE, PS, PET, PA-6,6, PC, PMMA). While effective in removing organic matter and preserving plastics, the tested particles were again relatively large (2.8–6.3 mm), limiting the applicability of the results to MP fractions relevant for coral ingestion.

Taken together, these studies represent important progress in protocol validations but reveal recurring limitations, including restricted particle diversity, unrealistic particle sizes, lack of combined chemical testing, and insufficient quantitative reporting, consequently limiting cross-study comparability and standardization. Indeed, the

International Organization for Standardization (ISO) has established several standardized protocols, such as those for MP monitoring in clean and drinking water, and for evaluating MP toxicity or biodegradation by microorganisms. However, attempts to extend these standards to biological samples have so far produced limited results (Brožová et al., 2025; Gambardella et al., 2019). In this context, this study aims to identify and validate a suitable chemical digestion protocol for the isolation of small-sized MPs (50–1000 µm) from Anthozoa, in accordance with standardized protocols for assessing the reliability of MP detection in marine biota (Laface et al., 2023; López-Rosales et al., 2026; Schirinzi et al., 2020; Tsangaris et al., 2021). To this end, two digestion protocols were comparatively tested to identify the one providing the highest digestion efficiency. The selected protocol, adapted from a previously unvalidated methodology developed for the northern star coral *Astrangia poculata* (Allen et al., 2017) later applied to the red coral *Corallium rubrum* (Corinaldesi et al., 2021) and to four shallow-water reef-building scleractinian corals (Reichert et al., 2022), was then optimized and validated for MP isolation from the bamboo coral (Anthozoa: Octocorallia) *Isidella elongata* (Esper, 1788) species. The protocol was tested on three polymer types (PE, PP, PS) and five size classes (10–1000 µm) to verify its suitability for MP isolation, ensuring efficient removal of biological material while preserving the physical and chemical integrity of the particles across polymers and size classes. The effectiveness of the protocol was evaluated both in the presence and absence of biological material, assessing recovery rates, morphological alterations of the particles and their chemical integrity through FT-IR spectroscopy.

This species was used as biological material during this study, since it is a key species that forms extensive facies on deep muddy bottoms of the Mediterranean Sea, significantly enhancing the biodiversity of these biotopes (Angiolillo et al., 2024; Carbonara et al., 2020; Gerovasileiou et al., 2019; Mastrototaro et al., 2017) and, despite its ecological importance, no studies to date have investigated the impact of MPs on this species. This study, building on previous efforts by adapting the methodology to *I. elongata* and conducting comprehensive validation, provides the first validated digestion protocol specifically implemented for octocorals and cold-water corals, contributing to the expansion of MP research in Mediterranean anthozoans.

Materials and methods

Selection of protocol for MP extraction from I. elongata

To assess the most suitable protocol, this research first tested the Digestion Efficiency (%DE) of two already existing methods for plastic extraction from biota. The selection of digestion protocols was guided by the compositional characteristics of the target species, whose tissues include both soft organic matter and a calcium carbonate skeletal component (Saucier et al., 2021), requiring a two-step digestion for the two matrices. These included one of the protocols already used for anthozoans and not yet validated. The two selected potential protocols were modified by adjusting some processing parameters to reduce the action of acids, including concentration, agent ratio, exposure time and temperature, to preserve the polymers and the small particle sizes intended for validation (Lusher et al., 2017). The adjustments also aimed to avoid unnecessary steps, reducing sample handling, contamination risks, and particle damage, while maintaining efficiency. The biological material used to validate the method was collected during an experimental trawl survey carried out in the Gulf of Squillace (Ionian Sea) in September 2023.

The first method was adapted and optimized based on previously published protocols developed for anthozoans (Allen et al., 2017; Corinaldesi et al., 2021; Reichert et al., 2022) (Protocol 1) (Table S1.1): *I. elongata* subsamples were weighed and placed into beakers, which were covered with aluminum foil. To digest organic matter, 10 mL of sodium hypochlorite solution (NaClO 8.25%) per 1 gram of weight of sample was added (1:10 w/v) in the beakers (1st digestion step). The samples were kept at room temperature for 24 hours, occasionally stirring to ensure thorough contact of the sample with the digestion solution. Following NaClO treatment, samples were filtered through Whatman GF/C filters (1.2 µm pore size) using a vacuum filtration apparatus and rinsed with Milli-Q water. To remove any residual calcium carbonate content, 40 mL per gram of sample of 10% hydrochloric acid (HCl) was applied directly to the filter for 30 minutes at room temperature (2nd digestion step). After this second digestion step, samples were filtered again, rinsed with Milli-Q water, and stored at room temperature in Petri dishes.

A second digestion method was adapted from Schirinzi et al. (2020) with minor modifications (Protocol 2): *I. elongata* subsamples were weighed and treated with 10% potassium hydroxide (KOH) solution in a 1:10 (w/v) ratio (1st digestion step). The beakers, covered with aluminum foil, were incubated at 50°C for 6 hours. Samples were then filtered through Whatman GF/C filters (1.2 µm pore size) and rinsed with Milli-Q water. The residual calcium carbonate content was removed by applying 40 mL per gram of sample of nitric acid (HNO₃ 5%) directly on the filter for 2 hours (2nd digestion step). The samples were then filtered, rinsed, and stored at room temperature in Petri dishes.

The whole procedures were performed under a fume hood to minimize potential contamination. To minimize any loss of biological material and MP particles retained on the glassware involved in the digestion and filtration process, including beakers and filtration cups, were thoroughly rinsed with a 1:1 solution of ethanol and Milli-Q water according to Concato et al. (2025).

For both protocols (Protocol 1 and Protocol 2), three replicates were considered to assess the %DE. The filters were dried overnight at 50°C and weighed before and after each digestion step to assess the percentage of weight variation, and thus the efficiency of tissue removal. In addition, visual inspection of the filters was used as a qualitative measure of digestion efficiency. The %DE was calculated according to the formula:

$$\%DE = \frac{W_i - (W_a - W_b)}{W_i} * 100$$

where W_a and W_b represent the dry weights of the filter after and before filtration, respectively, and W_i corresponds to the weight of the tissue subjected to digestion (Karami et al., 2017; Schirinzi et al., 2020). The protocol with the highest value of %DE was selected for the validation test.

Polymers preparation and test design

To perform the validation test, we chose to use the most common polymers in the marine environment (Andrady, 2017; Suaria et al., 2016). PE fragments were obtained from PE Powder of Sigma-Aldrich (Product No. 427772, density: 0.92 g/cm³), whereas industrial polypropylene (PP) pellets and foamy polystyrene (PS) spheres were processed

into irregularly shaped fragments using a Mixer Mill Retsch MM400 cooled with liquid nitrogen to avoid polymer alterations from heating. Plastics were chemically analyzed using Fourier transform infrared spectroscopy in Attenuated Total Reflection mode (ATR-FTIR; Shimadzu IRTracer-100) to confirm their polymeric nature and establish a self-generated spectral library containing only a reference spectrum of each polymer analyzed (PE, PP, PS). Fragments were manually isolated, photographed and measured with a stereomicroscope Zeiss Discovery V.8 coupled with Axiocam 208 color microscope camera and analyzed using ZEN 3.1 blue Edition software and then categorized into five size classes chosen according to the prey size range of the target species: a) 50-250 µm, b) 250-450 µm, c) 450-650 µm, d) 650-850 µm, and e) 850-1000 µm. Mean length and standard deviation of particles in each category are reported in Table S1.2.

Among the two tested protocols, the more efficient was tested on the five-size class for each polymer (PE, PP, PS). In detail, *I. elongata* tissue samples (1g in weight) were placed in a glass beaker, spiked with two fragments for each size class (test with tissues, TS). The same protocol was tested without tissue as a control (CTRL), adding one fragment for each class size according to Schirinzi et al. (2020) and Laface et al. (2023). These tests were carried out in duplicate for the treatment (TS) and in triplicate for CTRL, resulting in 105 MPs across a total of nine beakers.

Validation phases

After the exposure to both digestion steps of Protocol 1 for the digestion of organic matter and calcium carbonate contents, respectively, filters were removed and observed under the stereomicroscope. The number of recovered fragments was recorded and counted for each test (TS and CTRL), polymer (PE, PP, PS) and size class (a, b, c, d, e) to calculate the percentage of recovery as follows:

$$\frac{\text{Number of recovered MPs}}{\text{Number of spiked MPs}} * 100$$

Recovered MPs were photographed and measured after both digestion steps (Fig. 1.3A). Any changes in size and morphology were assessed by comparison with the respective pre-digestion samples. Morphological alterations were categorized as high, moderate, or low for changes in surface appearance (degradation), changes in shape

(deformation), changes in color (discoloration), and detachments of small fragments from the initial particle (fragmentation) according to Laface et al. (2023) and Tsangaris et al. (2021) (Table S1.3). The percentage of MP changes for each step of the protocol of both tests (TS; CTRL) was estimated as follows:

$$\frac{\text{Number of MPs with morphological modifications}}{\text{Number of spiked MPs}} * 100$$

ATR-FTIR was used to compare the recovered fragments with the initial spectrum, assessing any changes in the polymer composition or structure after digestion (Fig. 1.3B). For the polymer identification of the smallest particles (< 100 µm), the analysis was integrated using an infrared (IR) microscope (Shimadzu, AIMSight). Polymer identification was accepted when the match score with the reference spectra was > 70% (Schirinzi et al., 2020; Tsangaris et al., 2021).

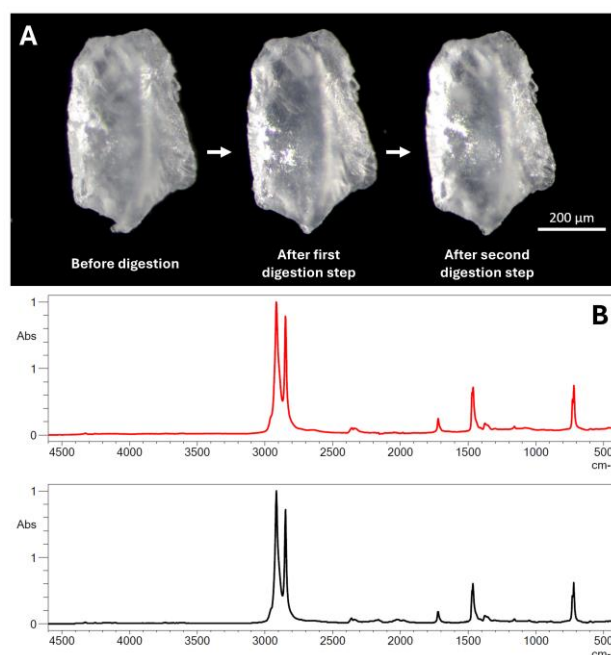


Figure 1.3. Example of: (A) a polyethylene particle before digestion, after the sodium hypochlorite and the hydrochloric acid treatments; (B) comparison between spectra before and after digestion.

Quality assurance (QA)

According to Pedà et al. (2022, 2020), the following measures were applied during all laboratory procedures: I) operators wore 100% cotton lab coats; II) all glass containers, tweezers and metal scissors were thoroughly rinsed with milli-Q water; III) all work surfaces were cleaned with ethanol; iv) tissue samples were covered with aluminium foil during digestion and when not in use, while filters obtained from digestion were covered with glass lids; v) sample filtration activities were conducted under a fume hood. As previously reported by Thiele et al. (2019) and Tsangaris et al. (2021), the use of procedural blanks to quantify possible airborne contamination was not considered necessary, as the MPs spiked during the test were fragments. Indeed, our samples did not include fibers, which are the main form of airborne contamination.

Statistical analysis

A Permutational Multivariate Analysis of Variance (PERMANOVA) was performed to evaluate differences in digestion efficiency between protocols, using Euclidean distances. A Poisson variance check was performed to assess the dispersion of the variance relative to mean and to evaluate whether the observed variability in recovery rates among replicates could be attributed to random fluctuations expected from Poisson-distributed particle counts (Corami et al., 2021). The effect of polymer type, particle size class, and presence or absence of biological material on MPs recovery after the digestion protocol was analyzed using a logistic regression model, setting as the reference level 'Polyethylene' for polymer type, 'a' for particle size class, and 'CTRL' for beaker type. Model assumptions were verified prior to interpretation, confirming the absence of multicollinearity (all VIF < 1.01) and no overdispersion (dispersion ratio = 1.06, $p = 0.75$). Odds ratios and 95% confidence intervals were calculated to estimate effect sizes. The contribution of polymer type, particle size class, and presence or absence of biological material on size variation was assessed through a PERMANOVA analysis, using Euclidean distances. To investigate potential significant differences in the morphological modification of particles between different polymers and/or size classes, a Pearson's Chi-squared test was conducted, followed by a Posthoc residual analysis to identify which

specific groups contribute to significant results. Effect size was quantified using Cramér's V. These analyses were performed using R and RStudio software.

Results

Digestion efficiency test

The two protocols were compared in terms of digestion efficiency (%DE). Protocol 1 resulted in a %DE of $97.55\% \pm 0.12$ (mean% $\pm$ SEM), while Protocol 2 yielded $96.36\% \pm 0.42$ (Fig. 1.4). Visual inspection of the filters showed better conditions after the application of Protocol 1 with clearer filters suggesting a more complete digestion of biological material (Fig. S1.1). In both cases, only small tissue fragments remained on the filters, especially the gorgonin nodes, partially intact, while no skeletal debris was retained. According to PERMANOVA analysis, the two protocols explained 64.5% of the variance in digestion efficiency ($R^2 = 0.64567$, $F = 7.289$), but there was no significant difference between the two protocols ($p = 0.1$) (Table S1.4). Although the test did not show significant statistical differences, Protocol 1 was selected and validated for the extraction of MPs from *Isidella elongata* tissues as a result of the higher %DE and the qualitative comparison.

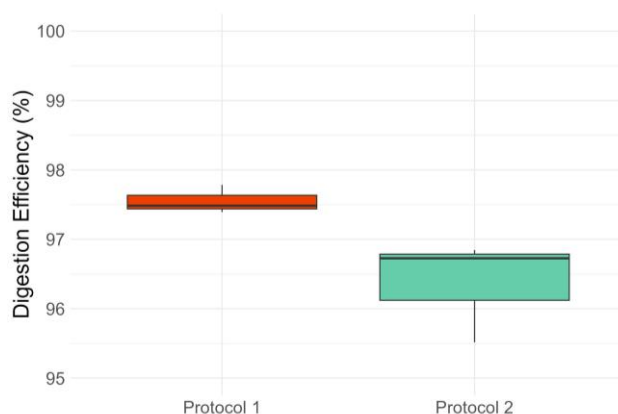


Figure 1.4. Comparison of digestion efficiency between Protocol 1 and Protocol 2.

Recovery rate of the polymers

A total of 91 and 92 out of 105 spiked particles in all replicates were recovered from the first and second digestion steps, respectively. After NaClO treatment, the recovery

rate varied between particle types: PE 94.3%, PP 86.6%, PS 77.1%. After the HCl step, recovery rates for PE, PP, and PS stood at 82.9%, 91.4%, and 88.6%, respectively. Recovery of particles increased with size class, with 81.0% in classes *a* and *b*, 85.7% in class *c*, and 95.2% in classes *d* and *e*. Recovery rates after digestion for each polymer and size class are shown in Figure 1.5. The results of the Poisson variance check (Table S1.5) showed that the variance-to-mean ratios were generally lower than 1, indicating stable performance of the protocol and variability consistent with that expected from Poisson-distributed counts. The only deviation was observed in PP size class *a*, where the loss of a few particles likely resulted in a reduction in recovery percentage.

However, the logistic regression model indicated no significant differences in recovery rates among the various tested polymers or across the different size categories ($p > 0.05$) (Table S1.6). In contrast, the presence of biological material had a significant positive effect on recovery ($p = 0.0435$), with particles in TS treatments reaching 93.3% of recovery, compared to 80.0% for CTRL treatments. The model fit was supported by the absence of multicollinearity ($VIF < 1.01$) and no overdispersion (dispersion ratio = 1.06, $p = 0.75$).

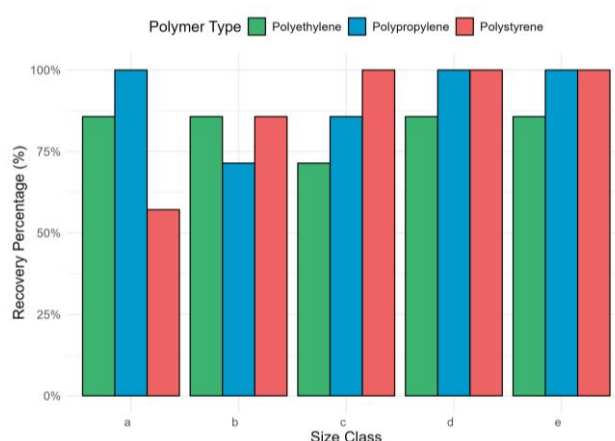


Figure 1.5. Recovery rates after chemical digestion sodium hypochlorite and hydrochloric acid for each polymer type and size class.

Morphological alteration of the polymer

The visual representation of PE, PP, and PS MPs (five size classes, control vs. tissue) throughout the digestion process is provided in the supplementary material (Figs. S2-4). Mean ($\pm$ standard deviation) length of each polymer and size class before and after digestion are reported in Table S1.3. At the end of digestion, the length decreased by $3.82 \pm 5.57 \mu\text{m}$ (mean $\pm$ SEM) and $3.72 \pm 2.88 \mu\text{m}$ (mean $\pm$ SEM) in CTRL and TS tests, respectively. The most affected polymer was PS with a reduction of $6.73 \pm 5.35 \mu\text{m}$, while among size classes, class *d* reached $10.59 \pm 4.86 \mu\text{m}$. The PERMANOVA analysis revealed no statistically significant differences in the variation of length among polymer types, size classes, or with and without biological material ($p = 0.941$, $R^2 = 0.0137$) (Table S1.7). Size variations in length of recovered particles after digestion for each polymer and size class in CTRL and TS are reported in Figure 1.6.



Figure 1.6. Boxplots reporting the variation length for each polymer and size class after digestion with sodium hypochlorite and hydrochloric acid in CTRL and TS treatments.

93.4% of the particles recovered after NaClO treatment showed no or low morphological alterations, while only six PS particles showed moderate fragmentation. Following HCl treatment, 77.2% of the recovered particles displayed no or low

morphological alterations, with no changes in color observed in any replicate. Among the remaining 22.8% that exhibited alterations, two particles of size class *a*—one PE and one PP—experienced high degradation, while three particles of size class *a*—two PE and one PP—were subjected to moderate and high deformation. Fragmentation was the most common morphological alteration, with moderate and high alterations affecting 15 PS particles across all size classes and a single PE particle from size class *a*. The Pearson's Chi-squared test revealed a significant relationship between the level of fragmentation and the polymer type ($X^2 = 31.45$, $df = 4$, $p < 0.001$), with the main contributor being PS ($p < 0.001$). The relationship between polymer type and fragmentation level was confirmed by a moderate effect size (Cramér's $V = 0.39$). Representative examples of the different types of morphological alterations observed after digestion treatments are shown in Figure 1.7.

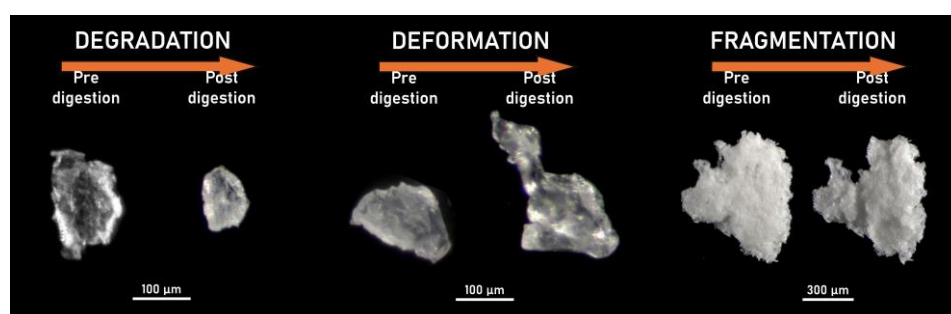


Figure 1.7. Representative examples of morphological alterations observed in MPs after chemical digestion treatments, including degradation of polypropylene, deformation of polyethylene, and fragmentation of polystyrene.

FT-IT identification

Polymer identification (match score $> 70\%$) was achieved in 88 of the 92 recovered particles, while three PP fragments from CTRL and one PE fragment from TS remained unidentified, all belonging to size class *a*. The mean match score after chemical digestion varied among polymers, with PE at $94.0\% \pm 1.5\%$, PP at $90.0\% \pm 1.7\%$ and PS at $91.7\% \pm 0.9\%$. The mean score for polymer identification increased progressively with size class, from $80.3\% \pm 2.8\%$ in class *a* to $96.4\% \pm 0.2\%$ in class *e* ($R^2 = 0.7741$).

The match score between the FT-IT spectra of each recovered polymer and those of the corresponding reference particles are reported in Table S1.8. A comparison of representative FT-IR spectra for each polymer between reference particle samples and particles after digestion in CTRL and TS treatment is reported in Figure S1.5.

Discussion

This study aimed to identify and validate, for the first time, a digestion protocol for extracting MPs from deep-sea cold-water octocoral using as biological material the bamboo coral *I. elongata*. Cold-water corals are key components of deep-sea “marine animal forests” (*sensu* Rossi et al., 2017), as they can create three-dimensional habitats that enhance biodiversity and ecosystem functioning by modulating the availability of resources and providing niches for other organisms to live, feed, and shelter (Nelson and Bramanti, 2020; Paoli et al., 2017). Among them, *I. elongata* plays a key ecological role in Mediterranean bathyal muddy bottoms and has been included in several international conservation frameworks due to its high vulnerability to human pressures (Georges et al., 2024; Mastrototaro et al., 2017). Although this species is listed as “Critically Endangered” on the IUCN’s European Red List of Threatened Species, especially due to the impacts of bottom trawling fishery (Cartes et al., 2013; Pierdomenico et al., 2018), the abundance and long-term effects of MPs on *I. elongata* and other cold-water corals remain largely unexplored, highlighting the need for dedicated research on this emerging threat in deep-sea ecosystems.

Therefore, the present study provided a reliable methodology for the isolation and identification of MPs from anthozoan tissues. Compared with previous validation efforts, it addresses key methodological gaps in Anthozoa-related studies (Lim et al., 2022; Reichert et al., 2022; Santana et al., 2022; Savage et al., 2022), including the limited number of tested polymers, the use of unrealistic particle sizes, and the lack of quantitative and multi-parametric assessments. The proposed protocol was tested on three of the most abundant polymer types found in marine environments and ingested by biota (PE, PP, PS; Andrady, 2017; De Sá et al., 2018; González-Ortegón et al., 2024; Palmieri et al., 2024; Rodrigues et al., 2019; Suaria et al., 2016) and across a wide range of size classes (50–1000 µm), thus including the size ranges realistically ingested by coral polyps. This study evaluated the combined effects of a two-step digestion involving 8.25% NaClO and 10% HCl and examined their potential application. The chemical compatibility of each reagent with the tested polymers was systematically verified (Laface

et al., 2023; Schirinzi et al., 2020; Tsangaris et al., 2021), integrating literature evidence (Lusher et al., 2017) with direct morphological and spectroscopic observations.

Protocol selection

Chemical digestion protocols previously applied for the extraction of MPs from biota were identified from the literature and subsequently adapted to the target species, considering the aggressiveness of chemical reagents to polymers, time exposure, time consumed and airborne contamination. In addition to these analytical considerations, factors such as cost and ease of use are also important when selecting suitable methodologies (Tsangaris et al., 2021). Moreover, the choice took into account the dual composition of *I. elongata* tissues, combining organic matter with a calcium carbonate skeleton (Saucier et al., 2021), a characteristic typical of several coral species. Consequently, it was crucial to include an acidic digestion step capable of dissolving the calcium carbonate fraction without compromising the recovery and preservation of MPs.

The first identified protocol was developed by Allen et al. (2017) that combines an alkaline oxidative agent for the degradation of soft tissues (sodium hypochlorite, NaClO), with a strong acid involved in the dissolution of the calcium carbonate sclerites and axial skeletons (hydrochloric acid, HCl). The same reagent combination, with different concentrations, was also used in subsequent protocols by Corinaldesi et al. (2021) and Reichert et al. (2022). In our study, the step that involved 8.25% NaClO at room temperature for 24 hours (Allen et al., 2017) was kept unchanged, as this concentration proved more effective for digesting *I. elongata* tissues than the lower concentrations used by Corinaldesi et al. (2021) and Reichert et al. (2022), of 4.5% and 7%, respectively, which were found to be insufficient under our experimental conditions. The limited aggressiveness of NaClO toward most polymers, even at higher concentrations, is supported by Lusher et al. (2017), who reviewed the chemical resistance of polymers to chemical agents under various conditions. Regarding the acid digestion phase, the use of 37% HCl, as in Allen et al. (2017) and Corinaldesi et al. (2021), is reported to cause the loss or complete degradation of some plastic polymers (e.g., Polyamide, Polycarbonate, Polyethylene Terephthalate), whereas 10% HCl seems to be significantly less aggressive while maintaining efficiency (Lusher et al., 2017). Reichert et al. (2022) used a lower HCl

concentration (5.5%) combined with a prolonged exposure time (18 h), a condition we chose to avoid due to potential risks associated with extended reagent contact. Furthermore, the second 24-hour soak in 8.25% NaClO (Allen et al., 2017) appeared unnecessary for our objectives, as the first two steps returned sufficiently clean filters, without leaving major residues. All subsequent steps were avoided as they may increase the probability of airborne contamination risk and physical damage to MPs, especially for small-sized particles. Additionally, the centrifugation and the following removal of precipitate carried out in Allen et al. (2017) and Reichert et al. (2022) assume that all MPs have densities low enough to float in saturated NaCl, likely leading to the underestimation of non-buoyant polymers as PS, PET and PVC (Bond et al., 2018; Chubarenko et al., 2016; Lusher et al., 2017). Finally, regarding particle retention, Allen et al. (2017) applied 125 µm mesh sieving after each digestion step, a size that is likely to overlook smaller MPs. Reichert et al. (2022) used 50 µm sieves after the first step, which, although closer to our target smaller size, still poses potential underestimation problems. In contrast, Corinaldesi et al. (2021) opted for a simplified workflow with minimal handling steps and final filtration through 0.2 µm pore size filters, in line with our approach that prioritizes sensitivity and minimal sample loss. Table S1.1 reports all the steps required from the protocols applied in previous studies with the modification made for the protocol proposed in the present study. A second digestion protocol previously applied on gastrointestinal tracts of the common dolphinfish (*Coryphaena hippurus*) by Schirinzi et al. (2020) was adapted by introducing minor modifications. The first digestion step, involving 10% KOH at 50°C for 6 hours, was unmodified, except for the weight/volume (g/mL) ratio from 1:3 to 1:10. This modification was necessary to ensure complete coverage of the samples during digestion, as the original protocol was optimized for compact gastrointestinal content matrices, whereas the coral fragments used in this study had a relatively high volume/weight ratio. In addition, HNO₃ concentration was reduced from 20% to 5% and the exposure time from 1 to 2 hours. This adjustment was made considering the smaller and more acid-sensitive particles (Corami et al., 2021) expected in anthozoans, compared to the larger debris ingested by carnivorous fish (Sequeira et al., 2020). The extended exposure time ensured the complete dissolution of calcium carbonate from coral skeletons without compromising the integrity of MPs.

Both Protocol 1 and Protocol 2 achieved satisfactory results in terms of digestion efficiency, leading to the effective removal of soft tissues and calcium carbonate matrices. Although statistical analysis did not indicate a significant difference among protocols, the higher %DE and more complete removal of organic material prompted us to choose Protocol 1 for the validation tests (Fig. S1.1).

Validation test

The validation of protocol is an important step, especially in studies focused on the extraction of MPs from environmental matrices, in order to demonstrate replicability and improve data reliability, also with a view to harmonizing and standardizing the methods to be used as universal protocols in the near future.

Our findings showed that the spiked particles of all polymers and size classes were efficiently recovered after chemical digestion. The recovery rate after NaClO treatment was lower than that observed after the second step with HCl, as the presence of indigested axial skeletons and numerous sclerites made it harder to isolate the plastic particles from the filters. This result confirms the need to apply both steps for the digestion of these complex organisms, also because the only use of acid step would be efficient in the digestion of organic material (Cole et al., 2014; Lusher et al., 2017). TS replicates displayed significantly higher recovery rates than CTRL ones after acid treatment. Indeed, biological tissues can act as a buffer, mitigating the effects of chemical reagents on plastic particles, particularly during acid treatment (Laface et al., 2023; Schirinzi et al., 2020). In the absence of biological tissues, the acid may interact more aggressively with MPs, particularly those in smaller size classes, due to their higher surface/volume ratio. Indeed, a previous study (Lusher et al., 2017) demonstrated that several polymers are not resistant to high concentrations of hydrochloric acid (HCl), especially in conditions of high temperatures. It is worth pointing out that the small size of MPs makes recovery more challenging, as particles can adhere to laboratory glassware, mostly filtration glasses, without the operator noticing. This issue has also been highlighted in other studies on MP extraction from environmental samples (Karlsson et al., 2017; Schirinzi et al., 2020; Thiele et al., 2019). However, the variability observed in recovery rates among replicates

may also be explained by the relatively limited number of spiked particles, whose counts are inherently affected by Poisson distribution (Corami et al., 2021) (Table S1.5).

The chemical digestion process had no significant impact on the size of recovered MPs, where only minor deviations were reported in all the polymers and size classes. The observed dimensional variations are random and non-systematic, as shown in PERMANOVA output ($p = 0.941$, $R^2 = 0.0137$), suggesting that the protocol does not significantly alter the size of the particles. In addition, the positioning of particles under the stereomicroscope affects their measurements, causing a systematic variation of length, both positive and negative, as also highlighted by Tsangaris et al. (2021). Most of the particles have preserved their integrity, with low levels of degradation and deformation, and no discoloration recorded. Among the types of damage assessed, fragmentation was found to be the most critical alteration, reporting a statistically significant difference among polymer groups. The overall effect was attributable to PS, whose higher fragmentation rate was due to its natural brittleness and limited elongation at break (Andrady and Koongolla, 2022; Sorasan et al., 2022). PE and PP were less susceptible to mechanical and chemical stress and more resistant to disruption in digestion, maintaining their overall structural integrity. Fragmentation of PS during experimental processing can affect particle counts by generating smaller fragments, leading to an overestimation of MP abundance if measurements are limited to particle numbers rather than combined metrics such as size distribution or total mass.

The comparison between particle FT-IR spectra before and after digestion suggests that polymer composition and structure were well preserved after digestion, as also confirmed in similar studies using different biological matrices and polymer types (Hurley et al., 2018; Laface et al., 2023; López-Rosales et al., 2026; Schirinzi et al., 2020). Only 4.3% of the recovered particles from size class *a* did not achieve the identification, having a match score $< 70\%$ (Table S1.8). This can be explained by the loss of instrument accuracy proportionally to the decrease in particle sizes, making it more challenging to obtain accurate measures for smaller size categories.

Method applicability

In addition to its successful application to *I. elongata*, the digestion protocol proposed in this work has potential for application across other anthozoans. However, some care must be taken, considering that tissue and skeletal composition vary significantly among taxonomic groups. The method was originally developed for the scleractinian coral (*Hexacorallia*) *Astrangia poculata* (Allen et al., 2017) and subsequently applied to *Corallium rubrum* (Corinaldesi et al., 2021). These successful applications on species with distinct skeletal architectures demonstrate the protocol's versatility, although specific adjustments are needed depending on tissue and skeleton composition.

Among hexacorals, scleractinians appear to be the most suitable candidates for the digestion protocol, given their tissue organization combining soft tissues with a calcium carbonate skeleton in the form of aragonite. Black corals (*Antipatharia*) have an axial chitinous skeleton (Bo et al., 2012) which may pose additional challenges for chemical digestion (Kallenbach et al., 2021). Other hexacorallians such as *Zoantharia*, *Ceriantharia*, and *Actinaria* generally lack calcareous matrices and are composed almost exclusively of soft tissues (Daly et al., 2003); in such cases, the acid digestion step could even be omitted, although the higher organic content might require an increased oxidizing strength of chemicals.

Octocorals, while all containing calcite sclerites embedded in their tissues, display highly variable skeletal architectures. Some, such as the so-called soft corals, do not have a compact axial structure, while others possess an axial skeleton that varies among species: axial skeleton of gorgonians (sea fans) is mainly constituted by sclerotized collagen (Sánchez et al., 2019), red corals have a calcified axis (Le Roy et al., 2021), and bamboo corals' skeleton is articulated in calcitic internodes separated by proteinaceous nodes (Saucier et al., 2021). According to these structural differences, the protocol would require further adjustment before being applied to organisms with different tissue compositions. Nonetheless, the present method, originally designed for the scleractinian coral (*Hexacorallia*) *Astrangia poculata* (Allen et al., 2017), has already proven to be effective in *C. rubrum* (Corinaldesi et al., 2021). Its potential application to hexacorals,

such as scleractinian corals that produce calcified skeletons (in the form of aragonite), can also be considered.

When applying this protocol to field-derived samples for the assessment of MP abundance, appropriate quality assurance/quality control, including good field and laboratory practices and the use of field and procedural blanks, will be essential to minimize contamination introduced during sample processing or from laboratory manipulation and to quantify background contamination (Brander et al., 2020; GESAMP, 2019; Lusher et al., 2017; Tsangaris et al., 2021). Careful consideration should be given to the amount of biological material processed, since excessive sample volumes may lead to filter clogging and reduce filtration efficiency, although multiple replicates may be required to ensure representativeness (López-Rosales et al., 2026). In this respect, an initial sample mass between 0.5 and 1 g is recommended to maintain optimal filtration efficiency and minimize material loss.

Conclusions

MP pollution is currently one of the most significant scientific and environmental challenges. Continuous efforts are required to monitor and protect the marine environment and its biodiversity from this phenomenon. In particular, extreme environments such as the deep sea and their associated organisms remain largely unexplored and understudied, since the inaccessibility of deep areas and the difficulty of carrying out permanent observations. This study has a twofold implication: on one hand, it contributes to scientific progress by providing a suitable and validated protocol for the extraction of MPs from anthozoans, particularly on cold-water corals; on the other hand, it uses an ecologically important and vulnerable Mediterranean species of octocoral, *I. elongata* as the eligible species for protocol validation, which is likely affected by MP pollution but still poorly investigated. The versatility and transferability of the identified protocol represent a valuable tool for improving monitoring efforts under Descriptor 10 of the Marine Strategy Framework Directive (2008/56/EC) and guiding targeted conservation plans.

Furthermore, this research represents a baseline for addressing the standardization of digestion methodologies for anthozoans, and a starting point for future application to other deep-sea coral species, an aspect that has so far been almost neglected. The experimental design and findings ensured the robustness of the method, which involved a high number of replicates and a comprehensive validation test integrating recovery rate, particle size variation, morphological integrity, and FT-IR spectral consistency.

CRedit authorship contribution statement

Antonio Giova: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Cristina Pedà:** Conceptualization, Data curation, Methodology, Supervision, Validation, Visualization, Writing - review & editing. **Daniela Pica:** Resources, Supervision, Validation, Writing - review & editing. **Silvestro Greco:** Funding acquisition, Resources, Writing - review &

editing. **Teresa Romeo:** Funding acquisition, Project administration, Resources, Writing - review & editing. **Nunziacarla Spanò:** Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementay materials 1

Supplementary materials

Table S1.1. Pipelines of the existing protocols performed by Allen et al. (2017), Corinaldesi et al. (2021), Reichert et al. (2022) and the present study involving the same reagent combination of sodium hypochlorite (NaClO) and hydrochloric acid (HCl). “/” = step not performed

Digestion protocol from Allen et al. (2017)	Digestion protocol from Corinaldesi et al. (2021)	Digestion protocol from Reichert et al. (2022)	Digestion protocol from the <i>Present study</i>
Coral colonies soaked in 8.25% NaClO solution for 24 hours	Coral colonies soaked in 5 mL per branch (~2 cm ²) of 4.5% NaClO solution for 24 hours	Coral colonies soaked in 7% NaClO solution for 24 hours	Coral colonies soaked in 10 mL per gram of sample of 8.25% NaClO solution for 24 hours
Particulate material trapped on 125 µm sieves	Not specified	Particulate material trapped on 50 µm sieves	Particulate material trapped on 1.2 µm filter in vacuum filtration system
Coral skeletons moved to 37% HCl solution for 30 minutes	Coral skeletons moved to 5 mL of 37% HCl solution for 30 minutes	Coral skeletons moved to 5.5% HCl solution for 18 hours	Coral skeletons soaked in 40 mL of 10% HCl solution for 30 minutes directly on the filter
Particulate material trapped on 125 µm sieves	Particulate material trapped on 0.2 µm filters in vacuum filtration system	Particulate material trapped on 50 µm sieves	Particulate material trapped on 1.2 µm filters in vacuum filtration system
Residues soaked in 8.25% NaClO for 24 hours	/	/	/
Particulate material trapped on 125 µm sieves	/	/	/
Residues centrifugated at 581g for 10 min with ~20–40 mL of deionized water and saturated NaCl solution	/	Residues centrifugated at 479 g for 10 min with filtered seawater	/

Mineral detritus removed from the base of the vial with a pipette	/	Mineral detritus removed from the base of the vial with a pipette	/
Floating particles trapped on filters in vacuum filtration system	/	Floating particles trapped on 8 µm filters in vacuum filtration system	/

Table S1.2. Mean ($\pm$ standard deviation) length of polyethylene (PE), polypropylene (PP), and polystyrene (PS) particles used for the spiking experiments, grouped into five predefined size classes (a–e), before and after digestion treatment.

POLYMER	Size class	PRE DIGESTION	AFTER DIGESTION
		Length (mean $\pm$ st.dev.) Size range	Length (mean $\pm$ st.dev.) Size range
PE	a	169.5 $\pm$ 36.7 96.6 - 222	180.3 $\pm$ 81.3 88.6 - 322.1
	b	379.8 $\pm$ 31.3 324.4 - 420.9	393.2 $\pm$ 25.6 354.8 - 437.6
	c	565.5 $\pm$ 35.1 517.2 - 607.3	578.4 $\pm$ 35.6 509.6 - 607.3
	d	770.2 $\pm$ 42.2 706.6 - 822.8	776.6 $\pm$ 40.8 710.5 - 827
	e	938.3 $\pm$ 33.8 882.7 - 980.9	926.6 $\pm$ 36.6 863.3 - 965.9
PP	a	181.3 $\pm$ 41.8 120.8 - 233.9	169.9 $\pm$ 46.8 111.0 - 234.2
	b	368.4 $\pm$ 39.6 322.3 - 435.8	379.1 $\pm$ 40.9 331.0 - 443.7
	c	520.1 $\pm$ 43 487 - 613.4	526.2 $\pm$ 47.6 492.5 - 623.6
	d	748.4 $\pm$ 39.8 680.6 - 807.0	741.4 $\pm$ 38.8 680.5 - 807
	e	951.6 $\pm$ 38.6 881.8 - 993.1	946.8 $\pm$ 40.8 880.4 - 999.7
PS	a	200.2 $\pm$ 33.8 135.8 - 244.4	195.8 $\pm$ 37.9 138 - 238.9
	b	339.5 $\pm$ 54.5 282.9 - 427.4	326.6 $\pm$ 85.7 186.3 - 426.7
	c	502.5 $\pm$ 37.3 453.9 - 568.8	490.0 $\pm$ 52.0 408.1 - 573.8

d	747.6 ± 50.3 677.9 - 821.5	724.9 ± 60.7 601.8 - 800.1
e	908.2 ± 36.1 857 - 957.3	914.5 ± 45.9 850.9 - 987.1

Table S1.3. Description of morphological alteration criteria from Laface et al. (2023) and Tsangaris et al. (2021). Morphological alterations are categorized as high, moderate, or low for changes in surface appearance (degradation), changes in shape (deformation), changes in color (discoloration), and detachments of small fragments from the initial particle (fragmentation).

Degradation	HIGH	Total surface corrosion
	MODERATE	Surface cracking
	LOW	Slight surface cracking
Discoloration	HIGH	Color change to brownish
	MODERATE	Color change to transparent/yellowish
	LOW	Color change to transparent/opaque
Fragmentation	HIGH	Sample fragmentation >50%
	MODERATE	20-50% sample fragmentation
	LOW	Sample fragmentation < 20%
Deformation	HIGH	Total or partial sample melting
	MODERATE	Alteration of initial shape (sample still recognizable)
	LOW	Slight alteration of initial shape (sample recognizable)

Table S1.4. Summary of the results of the Permutational Multivariate Analysis of Variance (PERMANOVA) using Euclidean distances, performed to evaluate differences in digestion efficiency between Protocol 1 and Protocol 2.

	DF	SUM OF SQUARES	R ²	P-VALUE (>F)
GROUP	1	0.64567	7.289	0.1
RESIDUAL	4	0.35433		
TOTAL	5	1.00000		

Table S1.5. Summary of the results of the Poisson variance check for each polymer and size class. A variance-to-mean ratio > 1 indicates overdispersion (high variability), values ≈ 1 suggest that the data may follow a Poisson distribution, while a ratio < 1 indicates underdispersion (low variability).

Polymer	Size class	Mean	Variance	Count	Variance/Mean ratio
PE	a	0.857	0.143	7	0.167
	b	0.857	0.143	7	0.167
	c	0.714	0.238	7	0.333
	d	0.857	0.143	7	0.167
	e	0.857	0.143	7	0.167
PP	a	1.000	0.000	7	0.000
	b	0.714	0.238	7	0.333
	c	0.857	0.143	7	0.167
	d	1.000	0.000	7	0.000
	e	1.000	0.000	7	0.000
PS	a	0.571	0.286	7	0.500
	b	0.857	0.143	7	0.167
	c	1.000	0.000	7	0.000
	d	1.000	0.000	7	0.000
	e	1.000	0.000	7	0.000

Table S1.6. Summary of the results of the logistic regression model applied to the recovery of microplastic particles after chemical digestion. The model intercept corresponds to polyethylene (PE) of size class "a", in the control treatment (CTRL), with a positive recovery outcome. The coefficients estimate the effect of different polymers, size classes and treatments on the recovery outcome.

	ESTIMATE	STD. ERROR	Z VALUE	PR(> Z)	ODDS RATIO	95% CI
(INTERCEPT)	3.92E-01	7.38E-01	0.531	0.5957	1.48	0.36– 6.93
POLYM. POLYPROPYLENE	8.70E-01	7.88E-01	1.104	0.2696	2.39	0.54– 12.92
POLYM. POLYSTYRENE	5.25E-01	7.33E-01	0.716	0.4742	1.69	0.41– 7.71
BEAKER TS	1.33E+00	6.57E-01	2.019	0.0435 *	3.77	1.10– 15.32
SIZE.CLASS B	3.45E-16	8.20E-01	0.000	1.000	1.00	0.19– 5.20
SIZE.CLASS C	3.73E-01	8.68E-01	0.429	0.6679	1.45	0.26– 8.80
SIZE.CLASS D	1.63E+00	1.19E+00	1.37	0.1708	5.11	0.64– 108.41
SIZE.CLASS E	1.63E+00	1.19E+00	1.37	0.1708	5.11	0.64– 108.41
SIGNIF. CODES: '***' p<0.001; '**' p<0.01; '*' p<0.05						

Table S1.7. Summary of the results of the Permutational Multivariate Analysis of Variance (PERMANOVA) using Euclidean distances. The effects of polymer type, size class, and the presence of biological material on the percentage variation in particle dimensions after the final digestion step was tested.

	DF	SUM OF SQUARES	R ²	F	P- VALUE
MODEL	7	254.8	0.01368	0.345	0.941
RESIDUAL	174	18363.9	0.98632		
TOTAL	181	18618.7	1.00000		

Table S1.8. The confidence level of spectral matches between the FT-IR spectra of each recovered polymer and their corresponding reference polymers. Polymer identification was accepted for levels of certainty > 70%.

Size class	Beaker	Polyethylene		Polypropylene		Polystyrene	
		Replicate	FT-IR match	Replicate	FT-IR match	Replicate	FT-IR match
a	CTRL	PE_CTRL_a_1	61.4 %	PP_CTRL_a_1	85.1 %	PS_CTRL_a_1	
a	CTRL	PE_CTRL_a_2	92.9 %	PP_CTRL_a_2	88.5 %	PS_CTRL_a_2	78.1 %
a	CTRL	PE_CTRL_a_3	90.0 %	PP_CTRL_a_3	83.3 %	PS_CTRL_a_3	75.7 %
a	TS	PE_R1_a_1	91.9 %	PP_R1_a_1	58.9 %	PS_R1_a_1	
a	TS	PE_R1_a_2		PP_R1_a_2	89.5 %	PS_R1_a_2	88.0 %
a	TS	PE_R2_a_1	88.6 %	PP_R2_a_1	64.5 %	PS_R2_a_1	
a	TS	PE_R2_a_2	71.3 %	PP_R2_a_2	66.1 %	PS_R2_a_2	90.7 %
b	CTRL	PE_CTRL_b_1	95.9 %	PP_CTRL_b_1	91.3 %	PS_CTRL_b_1	84.3 %
b	CTRL	PE_CTRL_b_2	94.7 %	PP_CTRL_b_2	91.6 %	PS_CTRL_b_2	90.9 %
b	CTRL	PE_CTRL_b_3		PP_CTRL_b_3		PS_CTRL_b_3	
b	TS	PE_R1_b_1	95.2 %	PP_R1_b_1		PS_R1_b_1	89.4 %
b	TS	PE_R1_b_2	93.3 %	PP_R1_b_2	89.8 %	PS_R1_b_2	88.4 %
b	TS	PE_R2_b_1	96.0 %	PP_R2_b_1	89.6 %	PS_R2_b_1	88.9 %
b	TS	PE_R2_b_2	96.1 %	PP_R2_b_2	89.5 %	PS_R2_b_2	88.9 %
c	CTRL	PE_CTRL_c_1		PP_CTRL_c_1	92.9 %	PS_CTRL_c_1	93.2 %
c	CTRL	PE_CTRL_c_2	97.9 %	PP_CTRL_c_2	92.6 %	PS_CTRL_c_2	92.8 %
c	CTRL	PE_CTRL_c_3		PP_CTRL_c_3		PS_CTRL_c_3	91.1 %
c	TS	PE_R1_c_1	96.2 %	PP_R1_c_1	92.2 %	PS_R1_c_1	92.5 %
c	TS	PE_R1_c_2	96.6 %	PP_R1_c_2	92.8 %	PS_R1_c_2	93.0 %
c	TS	PE_R2_c_1	97.1 %	PP_R2_c_1	94.2 %	PS_R2_c_1	90.3 %

c	TS	PE_R2_c_ 2	97.2 %	PP_R2_c_ 2	90.8 %	PS_R2_c_ 2	93.4 %
d	CTR L	PE_CTRL_ d_1	97.8 %	PP_CTRL_ d_1	95.2 %	PS_CTRL_ d_1	94.1 %
d	CTR L	PE_CTRL_ d_2	97.8 %	PP_CTRL_ d_2	94.2 %	PS_CTRL_ d_2	95.1 %
d	CTR L	PE_CTRL_ d_3		PP_CTRL_ d_3	96.0 %	PS_CTRL_ d_3	95.3 %
d	TS	PE_R1_d_1	97.8 %	PP_R1_d_1	96.1 %	PS_R1_d_1	94.4 %
d	TS	PE_R1_d_2	97.5 %	PP_R1_d_2	95.5 %	PS_R1_d_2	95.6 %
d	TS	PE_R2_d_1	97.9 %	PP_R2_d_1	95.4 %	PS_R2_d_1	93.4 %
d	TS	PE_R2_d_ 2	98.0 %	PP_R2_d_ 2	93.1 %	PS_R2_d_ 2	94.9 %
e	CTR L	PE_CTRL_ e_1	97.9 %	PP_CTRL_ e_1	95.8 %	PS_CTRL_ e_1	94.9 %
e	CTR L	PE_CTRL_ e_2	97.9 %	PP_CTRL_ e_2	95.8 %	PS_CTRL_ e_2	95.3 %
e	CTR L	PE_CTRL_ e_3		PP_CTRL_ e_3	96.6 %	PS_CTRL_ e_3	95.7 %
e	TS	PE_R1_e_1	97.8 %	PP_R1_e_1	96.2 %	PS_R1_e_1	96.4 %
e	TS	PE_R1_e_2	97.7 %	PP_R1_e_2	96.4 %	PS_R1_e_2	95.7 %
e	TS	PE_R2_e_1	97.5 %	PP_R2_e_1	95.6 %	PS_R2_e_1	95.7 %
e	TS	PE_R2_e_ 2	97.7 %	PP_R2_e_ 2	95.4 %	PS_R2_e_ 2	95.9 %

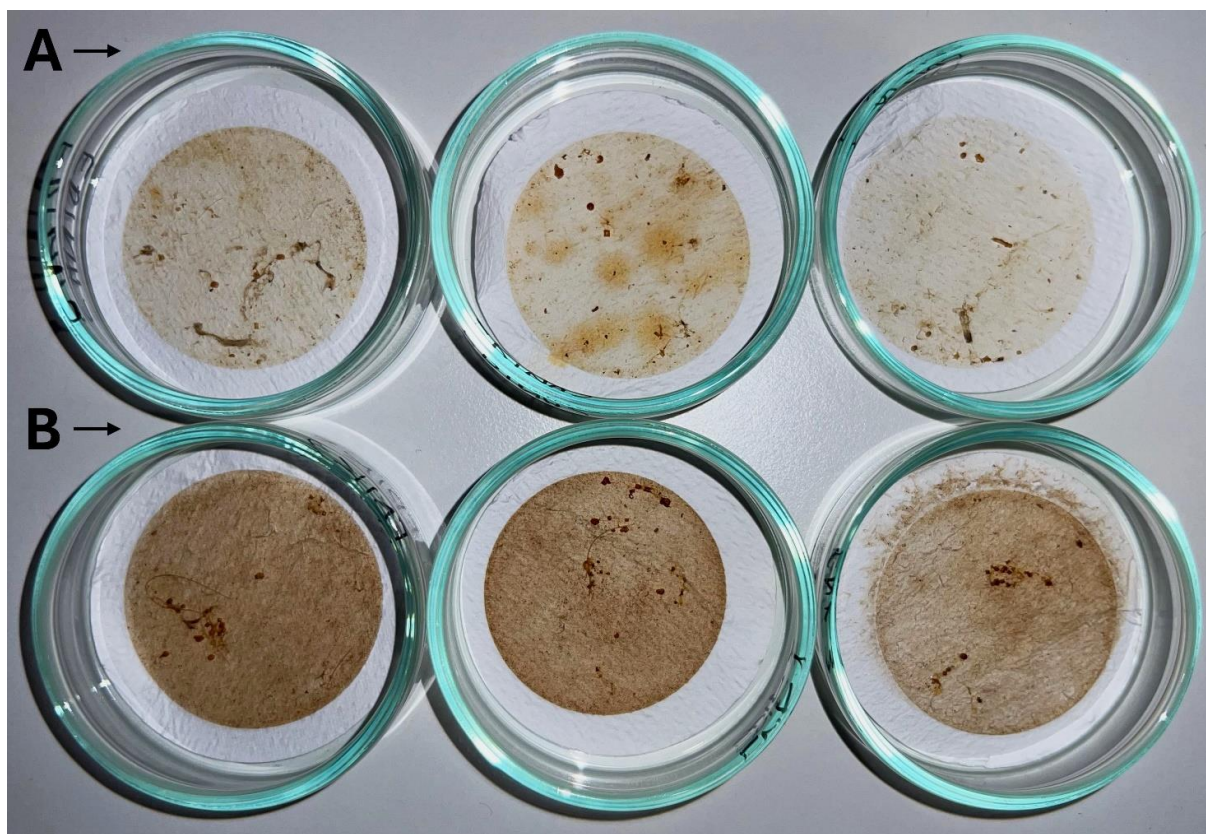


Figure S1.1. Visual comparison of filters resulting from the chemical digestion of *Isidella elongata* using the two protocols assessed for digestion efficiency: (A) Protocol 1 (sodium hypochlorite and hydrochloric acid); (B) Protocol 2 (potassium hydroxide and nitric acid).

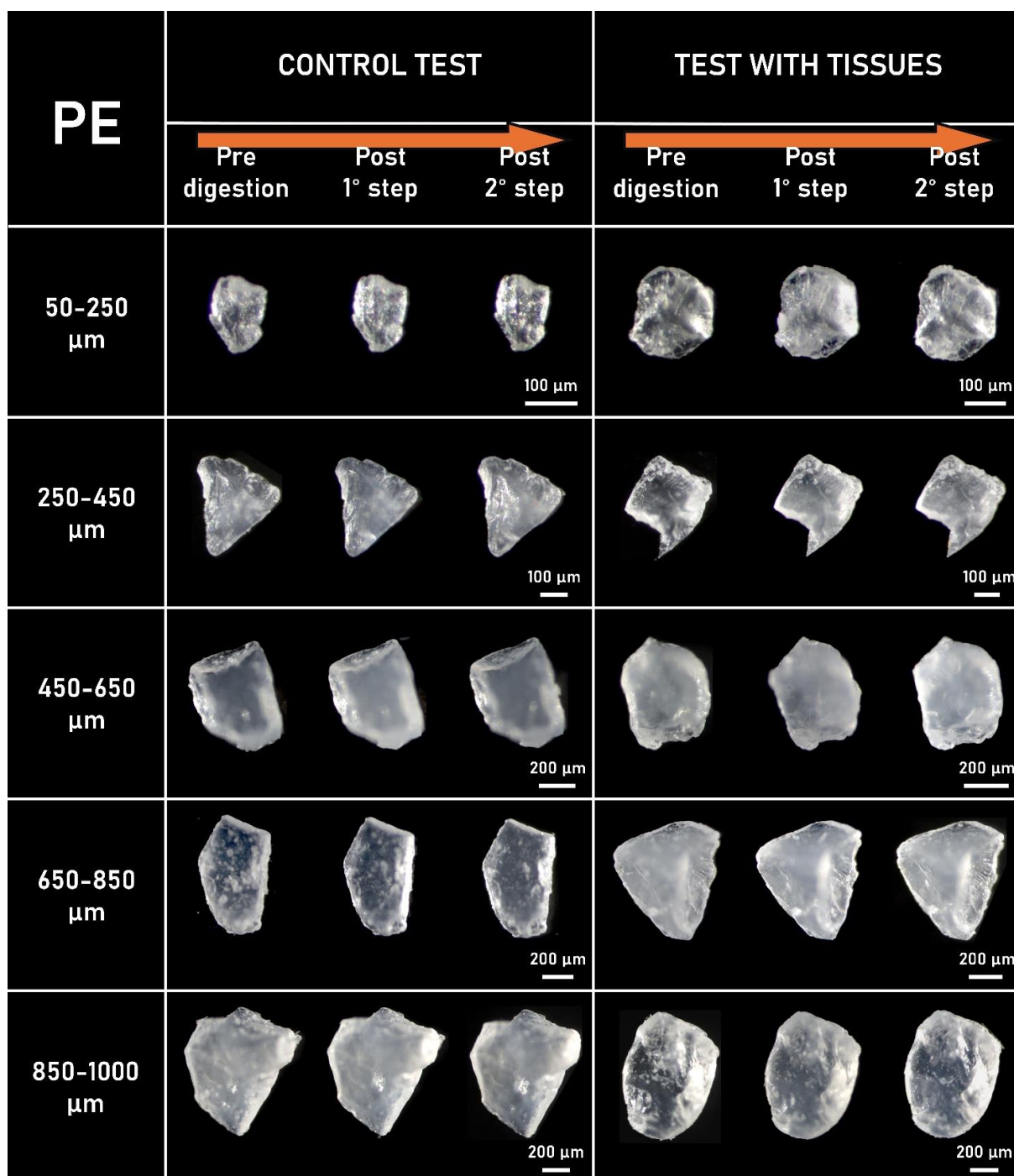


Figure S1.2. Representative example of polyethylene (PE) particles from each of the five size classes before chemical digestion and after each digestion step (sodium hypochlorite and hydrochloric acid) in both control (CTRL) and test with tissues (TS).

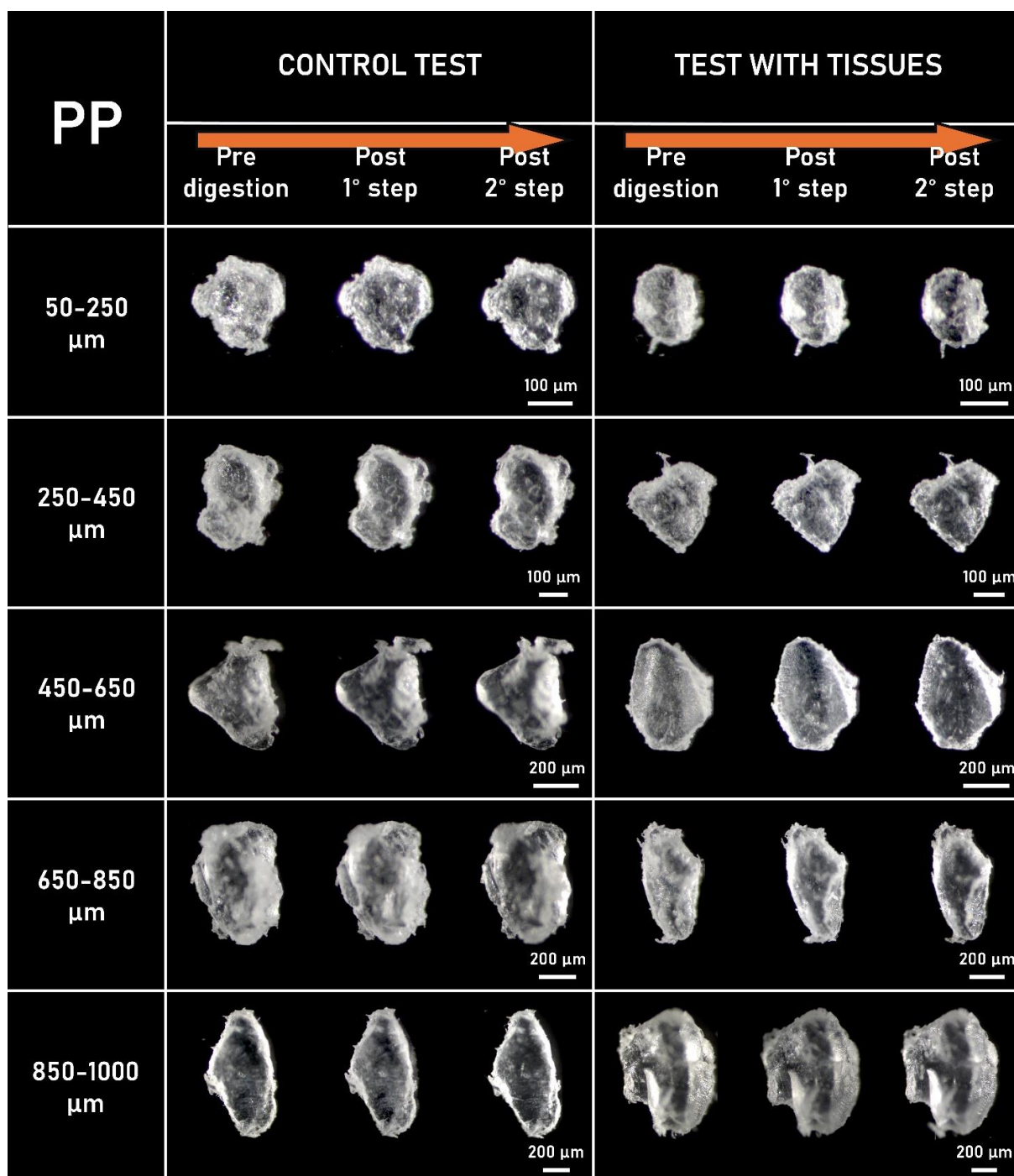


Figure S1.3. Representative example of polypropylene (PP) particles from each of the five size classes before chemical digestion and after each digestion step (sodium hypochlorite and hydrochloric acid) in both control (CTRL) and test with tissues (TS).

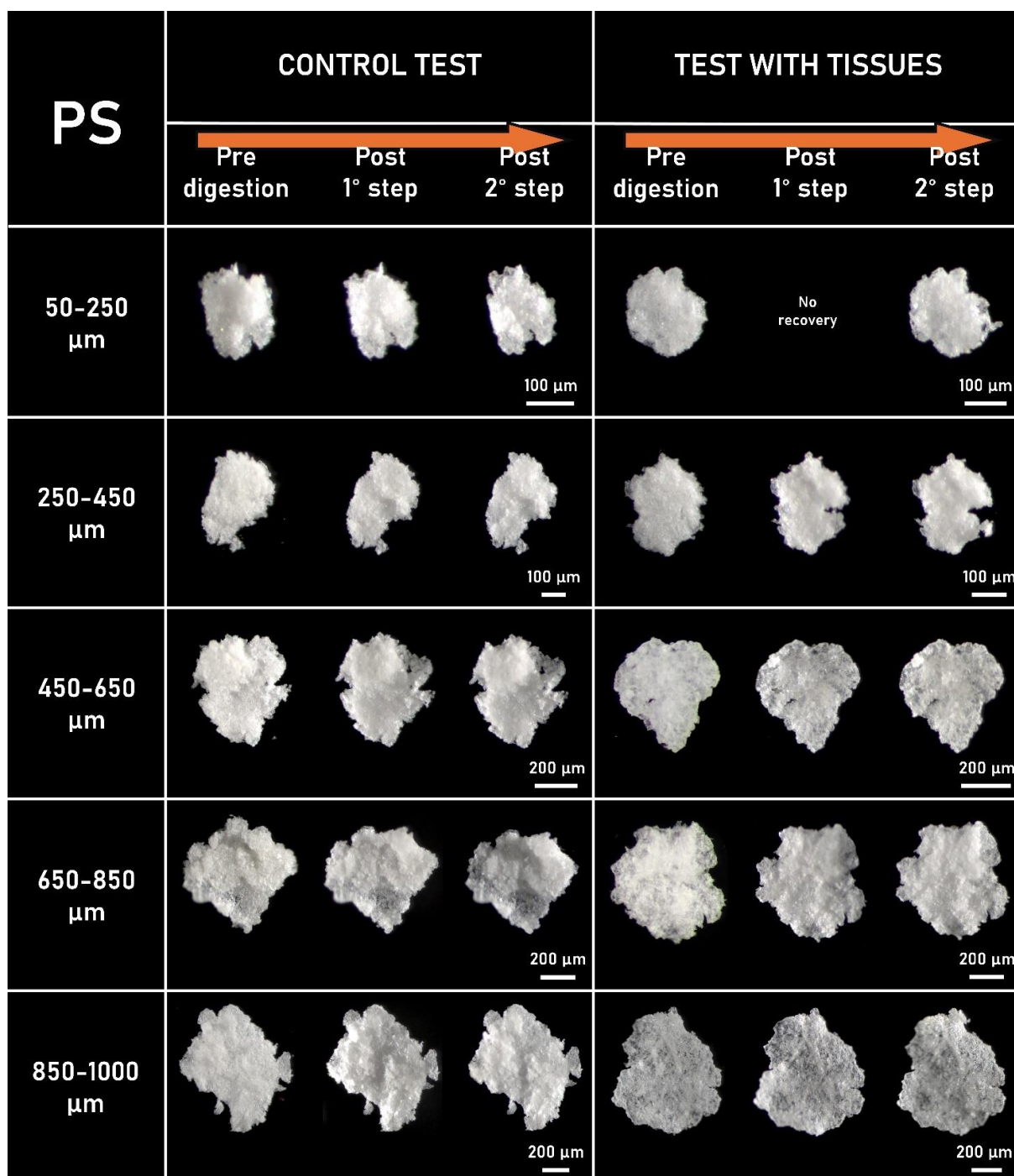


Figure S1.4. Representative example of polystyrene (PS) particles from each of the five size classes before chemical digestion and after each digestion step (sodium hypochlorite and hydrochloric acid) in both control (CTRL) and test with tissues (TS).

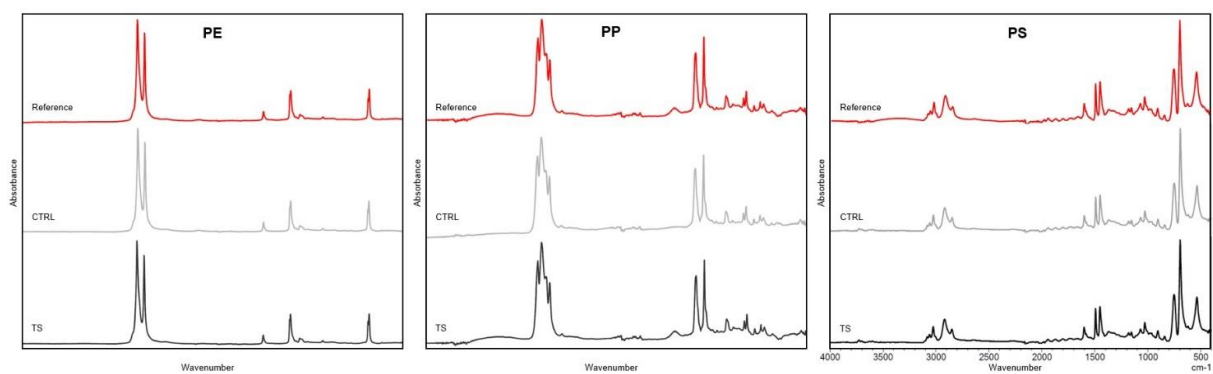


Figure S1.5. FTIR spectra comparison of PE, PP, and PS between reference particles (red lines) and particles subjected to the digestion with sodium hypochlorite and hydrochloric acid in CTRL (grey lines) and TS treatment (black lines).

Chapter 2

Microplastic Occurrence in Octocorals: A Field Study from the Deep Mediterranean Sea

Rationale for the study

Once the digestion protocol had been identified and implemented, the following step involved the processing of cold-water octocoral *Isidella elongata* (Esper, 1788) samples to assess microplastic (MP) uptake under natural environmental conditions.

This study is framed within the broader context of anthropogenic impacts on deep-sea ecosystems, which are increasingly recognized as vulnerable to a wide range of human pressures (Danovaro et al., 2010). Among habitat-forming organisms, cold-water corals play a key ecological role by enhancing habitat complexity and supporting diverse benthic assemblages including numerous species of commercial interest, thereby providing a range of ecosystem functions and services (Bo et al., 2014c; Rueda et al., 2019).

Cold-water coral habitats are increasingly threatened by multiple anthropogenic stressors. These include demersal fishing activities, habitat loss under future scenarios of ocean warming and acidification, and marine pollution. Among emerging contaminants, MPs have gained increasing attention in recent years as a potentially pervasive and harmful pollutant that deserves further investigation (Lartaud et al., 2020; Reichert et al., 2022). MPs are increasingly detected in deep-sea environments, yet their occurrence in habitat-forming octocorals under natural conditions remains largely unexplored.

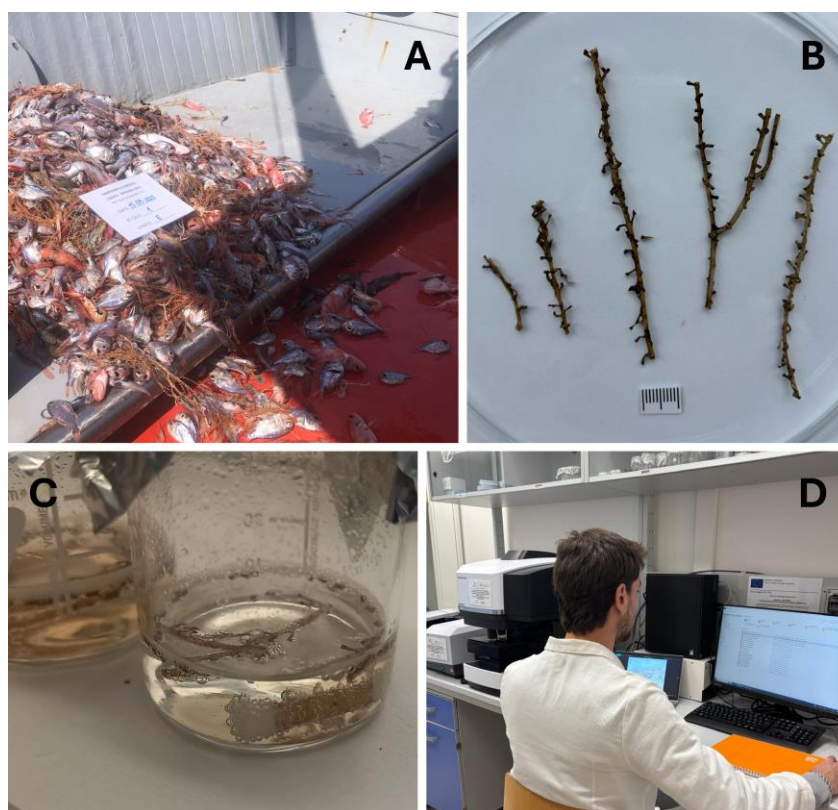


Figure 2.1. (A) Material collected during an experimental trawl haul; fish and *Isidella elongata* colonies are visible. (B) Branches of *I. elongata* used for microplastic assessment. (C) Chemical digestion of *I. elongata* branches with acids; CO₂ bubbles resulting from decalcification are visible. (D) Laboratory set up for polymer identification consisting of an ATR-FTIR Shimadzu IRTracer-100 and an infrared (IR) microscope Shimadzu AIMSight.

Within this context, the scientific question that this study aims to address is based on the near absence of research focusing on MP contamination in octocorals *in situ*, with only four studies currently available in literature and no one targeting Mediterranean species. Given the significant impacts of MPs reported for these organisms and the concerning projections of MP concentrations under future scenarios, establishing a baseline of knowledge on the actual level of contamination in octocorals is urgently needed.

Marine litter deposited on the seafloor surrounding the *Isidella elongata* collection sites, as well as MP contamination in fishes typically inhabiting *I. elongata* forests have also been analyzed. This approach allows the research to adopt a broader, ecosystem-based perspective, providing a more comprehensive view of MP contamination in deep-sea coral forest systems. Laboratory activities were conducted at the Sicily Marine Centre of Stazione Zoologica Anton Dohrn (Messina, Italy).

The results shown in this chapter represented the first assessment of MP contamination in deep-sea Mediterranean octocorals, also providing a wider perspective on the environment they habit and their associated fauna.

Tracing Marine Litter and Microplastics in the Deep Ionian Sea: messages from Seabed, Deep-Sea Fish and Cold-Water Corals

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Abstract

Deep-sea ecosystems host structurally complex and highly biodiverse habitats increasingly exposed to anthropogenic pressures. Among these, marine litter represents a growing concern addressed under Descriptor 10 of Marine Strategy Framework Directive. This study investigates the occurrence and composition of ML in an underexplored area of the deep Ionian Sea through an integrated approach combining data from seabed macrolitter, and plastic occurrence in the habitat-forming cold-water coral *Isidella elongata* and five associated deep-sea fish species. The material was collected during experimental trawls conducted in 2024 between 435 and 863 m depth. Seabed macrolitter and plastics isolated from biological samples through chemical digestion were classified following MSFD guidelines. Seafloor litter density reached 448.7 items km⁻², with plastic emerging as dominant category (84.8%), mainly consisting of single-use plastics. Fishing-related debris represented only 3.8% of the observations.

Plastics were found in 32.4% of the digestive systems of fish, with an average of 0.4 items fish⁻¹. A total of 585 microplastics were found in *I. elongata* tissues (40.3 items g⁻¹), of which 98% identified as paint. Our findings support the evidence that deep-sea environments act as waste accumulation zones, with potential implications for the health of benthic habitats. The occurrence of microplastics in *I. elongata* represents the first documented record for Mediterranean anthozoans and raises significant ecological concern. Understanding marine litter distribution patterns is essential for driving effective management and conservation measures of deep-sea ecosystems.

Keywords: Deep-sea, Seafloor litter, Microplastic ingestion, VMEs, teleosts, Mediterranean Sea

Highlights

- Marine litter pollution in the deep Ionian Sea was assessed by integrating data from seafloor litter, plastic contamination in deep-sea fish, and *Isidella elongata* coral.
- High densities of seafloor litter were recorded (448.7 items km⁻²), with plastics contributing for 84.8%.
- Microplastics were isolated from both demersal fish (0.4 items fish⁻¹) and *I. elongata* (40.3 items g⁻¹).
- First data of MP occurrence in Mediterranean anthozoans, reporting internal uptake and surface contamination.
- This ecosystem-based approach aligns with MSFD requirements identified for improving monitoring efficiency under Descriptor 10 of MSFD

Introduction

Deep seas represent the world's largest biome including more than 95% of the global biosphere, although they continue to be one of the Earth's most underexplored ecosystems (Danovaro et al., 2017, 2010; Paulus, 2021). However, since the early 2000s, visuals captured by underwater vehicles have opened the door for studying deep-sea wildlife in their natural settings, allowing for the identification of numerous habitats and a diverse associated biodiversity (Danovaro et al., 2014; Ramirez-Llodra et al., 2011). Concurrently, these investigations have uncovered the previously unnoticed effects of human actions on the seafloor, including the desolation caused by bottom trawling (Bo et al., 2014a; Clark et al., 2016) and the volume of debris impacting benthic ecosystems (Angiolillo and Fortibuoni, 2020; Consoli et al., 2019; Hanke et al., 2025; Pham et al., 2014).

Among anthropogenic pressures, marine litter (ML), and plastic debris above all, has emerged as a pervasive pollutant of marine ecosystems (Galgani et al., 1996; Hanke et al., 2025; Pham et al., 2014). Although floating litter represents the most visible fraction of plastic pollution, increasing evidence indicates that large amounts of debris are transferred through the water column and accumulate on the deep seafloor, particularly in topographically complex areas such as canyons and slopes (Angiolillo et al., 2015; Consoli et al., 2019, 2018a; Galgani et al., 1996; Martin et al., 2022; Pierdomenico et al., 2023). Physical processes, including sinking through the water column and turbidity currents (Chen et al., 2025; Zhao et al., 2025), together with biological transport pathways, such as biofouling, ingestion and vertical fluxes mediated by marine snow and fecal pellets (Cole et al., 2016; Fazey and Ryan, 2016), contribute to the redistribution of plastics to the seafloor. Once on the seafloor, ML can affect marine organisms through entanglement and coverage, frequently associated with abandoned, lost or derelict fishing gears (ALDFGs) (Angiolillo and Fortibuoni, 2020; Bo et al., 2014a; Consoli et al., 2020b, 2019, 2018a), affecting their ability to move, feed, and breathe (Kühn et al., 2015; Napper and Thompson, 2020; Worm et al., 2017; Zhang et al., 2025). In addition, many marine organisms can unintentionally ingest ML, especially microplastics (MPs, i.e., <5 mm size plastic items), either by mistaking it for food, or through incidental ingestion

during feeding activities (Derraik, 2002; Kühn et al., 2015; Laist, 1997; Werner et al., 2016; Worm et al., 2017). Indigestible debris such as plastics may accumulate in the digestive tract, potentially reducing individual fitness and impairing growth, reproduction and survival (Bjorndal et al., 1994; McCauley and Bjorndal, 1999; Van Franeker, 1985). While marine birds, turtles, and mammals have historically received most of the attention, the impacts of entanglement and ingestion on less charismatic taxa, such as fish and invertebrates, are now becoming increasingly evident (Consoli et al., 2019; Derraik, 2002; Laist, 1997; Pedà et al., 2025; Schirinzi et al., 2020; Sciutteri et al., 2023; Worm et al., 2017).

MPs constitute a growing and particularly concerning part of ML due to their persistence, small size and high bioavailability (Galgani et al., 2023; GESAMP, 2019). They enter marine ecosystems either directly by running off industrially manufactured microbeads of different sizes, widely used in consumer and personal care products (primary MPs) or from the degradation of larger plastic exposed to abiotic and biotic drivers (secondary MPs) (Andrady, 2017, 2011; Bergmann, 2015). MPs have been detected throughout the marine environment, from surface waters to deep-sea sediments, and in organisms spanning virtually all taxonomic groups (Lusher et al., 2017; Martin et al., 2022; Pedà et al., 2025).

Compared to shallower environments, deep-sea research remains scarce because of the high logistical and economic costs of deep sampling and the limited availability of dedicated cruises (Machin et al., 2026). For these reasons, data on the ingestion of plastic by deep-sea fishes and invertebrates remain fragmented across taxa and habitats, although it raises concerns about sub-lethal effects, trophic transfer and long-term ecological consequences (Werner et al., 2016; Woodall et al., 2014).

The present study aims to assess ML pollution in a poorly explored deep-sea area off the Gulf of Squillace in the Ionian Sea (Western Mediterranean Sea). Indeed, although many studies have estimated the density and composition of seafloor ML in the Mediterranean Sea, little information is available for this area (Angiolillo and Fortibuoni, 2020; Spedicato et al., 2019).

This study integrates data from both biological and environmental matrices, investigating (i) plastic occurrence in the bamboo coral *Isidella elongata* (Esper, 1788) and in digestion systems (DSs) of deep-sea fishes, used as biological indicators of MP contamination, and (ii) the occurrence and composition of seafloor ML as a proxy of anthropogenic pressure in the surrounding environment.

This work represents the first attempt to include cold-water corals (CWCs) within a multidisciplinary framework for assessing ML pollution in the Mediterranean Sea and reports the first evidence of MP occurrence in Mediterranean corals.

Materials and methods

Study area

The Gulf of Squillace is located in the north-western Ionian Sea and belongs to the basin of the Calabrian Arc, an area characterized by a generally narrow continental shelf and the presence of numerous submarine canyons along the coast (Carlucci et al., 2018; Maiorano et al., 2022; Ricci et al., 2019). The Gulf is shaped by the Squillace Canyon, which is structured by a system of channels and three main branches with a NW–SE trend, extending for about 50 km and connecting the shelf with the deep basin (Corradino et al., 2023; Masiá et al., 2024). This geomorphology provides a direct pathway for sediment transport and dense water cascading from coastal to deep waters, playing an important role in the functioning of deep-sea ecosystems (Maiorano et al., 2022). The circulation pattern of the Ionian Sea is characterized by a complex system of surface water arriving from the western Mediterranean through the Sicilian Channel (Modified Atlantic Water, MAW) and intermediate water from the Levantine basin (Levantine Intermediate Water, LIW), with a general cyclonic pattern strongly influenced by cold, dense deep-water masses of Adriatic origin entering through the Otranto Channel (Adriatic Deep Water, ADW) (Carlucci et al., 2018; Maiorano et al., 2010).

The area includes a complex set of habitats hosting important demersal fishery resources and several biodiversity hot spots, such as CWC habitats and submarine canyons (Capezzuto et al., 2010; Maiorano et al., 2010). As a result, the area is extensively exploited from fisheries, particularly bottom trawling, as part of FAO Geographical Sub-Area (GSA) 19.

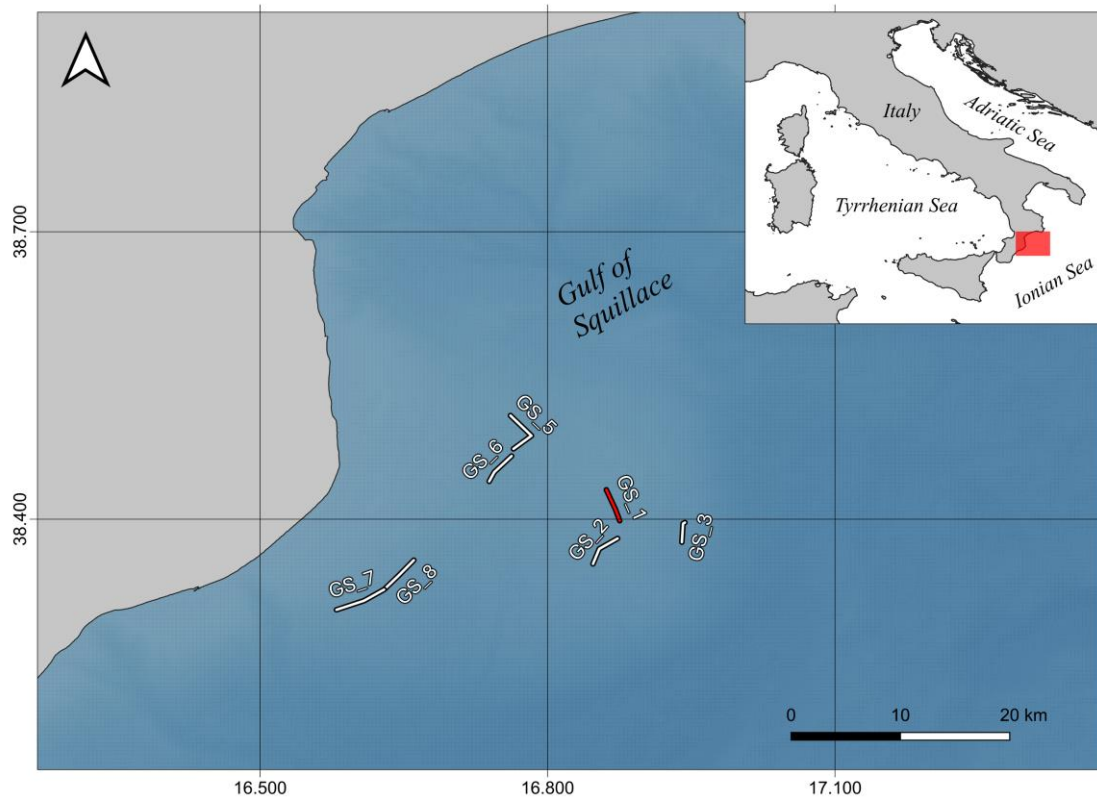


Figure 2.2. Geographic location of the bottom trawl transects. Seafloor litter and fish samples were collected along all transects, whereas colonies of *Isidella elongata* were collected during a single trawl haul, highlighted by the red transect.

Samples collection

Samples of seafloor ML (>2.5 cm, i.e., macrolitter) and by-caught fish and coral species were collected during an experimental bottom trawling survey on board an 18-meters vessel in September 2023 off the Gulf of Squillace, using a commercial bottom-trawl net characterized by an 8 × 1.8 m opening and a 50 mm mesh size. A maximum of two hauls per day were performed for a total of 15 hauls in 4 days between 440 and 850 m depth. Information on sampling activities is reported in Table 2.1.

Seafloor macrolitter categorization and analysis

The seafloor macrolitter was examined on board immediately after collection. Each item was isolated, photographed, measured and grouped in the following litter categories based on the Marine Strategy Framework Directive (MSFD, 2008/56/EC) protocol (Fleet et al., 2021; Sciutteri et al., 2023): *chemicals; cloth/textiles; food waste; glass/ceramics; metal; artificial polymer materials; paper/cardboard; rubber; and processed/worked wood*. Within each category, litter debris was identified to the best of the operators' ability to determine their use (source) by following the Joint List of Litter Categories for Macrolitter Monitoring prepared by the MSFD Technical Group on Marine Litter (Fleet et al., 2021). The list uses a classification system based on codes that define the litter type, the litter category, the use (source) of the item and whether it is a SUP (Single Use Plastic) or a Fishery related object. The Online Photo Catalogue of the Joint List of Litter Categories (<https://mcc.jrc.ec.europa.eu/main/photocatalogue.py?N=41&O=457&cat=all>) was used as a reference for the identification of the items. Items that could not be identified according to this classification system were reported as Unclassified. Litter densities (n/km² and kg/km²) were evaluated according to the length of the transect and the width of the fishing net. Data were reported as average $\pm$ standard deviation. In addition, the most abundant litter categories were identified and the percentage of ALDFG was also calculated. Litter was arranged in the following size classes: <25 cm², 25-100 cm², 100-400 cm², 400-2500 cm² and 2500-10000 cm².

Table 2.1. Summary of the sampling activity conducted in the study area along with the abundance and density of the seafloor macrolitter collected. For each haul the abundance and weight of the litter are reported, along with the densities expressed as n./100 m² and kg/100 m².

Haul ID	Mean Depth (m)	Haul Length (Km)	Surveyed Area (Km ²)	Litter Abundance (n.)	Litter Weight (Kg)	Litter Density (n./100 m ²)	Litter Density (Kg/100 m ²)
GS_1	501	5.271	0.042	19	2.91	0.045	0.007
GS_2	597	4.450	0.036	21	8.3	0.059	0.023
GS_3	846	2.475	0.020	10	0.03	0.050	0.000

GS_5	459	4.493	0.036	14	1	0.039	0.003
GS_6	447	3.811	0.030	17	0.03	0.056	0.000
GS_7	517	4.875	0.039	12	0.02	0.031	0.000
GS_8	511	4.402	0.035	12	0.32	0.034	0.001
TOTAL		29.778	0.238	105.000	12.610		
AVG ± SD						0.044 ± 0.011	0.005 ± 0.008

Plastic extraction and classification from deep-sea fish species

Five deep-sea fish species that are commonly found among bottom-trawl by-catch in Mediterranean Sea and display different feeding habits were selected and examined for litter ingestion: the grenadier hollow snout *Coelorinchus coelorhincus* (Risso, 1810), the shortnose greeneye *Chlorophthalmus agassizi* Bonaparte, 1840, the Mediterranean slimehead *Hoplostethus mediterraneus* Cuvier, 1829, the glasshead grenadier *Hymenocephalus italicus* Giglioli, 1884, the armed gurnard *Peristedion cataphractum* (Linnaeus, 1758). Only individuals without relevant damage due to fishing operations were considered for the analysis. Total Length (TL, cm) and Total Weight (TW, g) were recorded for all the specimens, after which they were immediately stored at 20°C. In the laboratory, the DS of each specimen was removed, washed with Milli-Q water, transferred to a glass petri dish and subjected to chemical digestion according to the protocol of Pedà et al. (2025, 2022). Plastic extraction was carried out using a 10 % potassium hydroxide (KOH) solution in a 1:5 (w/v) ratio, incubated in a stove at 50°C for 6 h and then, left at room temperature overnight. Digested samples were filtered through on fibreglass membranes (pore size 1.6 µm, GF/A Whatman) using a vacuum pump. Membranes were analyzed by visual inspection under a stereomicroscope Zeiss Discovery V.8. Then, the isolated items were counted, weighed (in grams to the nearest 0.0001 g), measured (length and width in mm), and photographed using a stereomicroscope Zeiss Discovery V.8 coupled with Axiocam 208 color microscope camera and analyzed using ZEN 3.1 blue Edition software. Polymeric nature of particles larger than 300 µm was determined by Fourier transform infrared (FT-IR) spectroscopy using the Agilent Cary 630 spectrometer equipped with dedicated polymer reference libraries (Agilent Polymer Handheld ATR Library, Agilent Elastomer

Oring and Seal Handheld ATR Library, Clear Polymers, POLY_D, ATR Demo Library). Polymer identification was accepted when a spectral match with a confidence level $\geq 70\%$ was obtained (Pedà et al., 2025, 2022a). Due to the instrumental technical limitation, smaller particles ($<300\ \mu\text{m}$) or thin samples (i.e., fiber) could not always be analyzed by FT-IR; therefore, their plastic nature was verified using the hot needle test (Pedà et al., 2025, 2022a). Isolated plastics were classified based on their color, shape (pellet, film, filament, fiber, foam and fragment), and size range (macro: $>25\ \text{mm}$; meso: $5\text{--}25\ \text{mm}$; micro: $<5\ \text{mm}$, further in large MP: $0.1\text{--}5\ \text{mm}$ and small MP: $<0.1\ \text{mm}$) according to the MSFD (Galgani et al., 2023; Hanke et al., 2013). For each species examined, plastic abundance indices were calculated as follows:

- Litter and plastic percentage of occurrence (%O) was estimated as the proportion, on the total sample, of the individuals which ingested litter and plastics: ($\%O = \text{N. individuals which ingested litter and plastics} / \text{N. total samples} \times 100$);
- Average number of plastic items found in the DSs calculated on the total number of individuals ($\text{N. plastic items} / \text{N. all examined individuals}$);
- Average number of plastic items found in the DSs calculated on the total number of individuals who ingested plastics ($\text{N. plastic items} / \text{N. individuals who ingested plastics}$).

Table 2.2. Information on deep-sea fishes. Sample size, morphometric parameters, number of digestive systems (DS) with plastics, number of plastic items and percentage of occurrence are shown for each species. **H. italicus* length morphometrics were not measurable due to damage to the caudal fin, a condition frequently occurring during bottom-trawl fishing operations.

Species	Sample size	Total Length (TL, cm) AVG $\pm$ SD	Total weight (W, g) AVG $\pm$ SD	N. of DS with plastics	N. of plastic items	Percentage of occurrence (%O)
<i>Chlorophthalmus agassizi</i>	20	15.2 $\pm$ 1.5	23.8 $\pm$ 8.0	5	8	25
<i>Coelorinchus caelorhincus</i>	11	22.9 $\pm$ 2.1	47.8 $\pm$ 14.5	5	6	45.5
<i>Hoplostethus mediterraneus</i>	20	15.4 $\pm$ 1.4	48.7 $\pm$ 11.5	10	12	50

<i>Hymenocephalus italicus</i>	17	-*	5.2 ± 0.9	2	4	11.8
<i>Pertistedium cataphractum</i>	3	18.5 ± 4.1	32.0 ± 21.8	1	1	33.3
Total	7 ¹	-	-	23	3 ¹	32.4

Plastic extraction and classification from the cold-water coral Isidella elongata

Five colonies of *I. elongata* were collected for MPs investigation. Three subsamples of approximately 1 g were obtained randomly from each *I. elongata* colony, reaching a total of 15 subsamples. Each subsample was photographed, and weight, total branch length and number of polyps were recorded to account for potential correlation in MPs abundance among different coral compartments. Table 2.3 provides information on each sample and replicates with their weight and the number of polyps. Prior to chemical digestion, all samples were rinsed with Milli-Q water using a 100 µm stainless steel mesh to remove particles adhered to surfaces by contamination. The biological material was subjected to chemical digestion following the protocol implemented in Giova et al. (2025). Biological subsamples were placed into beakers and covered with aluminum foil. To digest organic matter, 10 mL of sodium hypochlorite solution (NaClO 8.25%) per 1 g of weight of tissue sample were added (1:10 w/v) in the beakers (1st digestion step). The samples were kept at room temperature for 24 hours, occasionally stirring to ensure thorough contact of the sample with the digestion solution. Following NaClO treatment, samples were filtered through Whatman GF/C filters (1.2 µm pore size) using a vacuum filtration apparatus and rinsed with Milli-Q water. To remove any residual carbonate content, 40 mL per gram of sample of 10% hydrochloric acid (HCl) was applied directly to the filter for 30 minutes (2nd digestion step). After this second digestion step, samples were filtered again, rinsed with Milli-Q water, and stored at room temperature in Petri dishes for further inspection. Particles isolated from fish and corals were chemically analyzed through a Fourier Transform Infrared spectroscopy analysis FT-IR in Attenuated Total Reflection mode (ATR-FTIR; Shimadzu IRTracer-100) to identify their polymeric nature. For the identification of smaller particles (<200µm), the analysis was integrated using an infrared (IR) microscope (Shimadzu, AIMSight). Sometimes the

samples could not be identified due to their small size and/or condition. In this case, the hot needle technique was used to confirm their plastic nature (Pedà et al., 2025, 2022a). Once identified, plastic particles were classified according to MSFD (see details above in the text).

Table 2.3. Information on *Isidella elongata* colonies and relative replicates. For each replicate, the weight, number of polyps and number of microplastics were recorded.

Colony ID	Replicate	Weight (g)	Total branch length (cm)	Number of polyps	Plastic items
IE_1	R1	1.08	33.389	178	3
	R2	1.01	46.229	191	6
	R3	1.03	45.081	281	4
IE_2	R1	1.03	53.94	215	7
	R2	1.03	48.858	193	5
	R3	1.01	41.363	165	5
IE_3	R1	1.02	34.143	118	3
	R2	1.08	47.777	186	12
	R3	1.01	31.654	151	2
IE_4	R1	1.01	45.28	188	2
	R2	1.02	50.403	207	338
	R3	0.99	55.42	259	18
IE_5	R1	1.02	66.521	232	113
	R2	1.05	49.746	204	37
	R3	1.04	51.709	225	27

Quality Assurance and Quality Control (QA/QC)

According to Pedà et al. (2022, 2020), the following measures were applied during all laboratory procedures: i) operators wore 100% cotton lab coats; ii) all glass containers, tweezers and metal scissors were thoroughly rinsed with milli-Q water; iii) all work surfaces were cleaned with ethanol; iv) tissue samples were covered with aluminum foil during digestion and when not in use, while filters obtained from digestion were covered with glass lids; v) sample filtration activities were conducted under a fume hood; vi) fibers were excluded from MP assessment *I. elongata*. This methodological choice was adopted to avoid the risk of airborne contamination during onboard sample handling and processing, as synthetic fibers are widely recognized as the dominant source of laboratory contamination (Hermsen et al., 2018; Kühn et al., 2020); vii) blank filters (moist with ultrapure distilled water) were placed in Petri dishes, kept open during all sample treatments and subjected to digestion steps, in order to exclude the airborne contamination (Pedà et al., 2022a; Schirinzi et al., 2020). Particles co-occurring in blanks and filters resulting from sample digestions were excluded from MP counting.

Results

Seafloor macrolitter assessment

ML was retrieved in all trawl hauls (Table 1) with a total of 105 items collected between 435 and 863 m in a trawled area of 0.24 km², accounting for a total weight of 12.6 kg. The average density of macro-litter was 448.7 ± 108.0 items/km², corresponding to an average density by weight of 48.9 ± 84.9 kg/km² (Table 2.1). Five MSFD litter categories out of nine were represented (Fig. S2.1; Fig. S2.2), with the most abundant being *artificial polymer materials* for both number of items (84.8%) and weight (60.1%). The remaining categories accounted for lower proportions of items and weight, including *cloth/textile* (10.5% of items; 6.0% by weight), *metals* (1.9%, 10.5%), *organic food waste* (1.9%, 0.2%) and *glass/ceramics* (1%, 1.6%).

Concerning MSFD subcategories related to the use (source), six out of thirteen were represented. Items classified as undefined use were the most frequent (82.9%), followed by those related to food consumption (8.6%) and fishing activities (3.8%). The remaining three subcategories (aquaculture, clothing and smoking related) were represented by single items, together reaching 2.9%. Within the *artificial polymer materials* category, single-use plastics (SUPs) accounted for 35.2% of the items, while fishing-gear-related items represented 4.8%. SUPs mainly consisted of plastic bags attributed to undefined use, together with food-consumption-related items such as snack packets, wrappers, cups and bottles.

Regarding size-frequency distribution, most of the items equally belonged to the <25 cm² and 25-100 cm² size classes, together accounting for 70% of the total recorded items. The remaining items were distributed among the size classes as follows: 18% in 100-400 cm² size class, 11% in 400-2500 cm² size class and 1% in 2500-10000 cm². *Artificial polymer materials* were the only litter category spanning all size classes, whereas *cloth/textile* items were restricted to size classes <400 cm². All other categories occurred within a single size class only (*glass/ceramics*: 400–2500 cm²; *metals*: 100–400 cm²; *organic food waste*: <25 cm²).

Litter distribution across size classes, MSFD Categories, use-related MSFD sub-categories, and belonging to SUP/fishery, is shown in Figure 2.3.

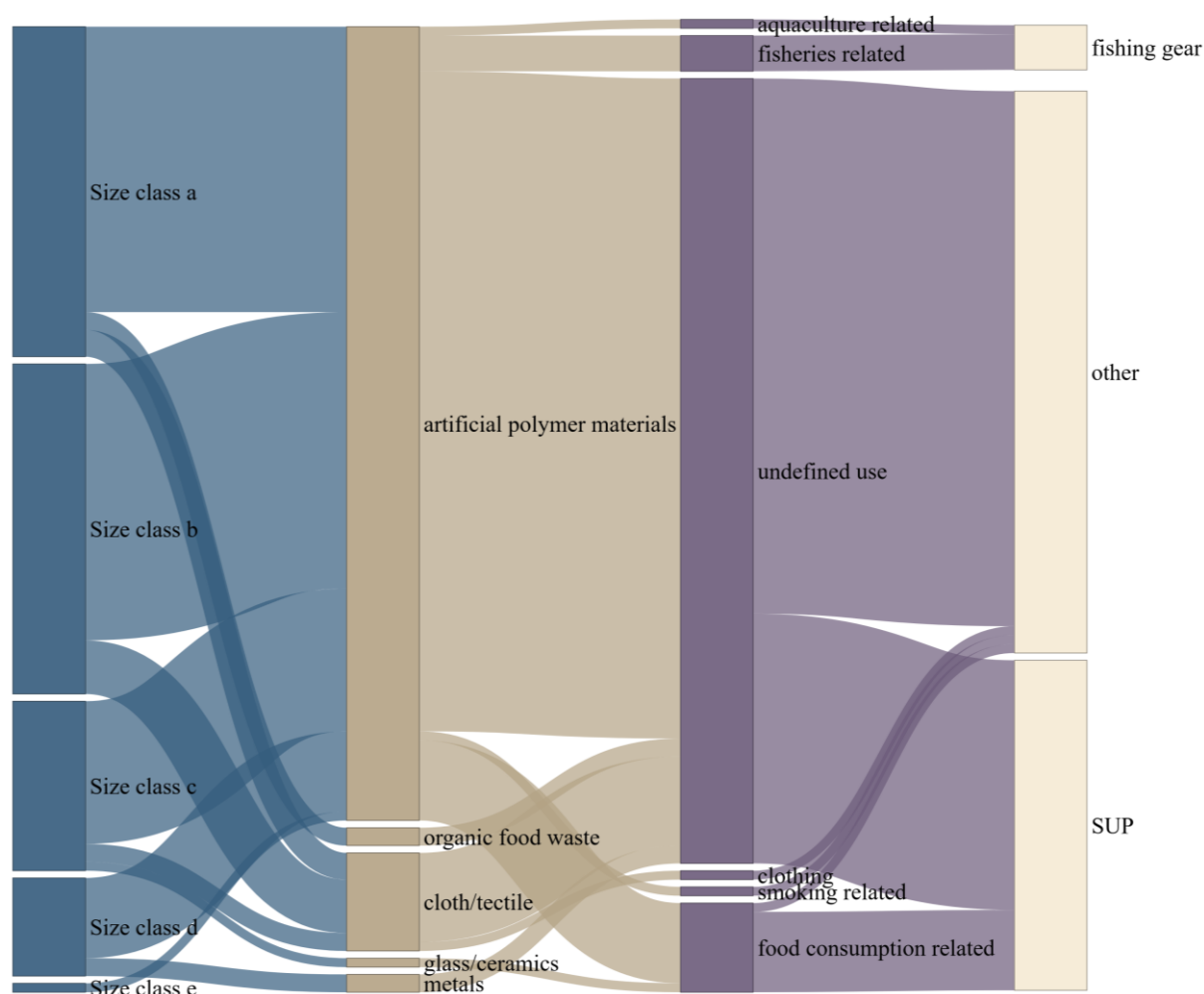


Figure 2.3. Flow diagram showing the distribution of seafloor litter across size classes, MSFD Categories, use-related MSFD sub-categories, and belonging to SUP/fishery. Node and flow thickness represent item abundances. Size class a: <25 cm²; Size class b: 25-100 cm²; Size class c: 100-400 cm²; Size class d: 400-2500 cm²; Size class e: 2500-10000 cm².

MP ingestion in deep-sea fish species

A total of 71 specimens belonging to five species were collected through bottom trawling sampling. Biomorphometric measurements (TL, TW) are reported in Table 2.2

for all species, with the exception for *H. italicus* for which TL were not measurable due to damage to the caudal fin, a condition frequently occurring during bottom-trawl fishing operations.

Plastic items were detected in all species investigated for a total of 31 items ingested by 23 specimens, corresponding to 1.23 items per specimen and an occurrence frequency of 32.4%. Specifically, 8 items were isolated from DSs of *C. agassizi* (%O = 25.0), 12 from *H. mediterraneus* (%O = 45.5), 6 from *C. caelorhincus* (%O = 50.0), 4 from *H. italicus* (%O = 11.8) and one single item from *P. cataphractum* (%O = 33.3). Number of plastic items per number of individuals analyzed for each species is reported in Figure 2.4.

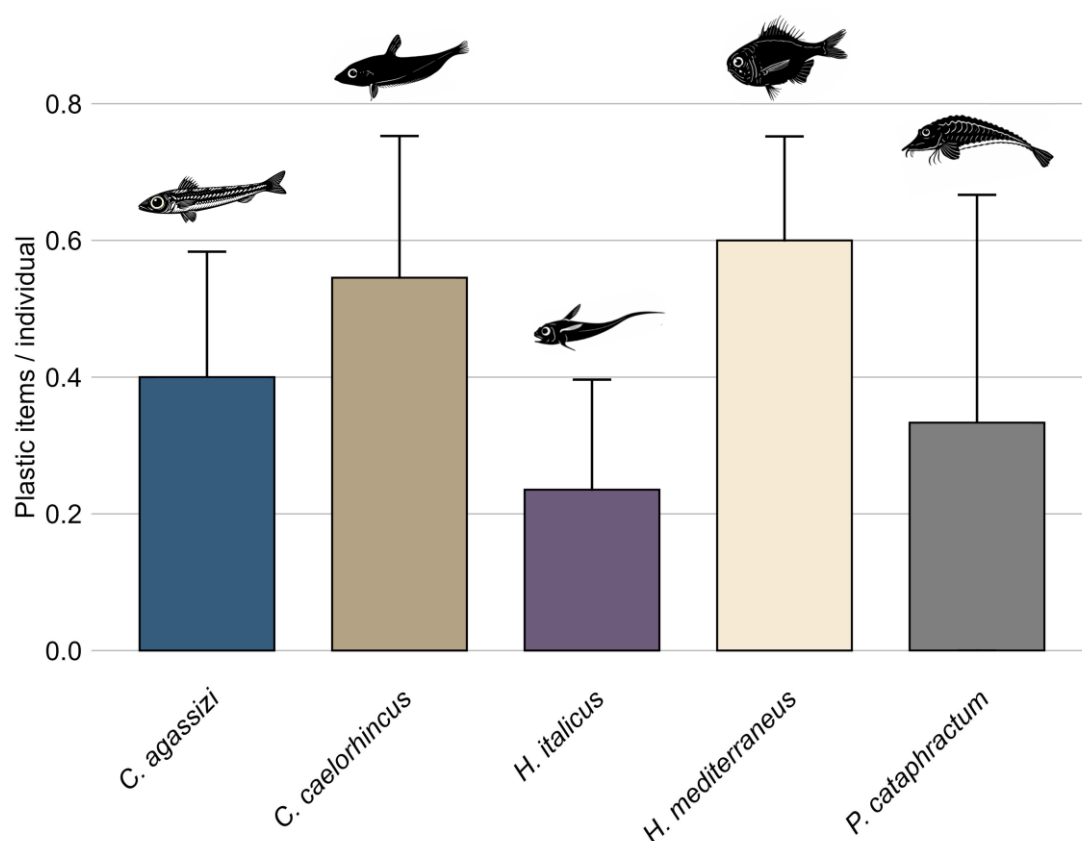


Figure 2.4. Number of plastic items per number of analyzed individuals (average + SE) for *Chlorophthalmus agassizi*, *Coelorinchus coelorhincus*, *Hymenocephalus italicus*, *Hoplostethus mediterraneus* and *Peristedion cataphractum*.

Most ingested plastics were classified as MPs (size range 0.03–4.81 mm), including 11 (35.5%) large MPs and 19 (61.3%) small MPs, while only one mesoplastic (9.77 mm; 2.3%) was found in the DS of *C. caelorhincus* (Fig. 2.5). Regarding plastic shape, fibers and fragments were the most frequent plastic items found in DSs, accounting for 15 (48.4%) and 14 (45.2%) occurrences, respectively. The remaining items consisted of one pellet (3.2%) isolated from *H. mediterraneus* and one film (3.2%) from *C. caelorhincus* (Fig. 2.5).

Blue was the most frequent color of the ingested plastics (n=15; 48.4%), followed by transparent (n=6; 19.4%), black (n=4; 12.9%), green (n=3; 9.7%), red (n=1; 3.2%), white (n=1; 3.2%) and brown (n=1; 3.2%) (Fig. 2.5).

FT-IR spectroscopy analysis revealed the presence of 5 synthetic polymers (16.1%), while the chemical nature of 17 items remained undefined (54.8%; Fig. 2.5, Fig. S2.3). Polyethylene Terephthalate (PET)/polyester (n =5; 16.1%) was detected between *C. agassizi* and *P. cataphractum*, whereas rubber particles (n = 4; 12.9%) were isolated from *C. agassizi* and *C. caelorhincus*. Additional polymers found in DSs included polyethylene (PE, n=2; 6.5%), polyurethane (PU, n=2; 6.5%) and polystyrene (PS, n=1; 3.2%), all observed in *H. mediterraneus* (Fig. 2.5).

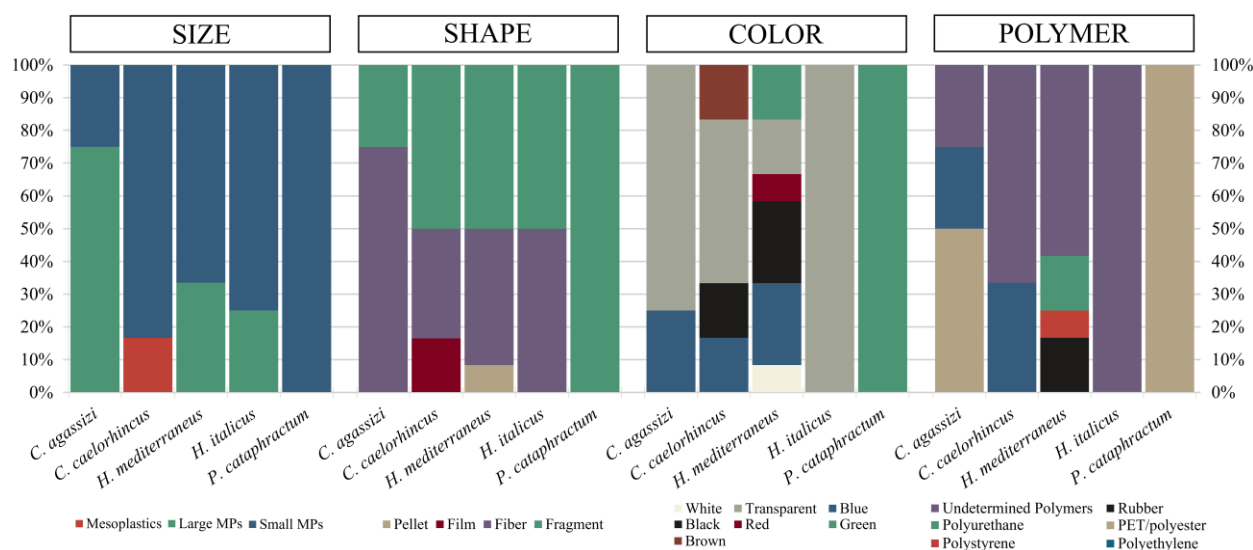


Figure 2.5. Relative composition (%) of plastic items ingested by five fish species (*Chlorophthalmus agassizi*, *Coelorhynchus caelorhincus*, *Hoplostethus mediterraneus*, *Hymenocephalus italicus* and *Pertistiedion cataphractum*), shown as 100% stacked bar charts. Four panels describe plastic characteristics by size (mesoplastics, large MPs, small MPs), shape (pellet, film, fiber, fragment), color (white, transparent, blue, green, black, red), and polymer type (undetermined polymers, rubber, polyurethane, PET/polyester). Only categories present in the data are displayed.

MP ingestion in the bamboo coral *Isidella elongata*

A total of 1558 particles were visually detected and documented during *I. elongata* filters inspection (average 103.9 particles/replicate), prior to chemical identification. Blank filters were checked and particles co-occurring within samples were removed from MP count. In addition, 105 particles representative of the overall dataset were selected for FT-IR analysis. As a result of chemical identification and blank filters inspection, 62.6% of particles, including contamination and organic or inorganic biogenic material, were excluded.

Finally, a total of 582 particles were confirmed as synthetic, with a mean abundance (avg $\pm$ SD) of 116.4 ± 152.1 items per colony, corresponding to 43.52 ± 54.08 items g^{-1} and 18.0 ± 23.0 items per 100 polyps. Paint was by far the most frequent polymer found with a

total of 572 items (98%; Fig. 2.6A), while only a small number of other polymers were identified, including polypropylene (PP, $n = 2$), polyethylene (PE, $n = 1$), rubber ($n = 1$), resin ($n = 1$), and five particles that remained undefined (Fig. 2.7; Fig. S2.4). During the assessment of coral compartments, paint was found to be also attached to coral colonies, including branches as well as polyps (Fig. 2.6B, C). Paint particles were associated with all examined colonies, with a mean abundance (avg $\pm$ SD) of 114.4 ± 151.8 items per colony, corresponding to 42.7 ± 53.8 items g^{-1} and 17.7 ± 23.0 items per 100 polyps. Other polymers accumulated within coral tissues were detected in 80% of the colonies, although in markedly lower numbers, with a mean (avg $\pm$ SD) of 2.0 ± 1.9 items per colony, equivalent to 0.8 ± 0.9 items g^{-1} and 0.3 ± 0.3 items per 100 polyps.

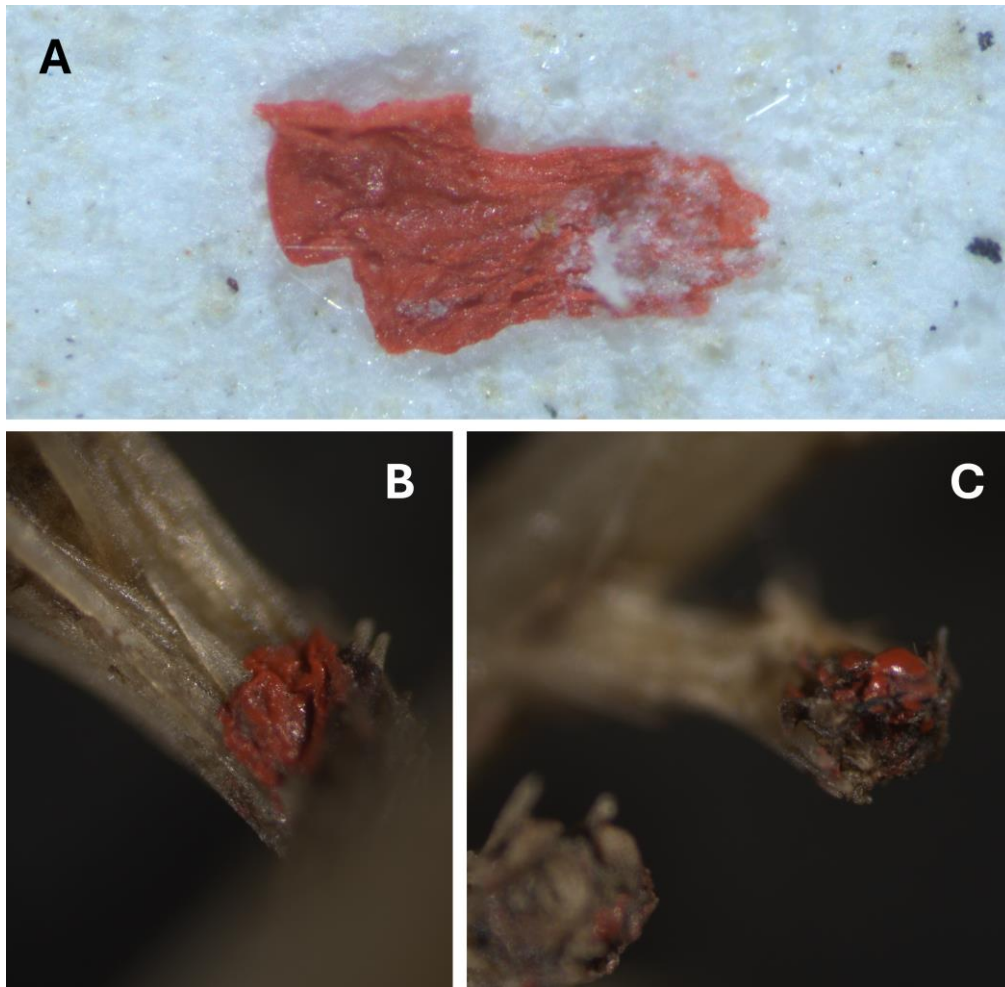


Figure 2.6. Occurrence and identification of red paint fragments associated with *Isidella elongata*. (A) Red paint fragment retrieved on the filter following chemical digestion of coral samples. Close up under the stereomicroscope of red paint dried on (B) the polyp body, (C) and on the apical part of the polyp.

The size of particles detected within coral tissues spanned from 39.1 to 331.4 μm (avg $\pm$ SE = 140.8 $\pm$ 31.1 μm), whereas red paint fragments ranged between 30.4 and 979.9 μm (avg $\pm$ SE = 123.6 $\pm$ 23.5 μm). In terms of particle shapes, fragments accounted for almost all observations ($n = 582$), with only three exceptions represented by two pellets and one film. Color distribution was strongly skewed towards red ($n = 570$), followed by minor observations of blue ($n = 4$), green ($n = 3$), black ($n = 2$), grey ($n = 1$), pink ($n = 1$), and purple ($n = 1$).

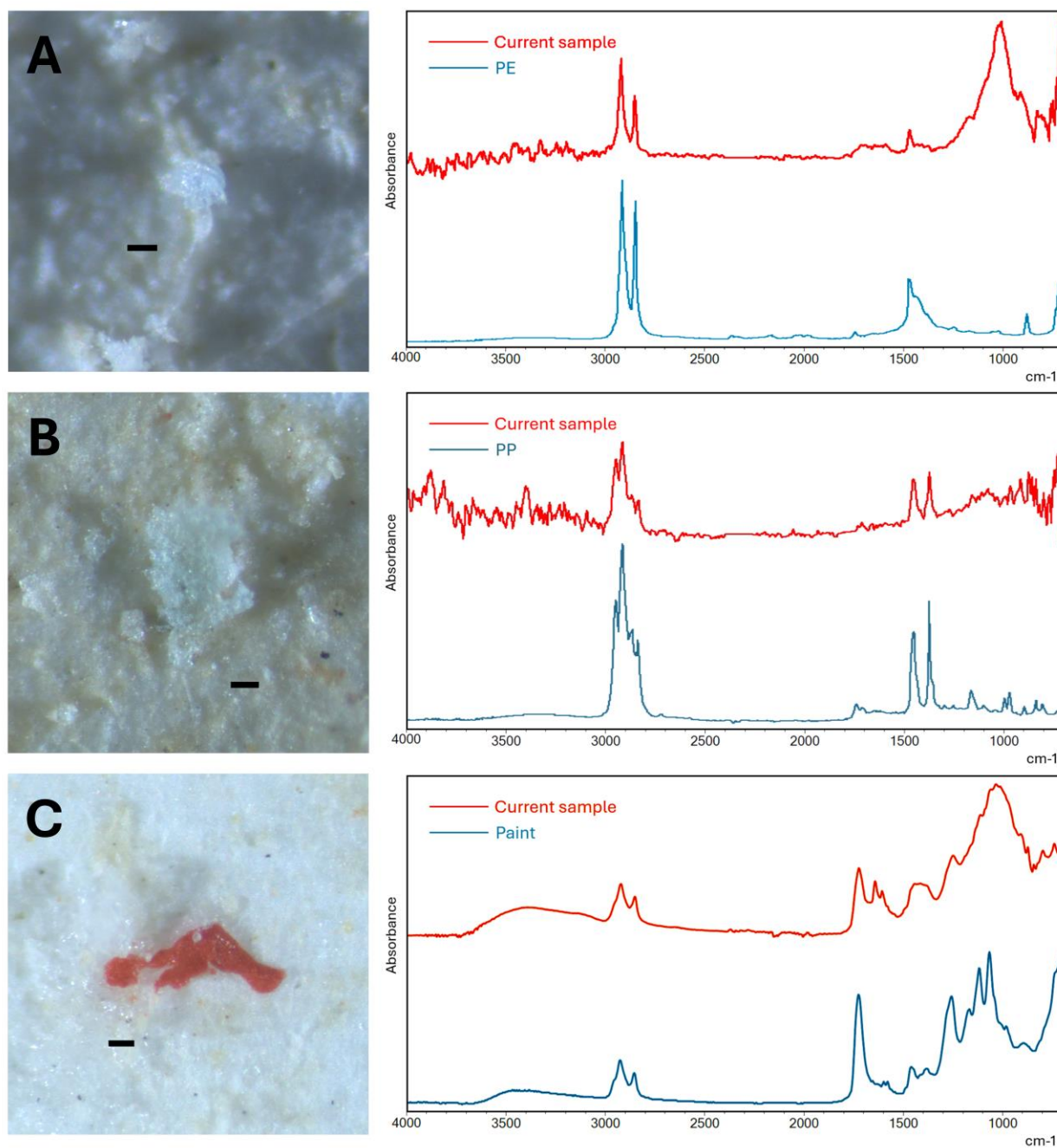


Figure 2.7. Occurrence and identification of red paint fragments associated with *Isidella elongata*. (A) Red paint fragment retrieved on the filter following chemical digestion of coral samples. Close up under the stereomicroscope of red paint dried on (B) the polyp body, (C) and on the apical part of the polyp.

Discussion

Although ML is widely recognized as a pervasive pollutant of marine ecosystems (Galgani et al., 1996; Hanke et al., 2025; Pham et al., 2014), its occurrence on the seafloor has been far less investigated than in surface waters, mainly due to the high costs and technical constraints of sampling in the deep sea (Pham et al., 2014; Ramirez-Llodra et al., 2011). Information on benthic marine litter has mostly been obtained through trawl surveys, typically as collateral data from campaigns aimed at quantifying fish stocks or benthic fauna (e.g., Galgani et al., 2000, 1996; Galil et al., 1995; Moore and Allen, 2000; Pham et al., 2014; Sánchez et al., 2013; Spedicato et al., 2019; Stefatos et al., 1999). More recently, the development of underwater vehicles has increased the possibilities for detecting and quantifying ML on the seafloor (Angiolillo and Fortibuoni, 2020; Bo et al., 2014a; Consoli et al., 2018a, 2018b; De Vincenzis et al., 2026; Orejas et al., 2009; Palummo et al., 2024; Valisano et al., 2019).

Meeting the requirements of the MSFD, the present study aimed to expand the effort in ML mapping in the Mediterranean Sea by assessing ML pollution in a poorly explored deep-sea area off the Gulf of Squillace in the Ionian Sea. The investigation adopted an integrated approach that combined data from MP occurrence in the habitat-forming CWC *I. elongata* and demersal fish species, as well as macrolitter pollution from the surrounding seafloor. Mediterranean CWC habitats act as feeding areas, shelters in front of predators and/or breeding and nursery habitats for fishes, where they generally display higher density and diversity values than in adjacent bottoms (Biber et al., 2014; Husebø et al., 2002; Rueda et al., 2019). Likewise, *I. elongata* forests provide high three-dimensionality on otherwise homogeneous muddy bottoms, providing habitat for a rich associated fauna (Carbonara et al., 2020; Cartes et al., 2022; Maynou and Cartes, 2012; Palummo et al., 2025). The fish species investigated in this work are commonly found within *I. elongata* forests and may also be strictly associated to this habitat, as in the case of *H. mediterraneus* and *H. italicus* (Maynou and Cartes, 2012).

The results of seafloor litter assessment in the Gulf of Squillace revealed a density of 448.7 items/km² at depths between 435 and 863 m, findings that are consistent with previous reports of ML in the Mediterranean Sea. However, the densities observed appear relatively high when compared specifically with studies conducted at similar bathymetric ranges (Alvito et al., 2018; Cau et al., 2022, 2018; Fiorentino et al., 2015; Saladié and Bustamante, 2021; Sciutteri et al., 2023; Spedicato et al., 2019). Notably, higher values have been reported in the Gulf of Naples, where Rizzo et al. (2022) documented densities of 1140 items km⁻² between 400 and 600 m depth. Some other studies assessing ML revealed similar or higher litter densities, although at shallower depths (0-281 m) (Pasquini et al., 2016; Vlachogianni et al., 2017). The relatively high levels of ML recorded in the study area may be explained by the characteristics of the sampling location. The study area, particularly within the surveyed depth range, is not subjected to intense trawling compared to other location along the Italian coasts (EMODnet Human Activities). In addition, some hauls were conducted in experimental, non-trawled sites, where the absence of regular “sweeping” of the seabed may have favored the persistence and accumulation of litter on the seafloor. Differently, seabeds subjected to frequent trawling can experience removal, burial, or redistribution of debris. Furthermore, deep-sea environments, and particularly those located below continental shelves and submarine canyons, are widely recognized as accumulation zones for ML (Angiolillo et al., 2015; Consoli et al., 2019, 2018a; Galgani et al., 1996; Martin et al., 2022; Pierdomenico et al., 2023).

The study site is included in GSA 19 where few studies have been conducted on ML using different sampling approaches. As a result, ML assessment reported in this study contributes to knowledge of the state of pollution of the area. Data from experimental bottom trawl surveys conducted within the MEDITS framework (Spedicato et al., 2019) reported lower macro-litter densities around the northern Ionian Sea (36 items/km²), with plastics representing the dominant fraction of collected debris in line with what is reported in the present study (84.8%). The preponderance of plastic debris, much of which belongs to the SUP category (35.2%), confirms what has been reported by other

studies in the Mediterranean Sea (Angiolillo et al., 2021; Canals et al., 2021; Cau et al., 2022; Consoli et al., 2020a, 2020c; Crocetta et al., 2020; Garofalo et al., 2020; Rizzo et al., 2022; Sciutteri et al., 2023). Such patterns suggest that most plastic litter in the study area likely originates from land-based sources. Although the adjacent coastline is characterized by relatively low permanent population density, seasonal tourism markedly increases anthropogenic pressure, potentially enhancing solid waste production and stress on wastewater systems. In addition, seasonal rivers and watercourses (e.g., Assi, Stilaro, Corace, Crocchio, and Alli) may act as key vectors for the transport of urban and agricultural debris to the marine environment (Galgani et al., 1996; Pham et al., 2014), particularly following intense rainfall events.

Studies based on ROV video analysis provided significantly higher mean densities of 35,600 items/km² (Angiolillo et al., 2023) and 38,300 items/km² (Costanzo et al., 2020). These differences highlight how methodological sampling methods can influence both the quantitative and qualitative characterization of ML (Vlachogianni et al., 2017). In contrast with the present study, which reported only 3.8% of litter related to fishing activities, both video-based studies identified ALDFGs as the most abundant litter category, accounting for 86.04% and 86.96% of the observed items. It is worth noticing that these studies have been conducted closer to the coast and at shallower depths where the impact of small-scale fisheries, which generally represent one of the main sources of ALDFGs at sea, is more intense (Battaglia et al., 2010; Sciutteri et al., 2023). Indeed, fishing pressure in the study area is mainly artisanal and coastal, although some fishing vessels from the nearby port of Roccella Ionica operate in the area overlapping the trawl hauls from GS5 to GS8 (Fig. S2.5; EMODnet Human Activities).

Impacts of plastic pollution on deep-sea fauna

The integrated approach applied in this study to assess the status of ML pollution in a deep area of the Ionian Sea also included the assessment of its potential impact on deep-sea biodiversity. Specifically, the possible ingestion of plastic was investigated in five deep-sea species, together with the assessment of plastic accumulation in corals, to better understand the ecological implications of ML on deep benthic ecosystems.

The deep-sea fishes examined in this study are generally discarded by fisheries and remain poorly investigated because of their limited commercial value, with the exception of *C. agassizi* and *H. mediterraneus* in some regions, as well as the logistical difficulties associated with deep-sea sampling. Plastic ingestion was recorded in all five species. Overall, the frequency of occurrence of plastic litter in DSs exceeded previously reported values for the same species in the Mediterranean Sea. *H. mediterraneus* was the species with the highest frequency of ingestion (50%), number of ingested plastics (N = 12), and plastic items/individual (0.6). Previous studies reported considerably lower frequencies for this species ranging from 1 in the eastern Mediterranean Sea to 2.9 in the southern Tyrrhenian Sea, respectively (Deudero and Alomar, 2015; Sciutteri et al., 2023). Eight plastic items were found in DSs of *C. agassizi*, corresponding to a percentage of occurrence of 25%, similarly to what observed in the southern Tyrrhenian Sea (%O = 21%, Panarello et al., 2019) although lower percentages (9.1%) were documented in the same area (Sciutteri et al., 2023). Plastic ingestion rates measured in *C. coelorhynchus* (%O = 45.5%) were not far from values observed by Esposito et al. (2022) and Scacco et al. (2022) in the Central Tyrrhenian Sea (%O = 35% and 43%, respectively). In our study, a plastic occurrence of 11.8% was recorded for *H. italicus*, the lowest among the investigated species, yet still higher than what was observed around the Balearic Islands (Alomar et al., 2020). To the best of our knowledge, this is the first study investigating litter ingestion in *P. cataphractum* in the Mediterranean Sea, for which we reported one plastic item among three specimens (%O = 33.3%). Although based on a limited sample size (n = 3), the detection of one plastic item (%O = 33.3%) provides preliminary evidence of exposure in this species and contributes to novel baseline information. Comparison among studies can be affected by the application of different methodological approaches for MP isolation. Indeed, while some studies, including the present one, have applied chemical digestion of tissues and isolation through filtration (Esposito et al., 2022), other authors relied on tissue dissection followed by manual sorting of plastic particles (Alomar et al., 2020; Panarello et al., 2019; Scacco et al., 2022; Sciutteri et al., 2023).

Interspecific differences in feeding habits may modulate exposure to plastic debris. Some species like *C. coelorhynchus* and *P. cataphractum* actively roots on the sediment in search of infaunal crustaceans, polychaetes and/or molluscs (Bottari et al., 2010;

Madurell and Cartes, 2006), experiencing increased exposure to MPs accumulated on the seafloor. *H. italicus* exhibits a more pelagic feeding strategy, preying mainly on mobile suprabenthic crustaceans loosely associated with the seafloor, likely experiencing less contact with sediment-associated particles (Madurell and Cartes, 2006). *H. mediterraneus* feeds on natatory benthopelagic prey, with a diet dominated by crustaceans and fishes, and may intercept particles suspended in the near-bottom water layer or ingest them secondarily through prey items (Madurell and Cartes, 2005). *C. agassizi* shows a mixed and opportunistic feeding strategy, exploiting a wide range of nectobenthic, suprabenthic and endobenthic prey, mainly fishes and crustaceans, with possible scavenging behavior as a secondary strategy (Anastasopoulou and Kapisiris, 2008). The mixed feeding habit may reflect on the exposure to mixed sources of MP particles. However, feeding strategy alone is unlikely to fully explain ingestion patterns, as local environmental conditions, including depth, turbidity, river runoff, distance from the coast, and the intensity of local anthropogenic pressures, also influence MP availability in deep-sea habitats (see Anastasopoulou and Fortibuoni, 2022 and references therein).

The present study reports the first evidence of plastic contamination in the bamboo coral *I. elongata* collected from a deep-sea area of the Gulf of Squillace (Ionian Sea) where this species has not been previously documented. However, available evidence suggests that the area offers suitable environmental conditions (Carbonara et al., 2020; Georges et al., 2024), and several populations have been reported on the adjacent muddy benthic plains (Carbonara et al., 2020).

I. elongata is a key species of Mediterranean bathyal muddy bottoms able to form extensive facies that significantly enhance the biodiversity of these biotopes (Angiolillo et al., 2024; Carbonara et al., 2020; Gerovasileiou et al., 2019; Mastrototaro et al., 2017; Palummo et al., 2025). Its life history traits (i.e., slow growth, recovery rates, recruitment, and long lifespan) are indicators of highly sensitive species (FAO, 2009). For these reasons, *I. elongata* belongs to the “Critically endangered” category in the IUCN Red List of Mediterranean Anthozoans and is listed in Annex II of the SPA/BD protocol of the Barcelona Convention. In addition, this species is a key component of soft-bottom coral

gardens, which are considered VMEs (Vulnerable Marine Ecosystems) in relation to several human activities (Angiolillo et al., 2024; Carbonara et al., 2020; FAO, 2009; Georges et al., 2024).

In this view, this study provides relevant information on MP contamination, which represents an additional, so far unquantified stressor for these ecologically critical habitats.

A total of 582 anthropogenic particles were found associated with *I. elongata*. The incidence and high occurrence of paint fragments (98% of total items) come alongside the retrieval of a paint can during trawling operations (Fig. S2). The paint inside the can was completely dry at the time of recovery. No traces of fresh paint were observed on the vessel, the net, or on any of the collected organisms, including those retrieved from inside the can itself. Our findings suggest that the paint particles detected on the corals are likely derived from the paint originally contained in the retrieved can, which was presumably discarded at sea causing paint spillage into the surrounding environment. Moreover, the conditions of the can (Fig. S2) indicated that it has not been in the marine environment for long, supporting the hypothesis of a relatively recent contamination event affecting both the corals and the surrounding environment at the sampling site. Coverage of coral surfaces (Fig. 5B, C) can result in clogging and smothering of polyps (Bruemmer et al., 2023). Such obstruction may compromise essential physiological functions such as breathing and feeding, and in severe cases, ultimately leading to organism mortality.

Particles embedded within coral tissues were detected in 80% of colonies. The observed size range (39.1-331.4 μm) overlaps with the prey size range of species from genus *Isidella* (Rodkina and Dautova, 2025). This observation supports the hypothesis that part of the accumulated fraction may derive from active or passive ingestion processes (Hall et al., 2015), while larger particles may also become incorporated as a consequence of external adherence and tissue overgrowth (Ding et al., 2019; Hierl et al., 2021). However, further histological investigations would be required to clarify uptake pathways in octocorals.

Unlike many MP studies in marine biota, fibers were not included in the final dataset. This methodological choice was adopted to minimize the risk of airborne contamination during onboard sample handling and processing, as synthetic fibers are widely recognized as the dominant source of laboratory contamination (Hermsen et al., 2018; Kühn et al., 2020). Their exclusion likely increased the robustness of the dataset, although leading to a conservative estimate of total MP content.

To date, no studies have investigated MP occurrence on Mediterranean octocorals and information from other basins are limited to few studies (Bian et al., 2024; Ding et al., 2019; Helmberger et al., 2025; Taylor et al., 2016). Inter-study comparisons are not always possible and should be interpreted with caution, as differences in analytical protocols (visual inspection vs. chemical digestion), data reporting, and the exclusion of fibers may substantially influence reported concentrations. The surface-associated MPs recorded in *I. elongata* ($42.7 \text{ items g}^{-1}$) greatly exceeds the values reported for the soft coral *Sinularia* sp. in the South China Sea ($0.99 \text{ items g}^{-1}$; Ding et al., 2019). Differently, the abundance of particles in coral tissues (0.8 items g^{-1}) is closer, although still higher, to MP density measured by Ding et al. (2019) ($0.27 \text{ items g}^{-1}$), and those later documented for the same genus in nearby coastal waters ($0.16 \text{ items g}^{-1}$; Bian et al., 2024). Some studies have targeted deep-sea octocorals. In particular, Taylor et al. (2016) detected four microfibers in a single sea pen specimen from the southwest Indian Ocean, while Helmberger et al. (2025) did not record MP occurrence analyzing 19 CWCs belonging to Class Octocorallia from the Central Pacific. Although not focusing on octocorals, Mateos-Cárdenas et al. (2024) reported $0.04 \text{ microparticles g}^{-1}$ in the CWC *Desmophyllum pertusum* from the northeast Atlantic Ocean, mainly composed by fibers.

Regarding polymer identification, plastic items were analyzed to determine their polymeric composition, allowing for a more accurate characterization of litter sources and potential environmental implications. The polymers identified in biological samples (fish and corals) from the study area were predominantly those most commonly found in the marine environment, such as PE, PP, PS, and PET (Andrady, 2017; De Sá et al., 2018; González-Ortegón et al., 2024; Palmieri et al., 2024; Rodrigues et al., 2019; Suaria et al., 2016). Our findings corroborate previous studies reporting similar polymer

compositions in other regions of the Mediterranean Sea (Pedà et al., 2020, 2022, 2025; Sciutteri et al., 2023). The detection of less common polymers, including polyurethane (PU), elastomeric materials (rubber), and synthetic resins, suggests that the studied organisms are exposed to a complex mixture of plastic debris as supported by seafloor litter data.

Conclusions

The ecosystem-based approach adopted in this study represents an important tool for assessing both ML density and the different types of related impacts on the seafloor, a challenging marine environmental compartment to explore. This integrated framework aligns with the needs identified for improving monitoring efficiency under Descriptor 10 of MSFD (Galgani et al., 2024), including the application of multispecies approach across all environmental compartments.

Our findings further support the evidence that deep-sea environments act as long-term sinks for ML (Galgani et al., 1996; Hanke et al., 2025; Martin et al., 2022; Peng et al., 2020), with potential implications not only for mobile fauna but also for habitat-forming species of high conservation value, such as *I. elongata*. Specifically, the occurrence of plastics in *I. elongata* provides a significant contribution to understanding the impact of MPs on Mediterranean octocoral species and highlights the need for further research on these organisms, given their ecological importance.

The detection of plastics in both surrounding seafloor and demersal fish highlights the widespread contamination at different trophic levels and environments. Moreover, by using corals as an indicator species for the first time, this study offers a more detailed understanding of anthropogenic impacts to CWC habitats. Considering the ecological role of Mediterranean CWC habitats as biodiversity hotspots and their recognized vulnerability under international and national frameworks, the observed levels of macro- and micro-litter raise additional concerns about cumulative stressors affecting these systems.

CRediT authorship contribution statement

Antonio Giova: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Cristina Peda:** Conceptualization, Data curation, Methodology, Supervision, Validation, Visualization, Writing - review & editing. **Giulia Lucido:** Data curation, Formal analysis, Writing - review & editing. **Simonepietro Canese:** Project administration, Resources,

Supervision, Writing - review & editing. **Francesco Longo:** Investigation, Resources, Writing - review & editing. **Daniela Pica:** Resources, Supervision, Validation, Writing - review & editing. **Silvestro Greco:** Funding acquisition, Resources, Writing - review & editing. **Teresa Romeo:** Funding acquisition, Project administration, Resources, Writing - review & editing. **Nunziacarla Spanò:** Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

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Supplementary materials



Figure S2.1. Marine litter retrieved during experimental trawl survey. (A) Plastic items. (B) Fishing line. (C) Glass bottle. (D) Tissues.



Figure S2.2. (A) Material retrieved during trawl haul GS_1 including demersal fish, *I. elongata*, and a red paint can. (B) Zoom in on some fish that got caught in the paint can during trawling net recovery. (C) Detail of paint can.

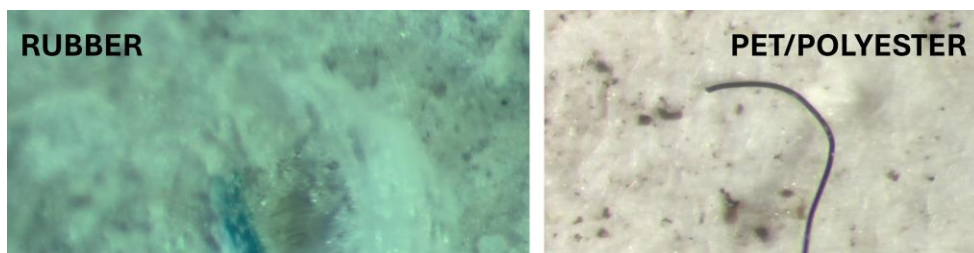


Figure S2.3. Examples of plastic litter found in digestive systems of demersal fishes. PET = Polyethylene Terephthalate, PS = Polystyrene, PU = Polyurethane.

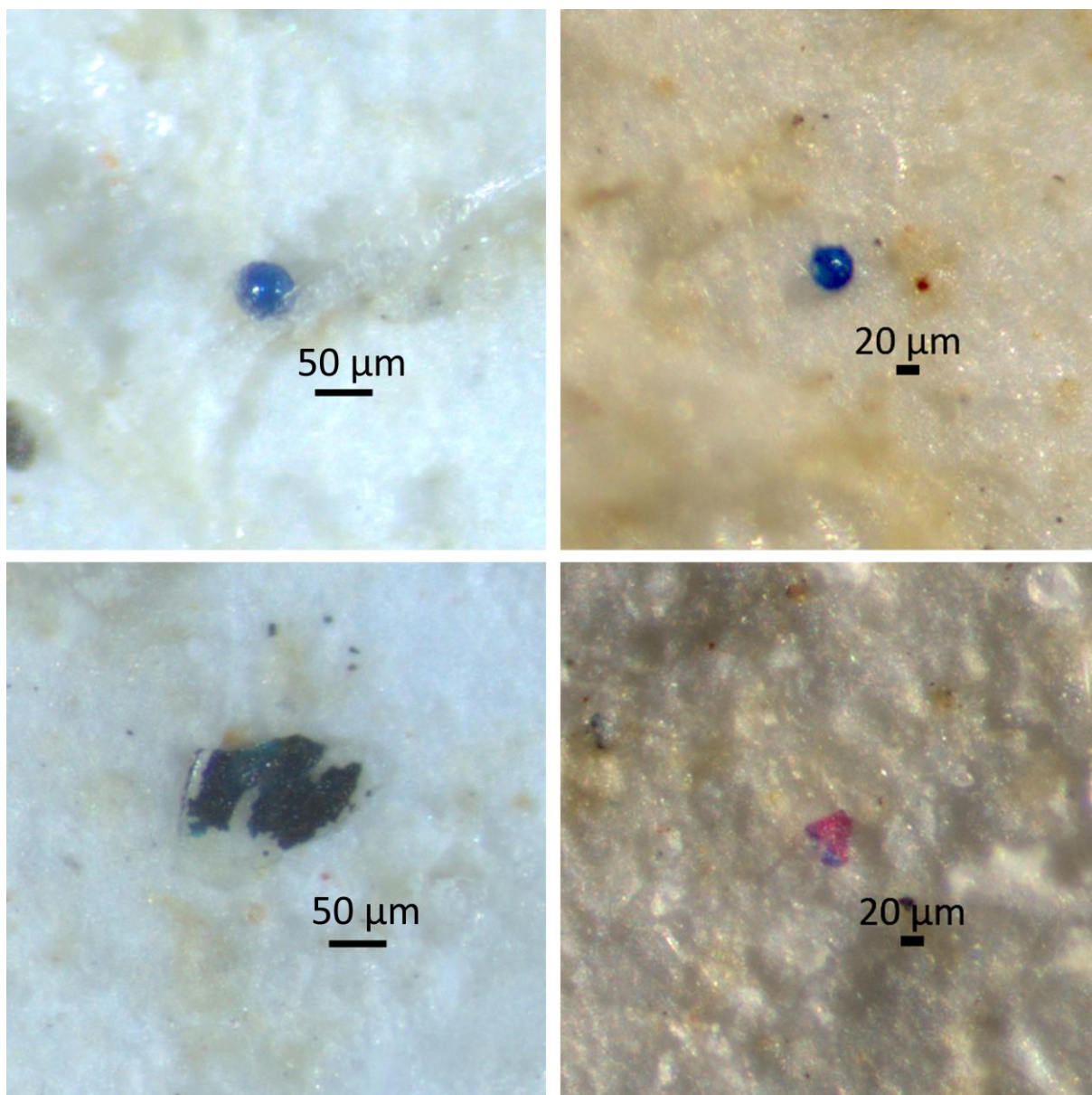


Figure S2.4. Examples of undetermined microplastic particles isolated from *Isidella elongata*

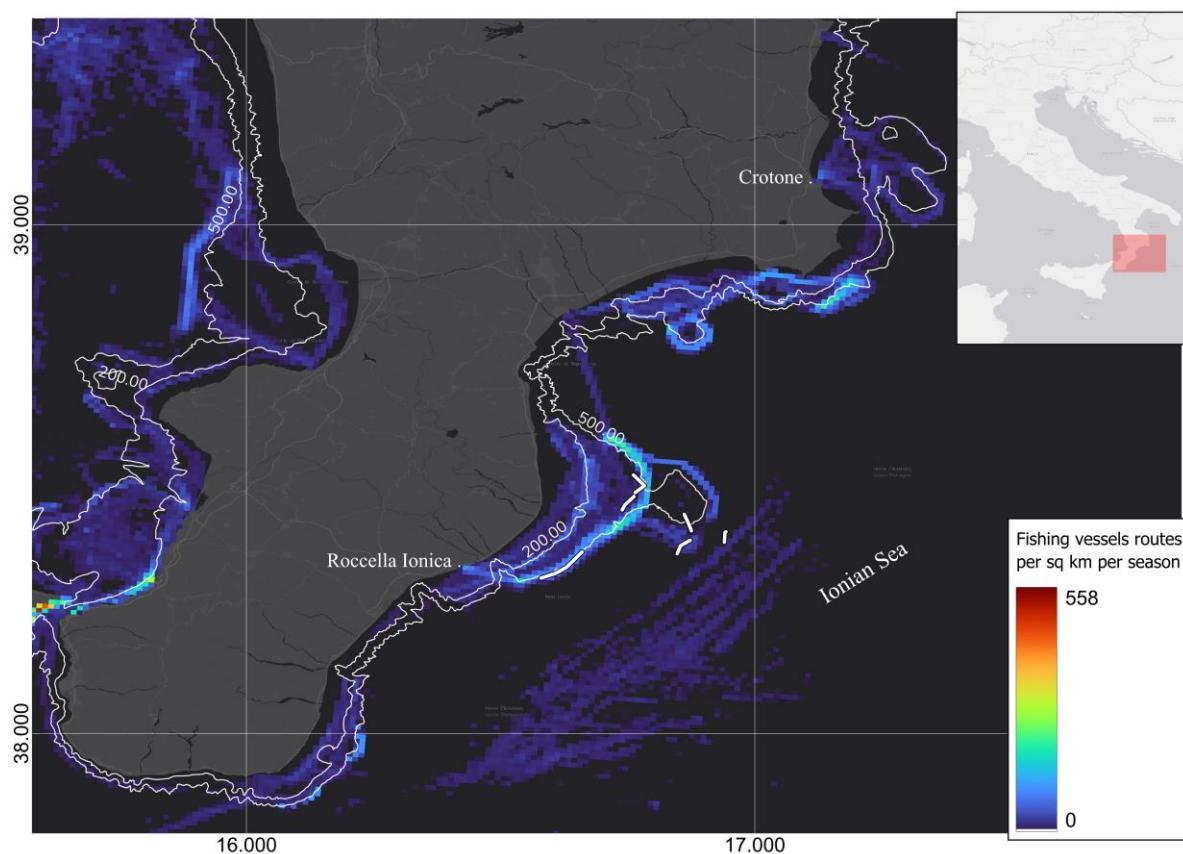


Figure S2.5. The route density map (routes $\text{km}^{-2} \text{ season}^{-1}$) at 1 km resolution from 2019 to 2024 overlapping the trawl hauls carried out in this study. Depth contours at 200 m and 500 m are shown. Data from the EMODnet Human Activities catalogue, initiative funded by the EU Commission (<https://emodnet.ec.europa.eu/geonetwork/srv/eng/catalog.search#/metadata/74eef9c6-13fe-4630-b935-f26871c8b661>).

Chapter 3

Effects of Microplastic Exposure on Octocorals

Rationale for the study

Following the identification of a targeted methodology for microplastic (MP) isolation and its application for assessing MP accumulation in octocorals in their natural environments, an experimental study addressing the biological effects of MP exposure in octocorals represented a logical next step to complete the picture of this project's aims. In particular, this study aimed to address key knowledge gaps on MP uptake and the potential effects in Mediterranean octocorals.

This study was carried out at the laboratory facilities of the Observatoire Océanologique de Banyuls sur Mer (Sorbonne University, France), identified based on their experience with experiments on Mediterranean anthozoans exposition to MPs under laboratory conditions.

Initially, the cold-water octocoral *Isidella elongata* (Esper, 1788) was selected as the target species for the exposure experiments, to maintain consistency with the previous phases of the project. However, practical constraints soon became evident. *I. elongata* is a rare species which collection requires dedicated oceanographic cruises, and its maintenance under laboratory conditions is extremely challenging. To date, no experimental studies have been conducted on this species, and its long-term survival in aquaria cannot be ensured. For these reasons, experimental activities were conducted on shallowerwater octocorals that are more accessible and whose maintenance is well established. Although differing in depth distribution, these species share key functional traits with deep-sea octocorals, including the suspension-feeding strategy and the habitat-forming role, allowing the investigation of biological responses to MP exposure.

The study involved two experiments targeting on two different species and life history stages. The first experiment focused on potential MP ingestion and egestion in the red gorgonian *Paramuricea clavata* (Risso, 1827), addressing the interaction between adult corals and MPs. The subsequent experiment investigated MP effects on larval survival and settlement of the white gorgonian *Eunicella singularis* (Esper, 1791) providing insight into potential impacts on early life stages. Both species are among the most representative habitat-forming gorgonians of rocky bottom communities on Western Mediterranean continental shelves (Angiolillo and Canese, 2018; Gori et al., 2007). *P. clavata* is a sciaphilous species which distribution covers the whole circalittoral depth range, while *E. singularis* is distributed on the upper circalittoral zone, and even in the shallower layers where it can live in association with symbiotic zooxanthellae (Gori et al., 2007).

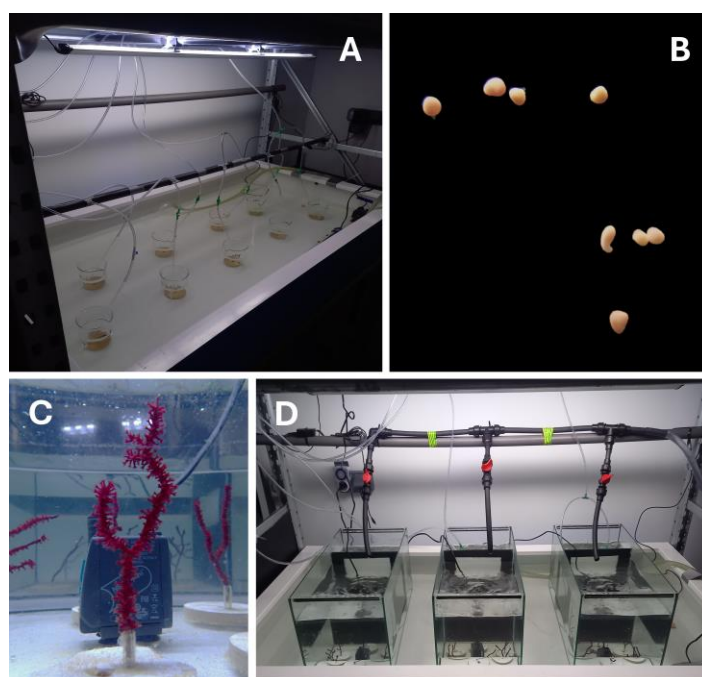


Figure 3.1. (A) Laboratory set up for the experiment of microplastic exposure on larvae of the white gorgonian *Eunicella singularis*. (B) Some *E. singularis* larvae observed under the stereomicroscope. (C) A branch of the red gorgonian *Paramuricea clavata* in the experimental water tank. (D) Laboratory set up for the experiment of microplastic exposure on adult colonies of *P. clavata*.

The experimental approach allowed the investigation of MP interactions with Mediterranean octocorals across multiple biological levels, from early life stages to adult colonies, providing a wide perspective on potential exposure pathways and biological responses throughout the octocoral life cycle.

Biological responses of two Mediterranean octocorals to microplastics exposure across different life history stages

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Abstract

Mesophotic gorgonian assemblages contribute to the structural complexity of Mediterranean coralligenous communities. They are increasingly threatened by well-documented anthropogenic stressors, whereas the potential effects of microplastics (MPs) remain largely underexplored. This study addresses this knowledge gap by investigating the responses of early life stages of *Eunicella singularis* and adult colonies of *Paramuricea clavata* to fluorescent polyethylene microbeads (200 and 2000 MP L⁻¹). Larvae of *E. singularis* were exposed for 42 days to MPs spanning from 10 to 1000 µm and monitored for survival, settlement rates, and MP ingestion in settlers. Mortality was significantly higher at the highest MP concentration, while settlement was not affected. Mucus production and particle adhesion were consistently observed. The increased larval mortality observed at high MP concentrations suggests a potential vulnerability of early life stages under extreme exposure scenarios, exacerbated by MP ingestion since the first life stages. Although the tested concentrations exceed most currently reported environmental levels, projected increases in plastic accumulation and the persistence of

MPs in benthic habitats may enhance long-term exposure risks. In adult colonies of *P. clavata*, ingestion was tested using microbeads of 200-250 µm during a 24-hour exposure followed by an additional 24 hours in MP-free water to assess potential egestion. No particle ingestion was detected under the tested conditions. This result may reflect the relatively large particle size compared to preferred prey, as well as the limited polyp activity recorded during the exposure period, potentially reducing feeding interactions. Overall, this study provides new experimental evidence on MP responses across different life stages of Mediterranean octocorals. Considering that comparable investigations are currently limited to a single study, these results contribute to establishing a baseline for assessing the ecological implications of MP pollution in Mediterranean habitat-forming anthozoans

Keywords: Microplastic ingestion, *Paramuricea clavata*, *Eunicella singularis*, Larvae, Habitat-forming species, Mediterranean Sea

Highlights

- Larvae of *Eunicella singularis* and adults of *Paramuricea clavata* were exposed to polyethylene microbeads at 200 and 2000 particles L⁻¹.
- Larval mortality increased significantly at high MP concentrations, while settlement was not affected.
- Microplastic ingestion was detected in newly settled polyps.
- Low polyp activity and no ingestion were observed in adult colonies of *P. clavata*.
- Results provide baseline experimental evidence on MP effects in Mediterranean habitat-forming octocorals.

Introduction

Gorgonians (Phylum Cnidaria: Subphylum Anthozoa: Class Octocorallia) are emblematic components of Mediterranean circalittoral communities and contribute to the structural complexity of coralligenous assemblages, recognized among the most biodiverse habitats worldwide (Ballesteros, 2006; Bavestrello et al., 1997; Gili and Ros, 1985; Weinberg, 1978). Gorgonians such as *Paramuricea clavata* (Risso, 1826), *Eunicella cavolini* (von Koch, 1887), *E. singularis* (Esper, 1791), *E. verrucosa* (Pallas, 1766) and the red coral *Corallium rubrum* (Linnaeus, 1758), characterized by long life spans and slow growth rates, can form mono- or multi-specific assemblages that generate structurally complex habitats commonly referred to as coral meadows, coral gardens or coral forests (Betti et al., 2020; Bo et al., 2015, 2014b; Buhl-Mortensen and Mortensen, 2004; Rossi et al., 2017; Stone, 2006). These aggregations represent a key component of marine animal forests (*sensu* Rossi et al., 2017), communities comparable in complexity, biodiversity, and structuring role to the terrestrial ones, but composed of suspension-feeding organisms (Bo et al., 2015, 2009; Buhl-Mortensen et al., 2010; Cerrano et al., 2010; Rossi et al., 2017). The three-dimensionality of marine animal forests provides secondary substrate and refuge for a rich associated fauna (Bo et al., 2014a; Cau et al., 2017; Cerrano et al., 2010; D'Onghia et al., 2017; Rueda et al., 2019), while enhancing benthic assemblage stability through local modification of hydrodynamics, light conditions and particle and larval retention (Ponti et al., 2018, 2014). As suspension feeders, octocorals play an important role in benthic-pelagic coupling by recycling particulate organic matter sinking from upper photosynthetic layers, directly influencing nutrient cycling and trophic pathways (Bo et al., 2014a; Corry et al., 2018; Fabricius, 2011; Gili and Coma, 1998).

Mesophotic gorgonian assemblages are threatened by several anthropogenic pressures, including recreational and professional demersal fishing activities that cause mechanical damage through gear contact and removal by demersal nets (Bavestrello et al., 1997; Betti et al., 2020), as well as recurrent marine heatwaves linked to global climate change that have triggered mass mortality events (Canessa et al., 2024; Coma et al., 2006; Garrabou et al., 2022; Gómez-Gras et al., 2021; Viladrich et al., 2022). While these stressors have been well documented in the last decades, microplastic (MP, plastic

particles <5 mm) pollution represents an emerging and invisible threat, whose ecological consequences are only beginning to be studied and remain challenging to anticipate.

MPs constitute a persistent fraction of marine litter that have been detected throughout the marine environment, from surface waters to deep-sea sediments, and in organisms spanning virtually all taxonomic groups (Lusher et al., 2017; Martin et al., 2022; Pedà et al., 2025). They enter marine ecosystems either directly by running off industrially manufactured microbeads of different sizes, widely used in consumer and personal care products (primary MPs), or from the degradation of larger plastic exposed to abiotic and biotic drivers (secondary MPs) (Andrady, 2017, 2011; Bergmann, 2015).

Corals can interact with MPs either passively, through adhesion to their surfaces, or actively, through ingestion (Huang et al., 2021; Pereira et al., 2024). Adhesion of MPs to the coenosarc has been associated with mucus overproduction, tissue necrosis and, in some cases, the incorporation of particles into skeletal structures (Corona et al., 2020; Ding et al., 2019; Hierl et al., 2021). MP ingestion can occur directly, since particles fall within the size range of their natural prey and are covered by organic matter (Hall et al., 2015; Reichert et al., 2024), or indirectly acquired through trophic transfer along the food web (Allen et al., 2017; Corinaldesi et al., 2021; Hall et al., 2015; Morais et al., 2020; Rotjan et al., 2019). Although laboratory studies have shown that corals are able to activate cleaning processes, such as egestion and mucus production (Allen et al., 2017; Hankins et al., 2018; Pereira et al., 2024; Reichert et al., 2018; Savage et al., 2022; Syakti et al., 2019), the maintenance of these mechanisms is energetically costly and leads to reduced feeding efficiency, and impaired growth and calcification (Chapron et al., 2018; Mouchi et al., 2019; Rades et al., 2022; Rotjan et al., 2019). At the physiological level, MP exposure has been associated with sublethal stress responses involving metabolic alterations, oxidative stress, and immune-related processes (Grillo et al., 2021; Lanctôt et al., 2020; Rocha et al., 2020; Tang et al., 2018; Xiao et al., 2021). Growing concern has also emerged regarding the potential effects of MPs on coral reproduction and early life history stages despite evidence being limited and sometimes controversial (Berry et al., 2019; Wilkins et al., 2024; Wilkins and Richmond, 2025).

Experimental evidence addressing MP ingestion and impacts in anthozoans comes from studies conducted on species belonging to the class Hexacorallia, whereas Octocorallia remains markedly understudied. Knowledge on the effects of MPs in Mediterranean octocorals is extremely limited, with only a single laboratory study to date conducted on the red coral *Corallium rubrum* (Corinaldesi et al., 2021).

The present study aims to address this knowledge gap by investigating the responses of two mesophotic gorgonian species to MP exposure across different life history stages. Two laboratory experiments were conducted to address the following questions: (i) whether MP exposure affects survival and settlement of *Eunicella singularis* planulae, including the potential uptake of MPs during early life stages; and (ii) whether adult colonies of *Paramuricea clavata* ingest MP particles during a 24-h exposure and their capability to eliminate ingested particles after a subsequent 24-h egestion period.

Materials and methods

Selected coral species

To achieve the goals of this research, two experiments were conducted separately under laboratory conditions using two selected species. Adults of *P. clavata* and *E. singularis* larvae were identified as experimental organisms for the laboratory study. These species were selected for their ecological importance in structuring complex three-dimensional habitats on the rocky bottoms of the collection site and the whole Mediterranean Sea. Biological samples were collected in 2025 at Cape Cerbère (42°26.489' N 3°10.802' E, north-western Mediterranean Sea, France), located at less than two kilometers from Cerbère-Banyuls Natural Reserve (Fig. 3.2).



Figure 3.2. Map of the study area showing the sampling location of *Eunicella singularis* and *Paramuricea clavata* (red dot) and the Cerbère-Banyuls Natural Reserve (green area).

Fluorescent low-density polyethylene (LDPE) MP beads (Cospheric, California, USA) were used in this research. Particles had a nominal density of approximately 1 g cm^{-3} and contained a fluorescent dye enabling visualization with an emission peak of 525 nm when excited at 485 nm. PE was chosen as it is recognized as the most abundant polymer found in marine environment and biota (Andrady, 2017; De Sá et al., 2018; González-Ortegón et al., 2024; Palmieri et al., 2024; Rodrigues et al., 2019; Suaria et al., 2016). MP size was selected according to the prey size range of the species (Coma et al., 1994). Three treatments were tested in both the experiments: (i) control (CTRL), treatment without MPs; (ii) low concentration treatment (LCT), reflecting current environmental levels ($200 \text{ particles L}^{-1}$); and (iii) high concentration treatment (HCT), simulating predicted future contamination scenarios ($2000 \text{ particles L}^{-1}$). The concentration of 200 MPs L^{-1} was selected to represent elevated but environmentally realistic exposure conditions, as comparable or even higher microplastic abundances have been reported in highly impacted marine and coastal environments, including harbours, estuaries, urbanized coastal zones, and surface microlayers, both within and outside the Mediterranean Sea (Anderson et al., 2018; Atwood et al., 2019; Song et al., 2014). Moreover, recent evidence suggests that conventional sampling approaches may substantially underestimate environmental MP concentrations, particularly for smaller particle size classes (Lindeque et al., 2020). The 2000 MPs L^{-1} represent a plausible scenario for MP concentrations by year 2100 in the Western Mediterranean, estimated to experience a 10-fold increase compared to 2010 levels (Everaert et al., 2020). PE microbeads were suspended in 96% ethanol (Karami et al., 2017) to prepare MP stock solutions at known nominal concentrations. Defined aliquots were then used to deliver controlled MP amounts to each treatment. Before each contamination, the suspension was homogenized by vigorous shaking the container. MP concentrations and MP delivery method were chosen according to Wolinski et al. (2025).

*Experiment 1: effects of MP exposure to larvae of *E. singularis**

Experimental design

Sexually mature colonies of *Eunicella singularis* were collected during the breeding season in June 2025 between 15 and 20 m depth. Colonies were maintained in open-circuit aquaria equipped with an air bubbling system at 17°C until the end of the larval release period. Larvae were collected for MP exposure after eight days using glass Pasteur pipettes, checked under a stereomicroscope, and randomly arranged into nine experimental glass beakers, with three replicates per treatment. Each beaker contained approximately 300 mL of 0.2 µm filtered seawater, 30 *E. singularis* larvae, and ceramic settlement tiles to promote larval settlement (Fig. 3.3).

To assess the effects of MP particles on larval survival, settlement, and potential uptake, *E. singularis* larvae were exposed for 42 days to three different treatments: CTRL, LCT and HCT using fluorescent LDPE MPs ranging from 10 to 1000 µm in diameter. Particle size distribution within each treatment was selected according to an inverse relationship between MP size and abundance observed in environmental matrixes (Leusch et al., 2023): 50.0% particles of 10–22 µm, 33.3% particles of 180–212 µm, and 16.7% particles of 850–1000 µm.

The system was kept under indirect white light exposure, taking into account the photopositive behavior of zooxanthellate *E. singularis* planulae (Weinberg, 1979). Continuous aeration ensured adequate oxygenation and maintained MPs in suspension throughout the exposure. Water renewals were performed every two days to limit bacterial growth, and MPs were replenished to maintain nominal concentrations in the treatment groups. During this manipulation, larvae were gently collected from each beaker and temporarily moved to clean Petri dishes containing MP-free filtered seawater for microscopic observation.

Assessing MP effects

The experiment lasted 42 days, including 32 days of exposure to ceramic tiles substrate and a subsequent 10-day period with crustose coralline algae (CCA) for settlement (Fig. 3.3). Data collected included: (i) larval mortality in water columns; (ii)

larvae metamorphosed in water columns; (iii) settled larvae; (iii) trans-epithelial MP uptake of larvae in water columns; (iv) settling rate; (v) number of ingested MPs per settler. For assessing MP uptake, larvae and settlers were kept in 10 % potassium hydroxide solution (10 % KOH, Sigma Aldrich) for 24 hours at 60°C and then filtered onto a 1.2 µm fiberglass filter (GF/C, diameter 47 mm, Whatman® Sigma-Aldrich) (Wolinski et al., 2025).

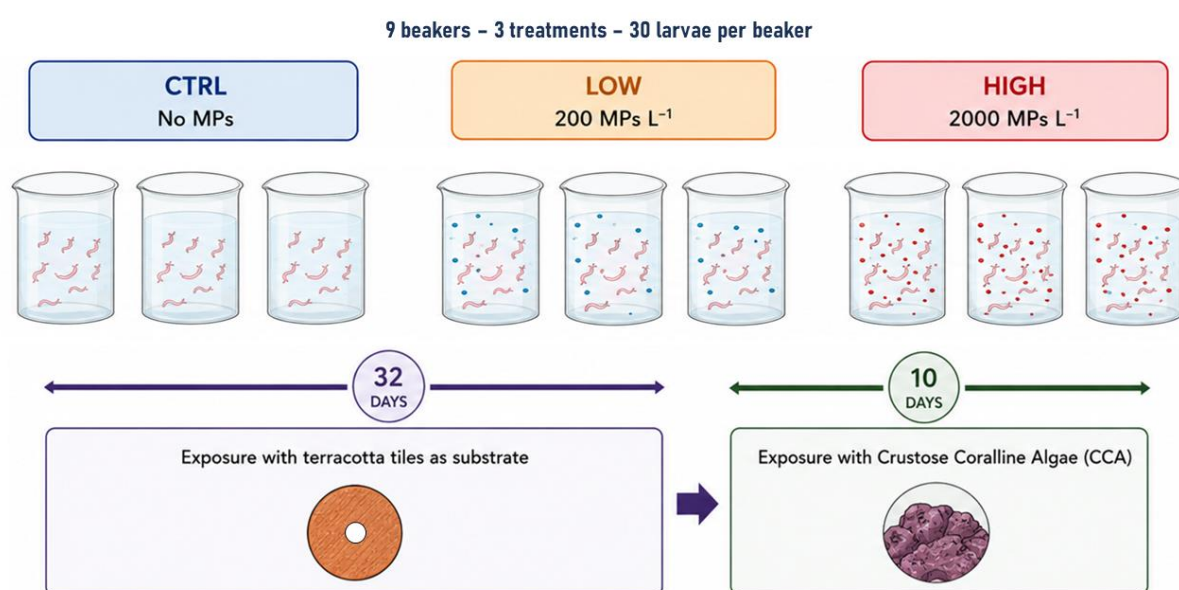


Figure 3.3. Graphical scheme of the experimental design for the exposure of *Eunicella singularis* larvae to microplastics.

Experiment 2: MP ingestion and egestion in *P. clavata* colonies

Experimental design

A total of 18 apical branches from six colonies of *P. clavata* (three branches each) were collected in August 2025 at Cape Cerbère at 25-35 m depth. After collection, the branches were transferred within one hour to the laboratory facilities of the Observatoire Océanologique de Banyuls-sur-Mer (Sorbonne University, France). Each fragment was fixed onto a ceramic tile using epoxy resin to ensure stability during aquarium handling.

Each branch originating from the same colony was placed into 3 experimental aquaria (20 L each), resulting in six coral fragments per tank (Fig. 3.4). Due to logistical constraints, limited material and space availability, each treatment was represented by a single aquarium; therefore, fragments within the same tank were considered pseudo-replicates.

Corals were acclimated for 30 days prior to experimental exposure. Aquaria were maintained at 18°C, with natural daily fluctuations restricted to 17.5–18.5°C. An open-system configuration was adopted, with continuous aeration provided by air bubblers and water circulation ensured by underwater pumps positioned to provide consistent water flow conditions across all coral fragments. Low intensity illumination was set for 9 h day⁻¹, simulating dim natural light conditions, and was measured before the experiment to ensure homogeneous exposure across all tanks. Colonies were fed every two days with a mixed diet of commercial phytoplankton, rotifers, and *Artemia salina* cysts to maintain polyp activity and overall health.

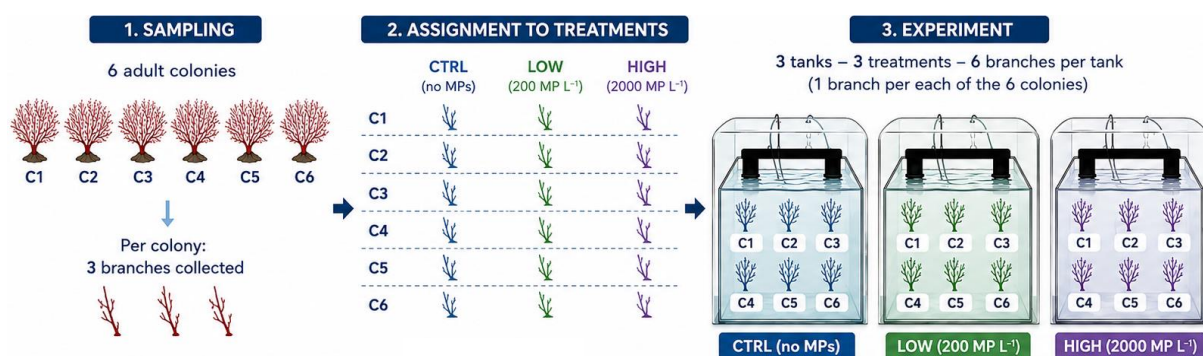


Figure 3.4. Graphical scheme of the experimental design for the exposure of *Paramuricea clavata* to microplastics.

P. clavata samples were exposed for 24 h to three different treatments: CTRL, LCT and HCT with fluorescent LDPE-MPs ranging in size from 200 to 250 µm in diameter, covering the size of the natural preys (Coma et al., 1994; Ribes et al., 1999). Exposures

were conducted in the same 20 L aquaria used for acclimation and each aquarium was assigned a treatment. Food supply was suspended during the exposure period.

Assessing MP ingestion and egestion rates

Polyp activity was monitored by acquiring images directly in the tanks using an underwater camera (OLYMPUS Tough TG-6) at baseline (T_0) and after 1 (T_1), 6 (T_6), 18 (T_{18}), and 24 (T_{24}) hours. Activity was recorded on a semi-quantitative scale as follows: 0 (all polyps closed), 0.5 (polyps partially active, tentacles retracted), and 1 (fully expanded polyps). At the end of the exposure, three 50 mL aliquots were collected from the water column of each tank to quantify the effective MPs suspended in mid-water around the branches.

After 24 h of exposure, half of the colonies from each treatment were collected for MP analysis. Sample colonies were rinsed and preserved in 70% ethanol until further processing. Despite careful rinsing, preliminary observations revealed that some particles remained adhered to the coral surface, likely retained within mucus layers or attached to external structures. To avoid overestimating ingestion rates, an additional rinsing step was performed directly on Whatman GF/C glass fiber filters (1.2 μm pore size) to remove externally attached particles prior to chemical digestion. Finally, ingested MPs were isolated using the digestion protocol routinely applied at the OOB (see Wolinski et al., 2025), with modifications implemented to adapt the procedure to the target species following Giova et al. (2025). Around 20 mL of 10 % potassium hydroxide (KOH) solution were added for each branch, ensuring the coverage of biological material, and incubated in a stove at 50°C for 12 h. A second step involved a 30 min incubation with hydrochloric acid (5%) at room temperature. Digested suspensions were filtered onto Whatman GF/C glass fiber filters (1.2 μm pore size).

The remaining colonies were transferred to clean, plastic-free 20 L aquaria for 24 h under the same physical and chemical conditions applied during acclimation (temperature, flow, aeration, and illumination), allowing for MP egestion. No additional food or MPs were supplied during the egestion phase. At the end of this phase, colonies were collected and processed for tissue digestion and MP recovery as described above.

MPs isolated from the colonies were inspected and quantified under the stereomicroscope. Particle abundance was normalized to the estimated number of polyps per colony. The number of polyps for each colony was indirectly estimated from total branch length and polyp density (polyps cm^{-1}). Total branch length was measured from photographs using a metric reference and analyzed with FIJI ImageJ software. Polyp density was estimated by counting polyps on both sides of two branches per colony over a measured linear length, selecting branches that balanced distal and apical portions (Fig. 3.5). The estimated number of polyps per colony was then calculated as the product of total branch length and mean polyp density.

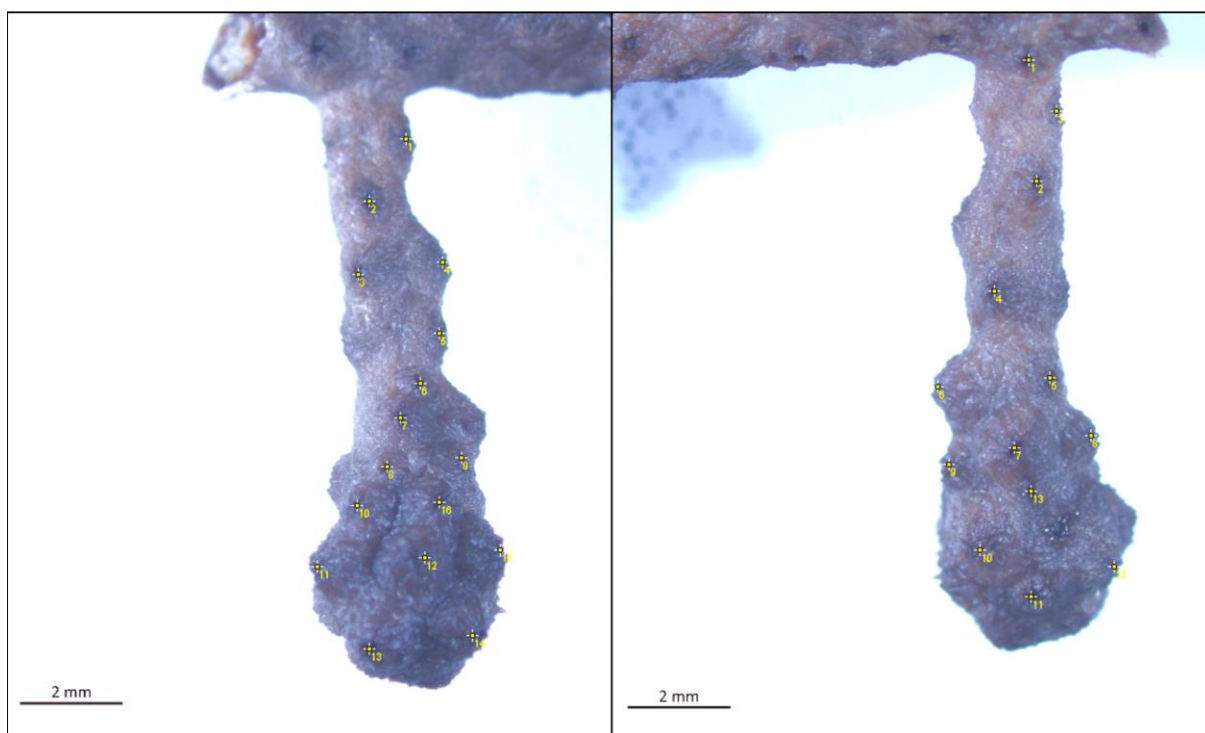


Figure 3.5. Polyp counting using ImageJ software with multi point tool. Pictures of front and back view were analyzed avoiding double counting.

Quality Assurance and Quality Control (QA/QC)

All manipulations and filtration steps were conducted using pre-cleaned equipment, thoroughly rinsed with a 1:1 mixture of Milli-Q water and ethanol to minimize impacts on corals, contamination and particle loss. A lamp Xite Fluorescence Flashlight (NIGHTSEA®, Massachusetts, USA) was used to follow fluorescent microbeads during sample manipulation and avoid MP loss during the experiment.

Statistical analysis

Polyp activity was analyzed using linear mixed-effects models (LMMs). Treatment, time, and their interaction were included as fixed effects, while Colony ID was included as a random intercept to account for within-branch correlation. The response variable was treated as continuous, given the ordered and equidistant structure of the scoring scale. Model significance was assessed using Type III ANOVA with Satterthwaite's approximation for degrees of freedom. When significant treatment effects were detected, pairwise comparisons were made using Tukey-adjusted estimated marginal means.

Larval mortality over time was analyzed using generalized linear mixed-effects models (GLMMs) with a binomial error distribution and logit link function. Mortality was modeled as cumulative counts of dead larvae over the initial number of larvae per replicate. Treatment, time, and their interaction were included as fixed effects, while replicate was included as a random intercept to account for repeated measurements. Model assumptions were evaluated through residual diagnostics and tests for overdispersion and zero inflation. Model significance was assessed using Type II ANOVA, and estimated marginal means were computed on the response scale with Tukey-adjusted pairwise comparisons. Following Wilkins and Richmond (2025), GLMMs were also used for settlement data analysis, with a binomial error distribution and logit link function, using treatment as fixed effect and replicate as random effect. Model assumptions were evaluated through residual diagnostics and tests for overdispersion and zero inflation. Estimated marginal means were computed to

compare treatments on the response scale. Model significance was assessed using Type II ANOVA.

Results

Effects of MP exposure on larval survival, settlement and uptake in E. singularis

During the 32-days exposure, larval mortality increased over time in all treatments (Fig. 3.6). However, mortality differed among treatments, reaching 12.2% in CTRL treatment, 6.7% in LCT and 24.4% in HCT. The generalized linear mixed-effects model revealed significant effects of treatment and time on mortality (both $p < 0.001$; Table S3.1), whereas the interaction between treatment and time was not significant ($p = 0.642$). This indicates that mortality increased over time across all treatments, but differences among treatments remained consistent throughout the experiment.

Post-hoc comparisons (Tukey-adjusted; Tab. S3.2) confirmed significantly higher mortality in HCT compared to both CTRL ($p = 0.014$) and LCT ($p < 0.001$), while no statistical differences were found between CTRL and LCT ($p = 0.07$), although mortality CTRL experienced higher mortality than LCT. The significant separation of the HCT mortality curve compared to other treatments became evident from day 16 onwards (Fig. 3.6).

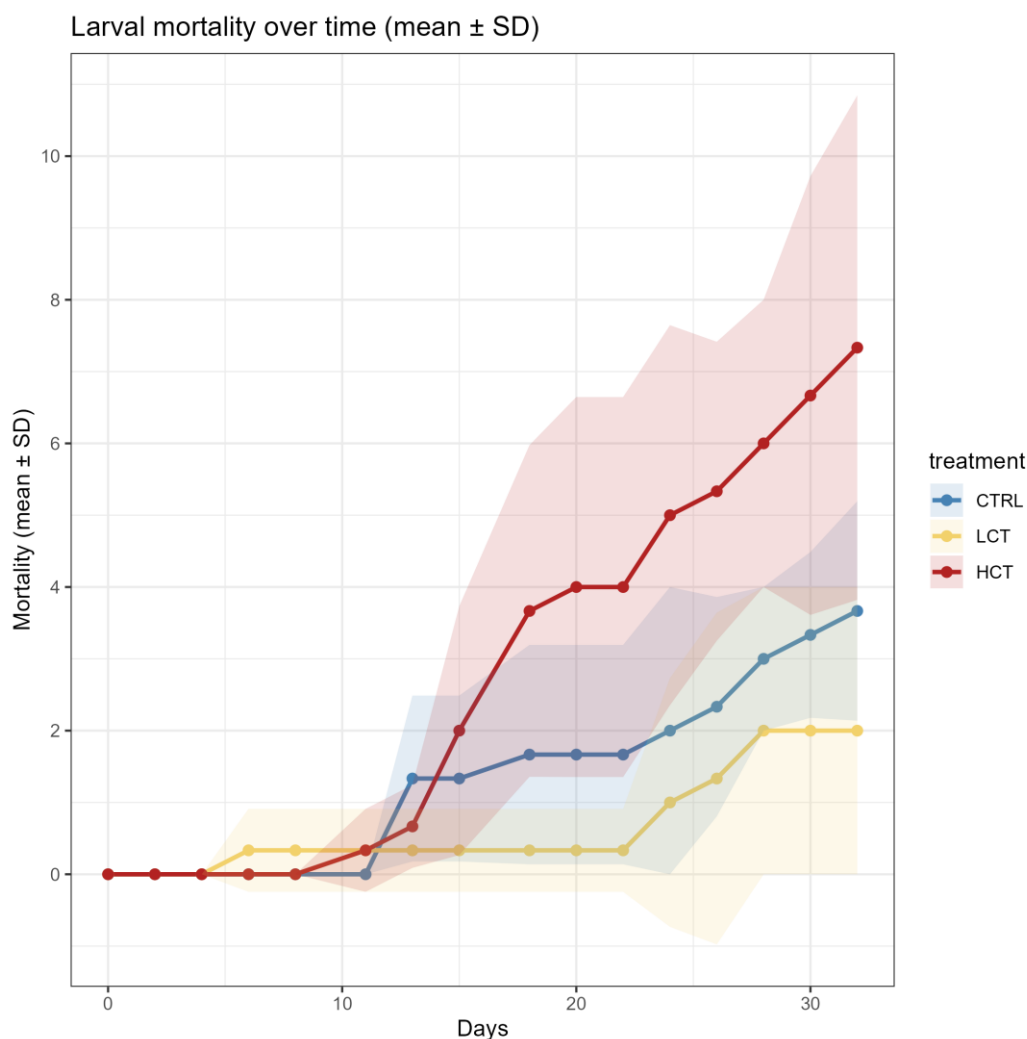


Figure 3.6. Mortality curves of *Eunicella singularis* larvae across 32-days exposure to microplastics. Mean mortality $\pm$ standard deviation is shown for each treatment. CTRL = plastic-free treatment; LCT = low concentration treatment; HCT = high concentration treatment.

Settlement during the 32-days exposure was negligible, with only one larva from LCT and three larvae from HCT (one for replicate) observed settling onto terracotta tiles or beaker glasses from day eight onwards (Fig. S3.1A). Differently, exposition to CCA promoted a higher settlement rate across all treatments. Settlement occurred in 35.6% of planulae in the CTRL treatment and 28.9% in both LCT and HCT (Fig. S3.1B-C). However, no significant effect of MP exposure on settlement was detected among treatments ($p > 0.05$; Tab. S3.3).

In addition to settlement, metamorphosis in the water column was observed in all treatments (Fig. S3.2). Metamorphosed planulae were first detected on day 4 in the CTRL treatment ($n = 8$), on day 11 in the LCT ($n = 6$), and since day 2 in the HCT ($n = 8$).

Adhesion to larvae was reported for both LCT and HCT throughout the whole experiment (Fig. 3.7A-C). In particular, four specimens from LCT and eight from HCT were observed with MP particles stuck in the mucus covering the planulae. Although mucus production was not quantitatively measured, it is worth noticing that mucus was observed in both exposure treatments even performing water changes every two days and, in some cases, resulted conspicuously in HCT beakers (Fig. 3.7D, E).

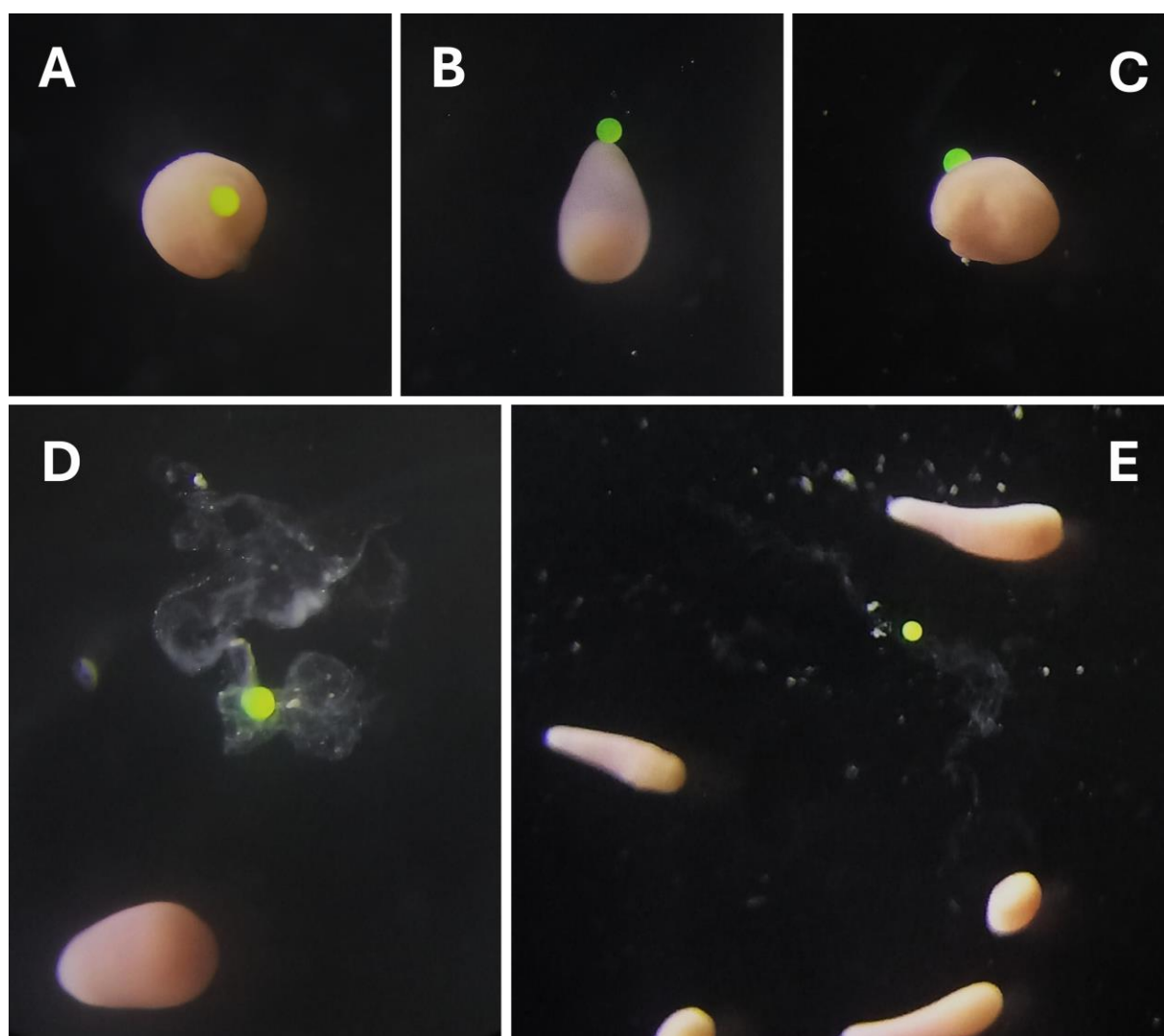


Figure 3.7. Close up on adhesion and mucus production phenomena. (A-C) Fluorescent polyethylene microbeads adhering to coral planulae surfaces. (D, E) Microbeads trapped by mucus agglomerates.

No trans-epithelial MP incorporation was reported for planulae. Differently, settlers exposed to MPs exhibited ingestion in all treatments. The highest mean MP uptake was recorded in HCT (1.00 particles settler⁻¹), followed by LCT (0.38 particles settler⁻¹), while only sporadic ingestion was observed in the CTRL (0.06 particles settler⁻¹). Size selectivity of particles was markedly oriented to $10\text{--}22\text{ }\mu\text{m}$ class (94.7%) and to a lesser extent to $180\text{--}212\text{ }\mu\text{m}$ class (5.3%).

MP Ingestion rates of P. clavata colonies

The mean number of polyps for treatment (AVG $\pm$ SD) was balanced, accounting for 547.0 ± 191.9 , 537.8 ± 121.2 , and 558.0 ± 110.9 in CTRL, LCT, and HCT respectively. Morphometric parameters of *P. clavata* branches are reported in Table S4.

Polyp activity was monitored at T₀, T₁, T₆, T₁₈, T₂₄ (Fig. 3.8). A strong effect of time was observed ($p < 0.001$; Tab. S5), indicating temporal changes in activity across the 24-hour period. Activity was highest at T₆, whereas lower and similar levels were recorded at T₁, T₁₈ and T₂₄. Polyp activity differed significantly among treatments ($p = 0.014$; Tab. S2), although this pattern did not show a linear dose-dependent response. Indeed, LCT showed significantly higher activity compared to CTRL ($p = 0.011$; Tab. S6), while no significant differences were detected between HCT and CTRL or between HCT and LCT. However, the interaction between treatment and time was not significant ($p = 0.186$; Tab. S5), with similar temporal trajectories among treatments.

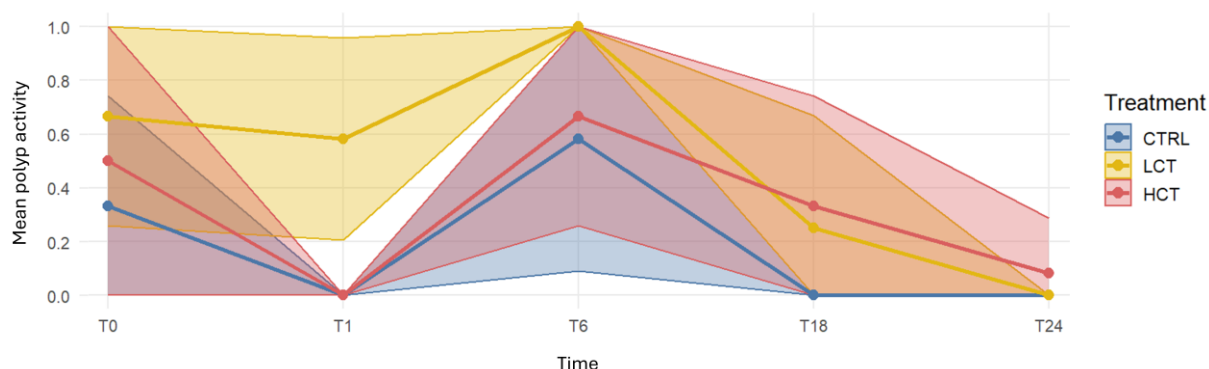


Figure 3.8. Mean polyp activity over time across treatments. Values range from 0 (fully closed) to 1 (fully open). Time is classified as hours from the beginning of the experiment.

The quantification of the effective concentrations of MPs suspended in mid-water around the branches provided far lower results compared to the theoretical concentration of spiked MPs. While MPs in CTRL treatment were confirmed as absent, concentrations in LCT and HCT reached 33.3 and 125.0 MPs L⁻¹, respectively.

Prior to chemical digestion for MP ingestion assessment, branches of *P. clavata* were rinsed directly on filters. The results of MPs adhering to surfaces were very unbalanced with 73 and 1 microbeads isolated from two branches subjected to HCT during the first 24 h of the ingestion assay (Table 3.1). After subsequent chemical digestion, performed to isolate MPs potentially present within coral tissues, no microbeads were detected in any of the analyzed samples (Table 3.1).

Table 3.1. Number of microplastic particles (MPs) adhered to colony surfaces and ingested by *Paramuricea clavata* under control conditions (CTRL), low concentration treatment (LCT), and high concentration treatment (HCT). Data are reported for each colony analyzed after 24 h exposure (ingestion assay) and after 48 h (24 h exposure followed by 24 h in plastic-free water for egestion assessment).

Experiment	Colony ID	Treatment	MPs on surface	MPs Inside polyps
24-h Ingestion	C_PCLA_1	CTRL	0	0
	C_PCLA_2	CTRL	0	0
	C_PCLA_3	CTRL	0	0
	L_PCLA_1	LCT	0	0
	L_PCLA_2	LCT	0	0
	L_PCLA_3	LCT	0	0
	H_PCLA_1	HCT	1	0
	H_PCLA_2	HCT	0	0
	H_PCLA_3	HCT	73	0
48-h Ingestion + Egestion	C_PCLA_4	CTRL	0	0
	C_PCLA_5	CTRL	0	0
	C_PCLA_6	CTRL	0	0
	L_PCLA_4	LCT	0	0
	L_PCLA_5	LCT	0	0
	L_PCLA_6	LCT	0	0
	H_PCLA_4	HCT	0	0
	H_PCLA_5	HCT	0	0
	H_PCLA_6	HCT	0	0

Discussion

This study experimentally investigated the effects of MP exposure on two Mediterranean mesophotic gorgonian species, addressing both adult and early life history stages. By combining an exposure experiment on *E. singularis* larvae with a short-term ingestion assay in *P. clavata*, the study aimed at contributing to the limited understanding of MP impacts in Mediterranean octocorals.

Gorgonians are key habitat-forming organisms of coralligenous assemblages, enhancing the three-dimensionality of the environment, supporting biodiversity, and regulating benthic–pelagic coupling. These species are recognized within national and international conservation frameworks and contribute to hard-bottom coral gardens, classified as Vulnerable Marine Ecosystems (VMEs; FAO, 2009). Despite their key ecological role, experimental evidence on MP effects in Mediterranean octocorals remains extremely scarce. To date, only one study has addressed this issue (Corinaldesi et al., 2021), and biological responses of early life stages have not been previously investigated.

Early life stage sensitivity to extreme MP exposure scenarios in E. singularis

Impacts of MPs to coral planulae have been previously examined on three stony coral species (Subphylum Anthozoa: Class Hexacorallia: Order Scleractinia) with different approaches (Berry et al., 2019; Wilkins and Richmond, 2025). Wilkins and Richmond (2025) assessed the effects of plastic leachates (i.e., additives released into seawater) on larval survival and settlement in *Porites* sp. and *Montipora capitata* following a 7-days exposure. Leachate solutions were prepared by incubating four different polymers in filtered seawater for one month at concentrations of 50, 100, and 200 particles L⁻¹. Both survival and settlement were negatively affected at all tested concentrations, with species-specific responses and clear dose-dependent patterns. Berry et al. (2019) examined the effects of PE and polypropylene (PP) (5–200 particles L⁻¹) on gamete fertilization, embryonic development, and larval settlement in the reef-building coral *Acropora tenuis*. The study found that early life processes were unaffected by MP contamination, reporting no significant impact on embryonic development and larval settlement.

In the present study, larval mortality increased over time in all treatments, indicating a general temporal effect independent of exposure condition. However, differences emerged among treatments. LCT (200 particles L⁻¹) showed lower mortality than CTRL (no plastics), although this difference was not statistically significant and likely reflects stochastic variability among replicates rather than a biological effect. In contrast, larvae exposed to HCT (2000 particles L⁻¹) experienced significantly higher mortality rates compared to both CTRL and LCT. Although both our study and that of Wilkins and Richmond (2025) documented that plastic-associated pollutants can impair larval survival, the application of distinct stressors limits direct comparison. Nevertheless, it is unlikely that the exposure time used would allow for leachates release, suggesting a different nature of the stressors. In our experimental system, physical/mechanical could have been the main driver for larval mortality, considering the wide size range and the high concentration used. In addition, significant mortality increases were detected only at the highest MP concentration tested and starting from day 16 of exposure. The ecological relevance of this delayed effect can depend on the duration of the pelagic phase under natural conditions. Pioneering studies have documented settlement competence within the first 30 hours after release (Weinberg and Weinberg, 1979), suggesting that a substantial fraction of larvae may settle before long-term exposure effects occur. Nevertheless, more recent observations indicate considerable variability in larval duration, with first settlers observed after 8 days and some individuals remaining longer in the water column for up to 57 days (Manea et al., 2025; Zelli et al., 2020). In this context, while early settlers may partially escape exposure, planulae with longer pelagic phases could experience sustained contact with MPs, potentially affecting the proportion of individuals successfully achieving settlement. In this regard, while no significant differences in settlement density on CCA were detected among treatments, as also described in Berry et al. (2019), settlement rate ranged between 28.9% and 35.6% within 10 days. This observation supports potential persistence of a large fraction of larvae in the water column. Moreover, MPs may continue to have an impact on settlers, although not specifically addressed in the present study, with consequences that have yet to be investigated.

The possibility of a trans-epithelial MP uptake in coral planulae was assessed, following observations of phagocytosis of MP particles in cell assays (Kwon et al., 2022; Park et al., 2020). However, no MPs were detected after chemical digestion of larvae. An explanation can surely be the size of the tested microbead. Indeed, even the smaller particles (10 μm) likely exceed the threshold for efficient epithelial internalization as also reported in Kwon et al. (2022). Differently, ingestion of MPs was documented in newly settled individuals, with average ingestion reaching up to 1 particle settler⁻¹ in HCT. Particle size selectivity was strongly oriented towards the 10–22 μm class, with very limited ingestion of larger particles (180–212 μm). The ingestion during the sensitive early life stages represents a concerning aspect that may lead to longer-term implications. Indeed, MP ingestion in corals has been demonstrated to cause sublethal stress responses (Grillo et al., 2021; Lanctôt et al., 2020; Rocha et al., 2020; Tang et al., 2018; Xiao et al., 2021) and activate energetically-costly mechanism, such as egestion and mucus production (Allen et al., 2017; Hankins et al., 2018; Pereira et al., 2024; Reichert et al., 2018; Savage et al., 2022; Syakti et al., 2019), resulting in reduced feeding efficiency, and impaired growth and calcification (Chapron et al., 2018; Mouchi et al., 2019; Rades et al., 2022; Rotjan et al., 2019). No statistical analysis was performed on MP ingestion in settlers due to the unbalanced structure of the dataset, as larvae settled at different times and in varying numbers across replicates during the 10-day period, leading to unequal exposure durations and sample sizes among treatments. Finally, the ingestion of a single particle in CTRL treatment likely reflects minor background contamination resulting from the aerial transport of the smallest size class (10–22 μm), despite the precautions adopted, including regular water renewal and daily monitoring of fluorescent microbeads under blue light. Although it is highly unlikely that such minimal background contamination influenced survival rates, this possibility cannot be completely excluded. However, water renewal and MP monitoring prevented their accumulation, making it unlikely that background contamination significantly affected the observed survival pattern.

Adhesion of MPs to larval mucus was observed in exposure treatments, especially in HCT (Fig. S3.5A-C). Although mucus production was not quantified, visible mucus and particle entrapment were frequently documented, even under regular water renewal (Fig. S3.5D, E). The observation of a cleaning response of larvae to MP exposure follows

previous findings linked to adult coral colonies (Allen et al., 2017; Hankins et al., 2018; Pereira et al., 2024; Reichert et al., 2018; Savage et al., 2022; Syakti et al., 2019). Although mucus production in planula larvae has not been previously observed as a response to MP exposure, it has been reported in reef-building corals exposed to high sedimentation (Ricardo et al., 2016). This observation supports the hypothesis of a strong physical/mechanical stress to coral planulae. However, without quantitative mucus measurements or energetic assessments, it cannot be determined whether this interaction caused energetical and physiological costs.

Metamorphosis in the water column occurred in all treatments (Fig. S3.2), with some variation in timing among groups. However, because this endpoint was not formally analyzed and because metamorphosis events were limited in number, these observations remain descriptive. However, the number of metamorphosed larvae at the end of the experiment was similar across treatments, despite variations in the timing with which the event was observed. The occurrence of larval metamorphosis in the water column, together with the sudden settlement after the addition of CCA, indicates that larvae were already competent during first experimental phase. For this reason, the near absence of settlement on ceramic tiles suggests that this substrate was largely avoided, highlighting a clear substrate-specific preference.

MP Ingestion in P. clavata

The short-term exposure experiment conducted on adult colonies of *P. clavata* did not reveal MP ingestion within 24 hours. Although the ability of corals to recognize and negatively select synthetic particles has been documented (Allen et al., 2017; Hankins et al., 2018; Pereira et al., 2024; Syakti et al., 2019), our results are not consistent with similar studies conducted on other anthozoan, including octocorals.

While anthozoans are capable of recognizing plastics and expelling them after few minutes (Pereira et al., 2024), it has also been shown that particle retention times can exceed 24 hours (Chapron et al., 2025; Pereira et al., 2024). In this regard, it is unlikely that short particle retention time may be the reason for the absence of MP within tissues.

Another factor could be attributed to the size of the particles selected for exposure assay. Coma et al. (1994) observed that the majority of the prey items consumed by *P. clavata* fell within the 100–200 μm range under natural feeding conditions. Although the particle size selected for the present exposure assay (200–250 μm) slightly exceeded this optimal range, it still remains extremely close to it. Moreover, *P. clavata* is known to feed on organisms reaching up to 800 μm (Coma et al., 1994; Ribes et al., 1999), indicating that the selected size spectrum still falls within its theoretical prey range. Taking together, these considerations lead us to exclude particle size as a primary factor explaining the absence of ingestion observed in this study.

Another reason that may influence particle ingestion is the use of virgin MPs rather than biofouled particles. Reichert et al. (2024) reported higher feeding responses and ingestion rates when MPs were colonized by biofilm compared to virgin particles, suggesting that surface conditioning may enhance particle uptake. Conversely, Allen et al. (2017) observed greater ingestion of virgin MPs compared to biofouled ones in a scleractinian species, highlighting species-specific variability in particle–polyp interactions. Overall, the contrasting evidence available in the literature indicates that particle conditioning alone cannot fully explain the absence of ingestion observed in the present study.

Polyp activity was monitored throughout the first 24-h experiment (Fig. 3.8). Activity resulted to be relatively low under all experimental conditions. Such reduced activity likely limited the interactions between polyps and MP particles, and thus prey capture attempts. Overall, low polyp activity could therefore be the main factor explaining the absence of ingestion observed, perhaps supported by other limiting conditions previously discussed.

However, a statistically significant increase in polyp activity was detected in the LCT treatment compared to CTRL and HCT. In this regard, a primary issue to address is the possibility of a tank effect resulting from pseudo-replication. Ideally, exposure experiments should rely on fully replicated aquarium systems, but logistical constraints related to space, costs, and material availability often limit such designs. To mitigate the

absence of independent tank replication, particular care was taken to standardize environmental conditions across treatments. Each aquarium hosted six branches originating from the same six parent colonies, with three branches per colony distributed among treatments, ensuring comparable initial physiological conditions. Temperature was uniformly controlled through shared thermostatic water baths, maintaining stable and identical thermal conditions. Water flow originated from a common source and was provided drop-by-drop to ensure continuous and comparable water renewal across tanks. Light was supplied by a single low-intensity source evenly distributed over the tanks and monitored to ensure homogeneous irradiance levels. Finally, internal pumps were placed in the same position, direction, and intensity in each tank to promote comparable hydrodynamic conditions among branches and treatments. In conclusion, while a residual tank effect cannot be entirely excluded, the precautions adopted substantially reduced this risk. Therefore, the possibility that low concentrations of MPs may stimulate higher polyp activity should be considered.

Further investigations are required to better understand the responses of *P. clavata* to MP exposure. Future studies should consider testing different experimental conditions, including a wider range of particle sizes, the comparison between virgin and biofouled particles, as well as longer acclimation and exposure times when necessary.

Conclusions

This study provides new experimental insights into the effects of Mediterranean mesophotic octocorals to MP exposure, addressing both early life history stages and adult colonies. By investigating larval responses in *E. singularis* and particle ingestion in *P. clavata*, we expand the currently limited body of evidence available for habitat-forming anthozoans in the Mediterranean Sea.

Given the ecological relevance of gorgonian forests in structuring Mediterranean coralligenous assemblages and supporting biodiversity, assessing their sensitivity to emerging stressors such as MPs is essential to anticipate potential consequences for ecosystem functioning and resilience. The increased larval mortality observed under high MP concentrations suggests that early developmental stages may represent a critical bottleneck under future pollution scenarios, potentially affecting recruitment effectiveness and long-term population dynamics.

Considering that experimental evidence on Mediterranean octocorals remains extremely scarce, these findings contribute to establishing a baseline for future research. Further long-term and multi-stressor investigations will be necessary to clarify how MP pollution may interact with other anthropogenic pressures and shape the future persistence of coral forests in a changing Mediterranean Sea.

CRediT authorship contribution statement

Antonio Giova: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Cristina Peda:** Data curation, Methodology, Supervision, Validation, Visualization, Writing - review & editing. **Franck Lartaud:** Conceptualization, Methodology, Supervision, Resources, Validation, Visualization, Writing - review & editing. **Lorenzo Bramanti:** Conceptualization, Methodology, Supervision, Resources, Validation, Visualization, Writing - review & editing. **Arianna Calderazzo:** Investigation, Data curation, Writing -

review & editing. **Lauryn Olla:** Investigation, Data curation, Writing - review & editing. **Erwan Peru:** Investigation, Supervision, Writing - review & editing. **Daniela Pica:** Supervision, Validation, Writing - review & editing. **Teresa Romeo:** Funding acquisition, Project administration, Resources, Writing - review & editing. **Nunziacarla Spanò:** Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

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Supplementary materials

Table S1. Summary of Type II Analysis of Variance (ANOVA) of the binomial general linear mixed model testing the effects of treatment, days, and their interaction on larval mortality.

Effect	Chisq	N. Df	Pr(>Chisq)
Treatment	71.5257	2	2.94e-16 ***
Day	159.1853	1	< 2.2e-16 ***
Treatment : Day	0.8862	2	0.642

Signif. codes: '***' p<0.001; '**' p<0.01; '*' p<0.05

Table S2. Tukey-adjusted pairwise comparisons (binomial general linear mixed model) of treatments for larval mortality.

Contrast	Estimate (log-odds)	SE	z value	p-value
CTRL – LCT	0.51	0.18	2.83	0.070
CTRL – HCT	–0.69	0.21	–3.29	0.014 *
LCT – HCT	–1.20	0.22	–5.45	<0.001 **

Signif. codes: '***' p<0.001; '**' p<0.01; '*' p<0.05

Table S3. Summary of Type II Analysis of Variance (ANOVA) of the binomial general linear mixed model testing the effects of treatment, days, and their interaction on larval settlement.

Effect	Chisq	N. Df	Pr(>Chisq)
Treatment	1.0128	2	0.6027
Day	36.4671	1	1.55E-09 ***
Treatment : Day	0.1507	2	0.9274

Signif. codes: '***' p<0.001; '**' p<0.01; '*' p<0.05

Table S4. Summary of morphometric parameters measured for each *Paramuricea clavata* branch. Total branch length was measured using with ImageJ; polyp number was estimated as the product of total branch length and mean polyp density.

Colony ID	Treatment	Total branch length (cm)	Polyp number
C_PCLA_1	CTRL	27.2726	759
C_PCLA_2	CTRL	21.0292	586
C_PCLA_3	CTRL	23.0698	642
C_PCLA_4	CTRL	8.87585	247
C_PCLA_5	CTRL	13.8928	387
C_PCLA_6	CTRL	23.72245	661
L_PCLA_1	LCT	15.07365	420
L_PCLA_2	LCT	26.09355	727
L_PCLA_3	LCT	15.32895	427
L_PCLA_4	LCT	16.76535	467
L_PCLA_5	LCT	20.83825	580
L_PCLA_6	LCT	21.7812	607

H_PCLA_1	HCT	15.62465	435
H_PCLA_2	HCT	15.2836	426
H_PCLA_3	HCT	23.36905	651
H_PCLA_4	HCT	21.85475	609
H_PCLA_5	HCT	24.76555	690
H_PCLA_6	HCT	19.32775	538

Table S5. Summary of Type III Analysis of Variance (ANOVA) of the linear mixed-effects model testing the effects of treatment, time, and their interaction on polyp activity.

Effect	Num DF	Den DF	F value	p-value
Treatment	2	15	5.74	0.014 *
Time	4	60	15.38	< 0.001 ***
Treatment × Time	8	60	1.47	0.186

Signif. codes: '***' p<0.001; '**' p<0.01; '*' p<0.05

Table S6. Tukey-adjusted pairwise comparisons of treatments for polyp activity.

Contrast	Estimate	SE	df	t-ratio	p-value
CTRL – HCT	-0.133	0.094	15	-1.420	0.356
CTRL – LCT	-0.317	0.094	15	-3.373	0.011
HCT – LCT	-0.183	0.094	15	-1.953	0.159

Signif. codes: '***' p<0.001; '**' p<0.01; '*' p<0.05

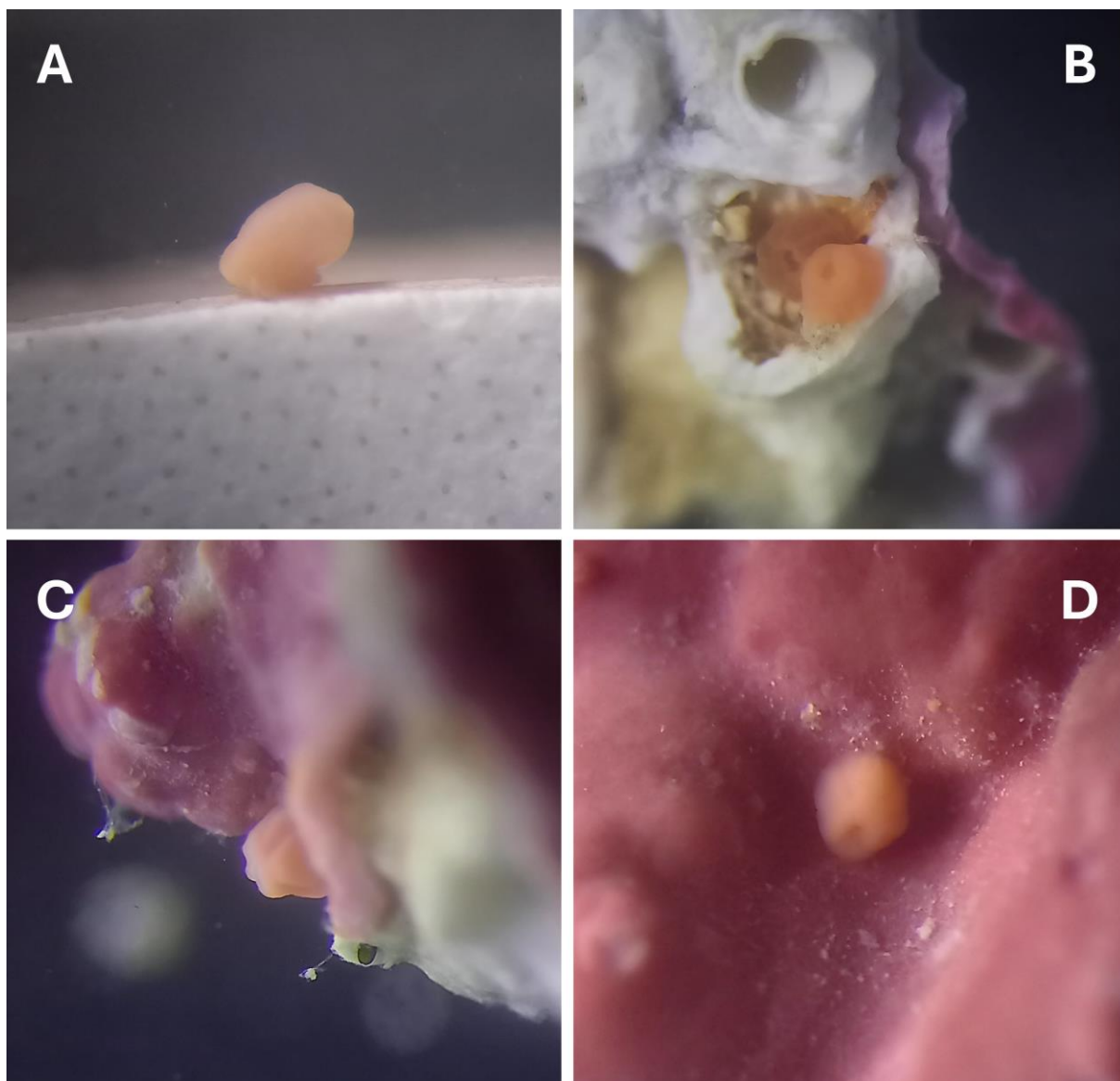


Figure S3.1. Settlers of *Eunicella singularis* observed throughout the experiment on (A) ceramic tile, (B) lower side of crustose coralline algae (CCA), (C) edge of CCA, and (D) upper CCA surface.

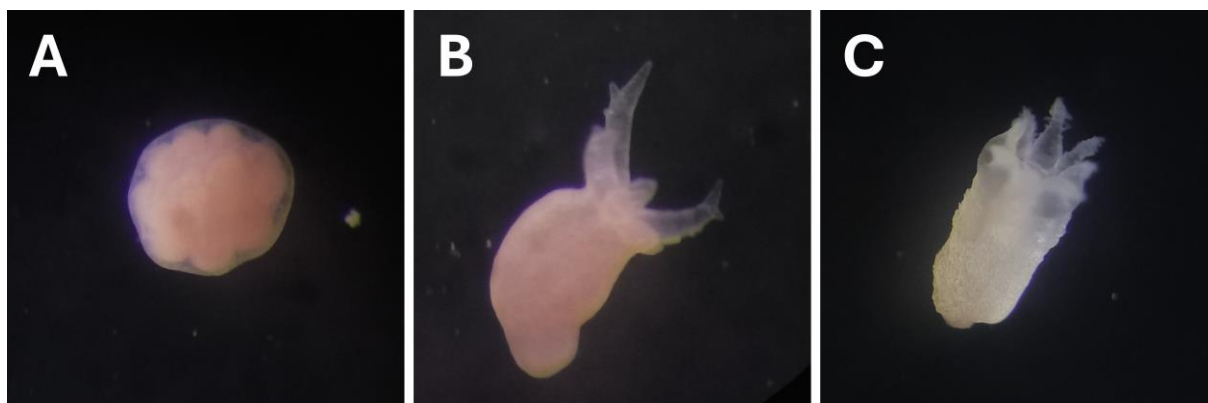


Figure S3.2. Metamorphosis of planulae occurring in the water column at different stages: (A) early phase of tentacle formation, (B) primary polyp under development, and (C) fully developed primary polyp.

Global considerations and Future Perspectives

This doctoral research covers several aspects of microplastic (MP) pollution in Octocorals, spanning from the development of standardized methodologies, the assessment of current field contamination patterns and the biological responses across life history stages. This multi-level approach provides a wide perspective of the MP research in Octocorals, contributing to fulfilling the existing knowledge gaps in the field.

Octocorals are key structural components of Mediterranean benthic ecosystems across a wide bathymetric gradient, from shallow waters to bathyal environments. Erect gorgonians contribute to the formation of three-dimensional habitats commonly referred to as coral forests or coral gardens (Bo et al., 2015, 2014b; Buhl-Mortensen and Mortensen, 2004; Rossi et al., 2017; Stone, 2006). These habitats enhance environmental heterogeneity, support biodiversity, modulate local hydrodynamic regimes, and facilitate benthic–pelagic coupling through suspension feeding activity (Buhl-Mortensen et al., 2010; De Vincenzis et al., 2026; Gomes-Pereira et al., 2017; Gómez-Gras et al., 2025; Ponti et al., 2018). Gorgonian forests provides secondary substrates and niches, forming a spawning, nursery and shelter ground for a rich associated fauna (Bo et al., 2014a; Cau et al., 2017; Cerrano et al., 2010; D’Onghia et al., 2017; Rueda et al., 2019). These organisms, and especially the habitats they create, are characterized by longevity, slow growth rates, and sporadic sexual reproduction. Considering the key ecological role, the biological traits, and the high sensitivity to anthropogenic stressors, most octocorals are recognized under several national and international conservation frameworks. These frameworks include IUCN Red List of Mediterranean Anthozoans, SPA/BD protocol of the Barcelona Convention, Bern Convention and Habitats Directive. In addition, the habitat created by octocorals are considered Vulnerable Marine Ecosystems in relation to several human activities (FAO, 2009).

Although some large-scale impacts of human activities on marine animal forests are evident and extensively documented, including the desolation caused by bottom

trawling (Bo et al., 2014a; Clark et al., 2016), the volume of debris impacting benthic ecosystems (Angiolillo and Fortibuoni, 2020; Consoli et al., 2019; Hanke et al., 2025; Pham et al., 2014) and mass mortality events due to marine heatwaves (Garrahou et al., 2022; Gómez-Gras et al., 2021; Viladrich et al., 2022), MP pollution has recently emerged as a concerning threat due to their small size, persistence in the environment and ubiquity.

However, MP research in Octocorals is in its infancy and substantial knowledge gaps span across multiple levels. First, methodological heterogeneity has hindered comparability among studies. Differences in digestion protocols, contamination control, particle identification techniques and size detection limits have generated uncertainty in quantifying MP uptake in anthozoans. The absence of standardized, and sometimes validated, approaches has likely led to both underestimation and overestimation of contamination levels. Second, environmental data on MP occurrence in habitat-forming octocorals is scarce. Information on particle abundance, polymer composition, size distribution and spatial variability within coral habitats is still fragmentary. While tropical reef-building scleractinians have received increasing attention, Mediterranean anthozoans remain largely under-investigated. Third, although experimental research on biological effects is comparatively more developed than other research lines, it remains far from comprehensive. Existing studies are predominantly focused on scleractinian corals, whereas octocorals remain markedly underrepresented. Narrowing the focus to Mediterranean species, available information is reduced to a single study (Corinaldesi et al., 2021). Moreover, early life stages have been largely neglected across Anthozoa as a whole.

To address these limitations, the present doctoral research was designed to address each of the identified gaps. As a first objective, the project aimed to assess *in situ* contamination in the deep-sea bamboo coral *Isidella elongata*. However, it became evident that no established or validated protocols were available for the isolation, identification and quantification of MPs in octocorals. This methodological lack represented a critical bottleneck, considering that without a reliable and standardized extraction protocol, any attempt to quantify MP occurrence would have been affected by analytical uncertainty. Consequently, the focus switched to critical selection,

systematic testing and validation of digestion procedures specifically applicable to octocoral samples. As a result, a two-step digestion protocol based on 8.25% sodium hypochlorite (NaClO) followed by 10% hydrochloric acid (HCl), previously applied to other marine species (Allen et al., 2017; Corinaldesi et al., 2021; Reichert et al., 2022) , was selected and subjected to a thorough validation process. The validation included recovery tests, as well as morphological and chemical alteration assessments on the three most abundant polymer types reported in marine environments and ingested by biota (polyethylene, polypropylene, and polystyrene; Andrady, 2017; De Sá et al., 2018; González-Ortegón et al., 2024; Palmieri et al., 2024; Rodrigues et al., 2019; Suaria et al., 2016) across a wide range of size classes (50–1000 µm), encompassing the size range realistically ingested by coral polyps. Compared with previous validation efforts, this approach addressed key methodological gaps in Anthozoa-related studies (Lim et al., 2022; Reichert et al., 2022; Santana et al., 2022; Savage et al., 2022), including the limited number of tested polymers, the use of unrealistic particle sizes, and the lack of quantitative and multi-parametric assessments.

The digestion protocol was then applied to environmental samples of *I. elongata* for laying the first brick for understanding the current state of MP contamination in Mediterranean corals. The analysis of MP occurrence in corals was integrated with the quantification of plastics in deep-sea fish species and macrolitter pollution from the surrounding seafloor. The study was conducted in a poorly explored deep-sea area off the Gulf of Squillace in the Ionian Sea (Western Mediterranean Sea). High seafloor litter density was recorded with plastic representing the dominant fraction, consistent with Mediterranean-wide patterns (Angiolillo et al., 2021; Canals et al., 2021; Cau et al., 2022; Consoli et al., 2020a, 2020c; Crocetta et al., 2020; Garofalo et al., 2020; Rizzo et al., 2022; Sciutteri et al., 2023). MP occurrence was documented in both demersal fish and *I. elongata* corals, documenting for the first time MP contamination in Mediterranean anthozoans. In addition, *I. elongata* was found partially covered in paint that can result in clogging and smothering of polyps (Bruemmer et al., 2023). Our findings further supported the evidence that deep-sea environments act as long-term sinks for ML (Galgani et al., 1996; Hanke et al., 2025; Martin et al., 2022; Peng et al., 2020), with potential implications for mobile fauna and habitat-forming species of high conservation

value. The study also contributed to the understanding of anthropogenic impacts to CWC habitats. Considering the ecological role of Mediterranean CWC habitats as biodiversity hotspots and their recognized vulnerability under international and national frameworks, the observed levels of macro- and micro-litter raise additional concerns about cumulative stressors affecting these systems.

The last step of this project involved the evaluation of biological responses to MP exposure under controlled conditions, targeting two Mediterranean mesophotic octocorals and two life history stages. In particular, planula larvae of *Eunicella singularis* and adult colonies of *Paramuricea clavata* were exposed to different MP concentration encompassing current high concentrations (200 particles L⁻¹) and predictive concentrations in future scenarios (2000 particles L⁻¹). The short-term exposure experiment conducted on adult colonies of *P. clavata* did not reveal MP ingestion within 24 hours. This result suggested limited interaction with MPs, probably driven by low polyp activity during the experiment. Further investigations are needed to understand responses of *P. clavata* to MPs. In contrast to the adult experiment, the mid-term larval exposure assay revealed measurable responses to MPs ranging from 10 to 1000 µm. Larval mortality in *E. singularis* increased over time across all treatments and significantly higher mortality was observed under the highest concentration tested after extended exposure (day 16 onwards). Our finding suggested that extreme contamination events or future scenarios may threaten reproductive success with concerning implications on gorgonian population dynamics. Settlement rate on crustose coralline algae was not significantly affected by MP exposure, yet ingestion was documented in newly settled individuals. Additionally, mucus production was frequently observed during the experiment in both exposure treatments, especially at higher MP concentrations. Overall, this study contributed to the very limited knowledge of the biological responses of Mediterranean corals to MP exposure.

The studies conducted under this research project directly address the requirements of Descriptor 10 of the Marine Strategy Framework Directive (MSFD, 2008/56/EC), which aims to achieve Good Environmental Status by reducing marine litter (ML) and its impact on marine ecosystems. Specifically, this study contributes to improving monitoring

efficiency under Descriptor 10 by applying a standardized digestion protocol, successfully tested on *I. elongata*, with potential extension to other anthozoans. This methodological approach supports the implementation of harmonized and comparable monitoring strategies, in line with the needs identified for strengthening MSFD assessments (Galgani et al., 2024). Moreover, the ecosystem-based framework adopted in the Gulf of Squillace promotes a multispecies approach across different environmental compartments, further aligning with the integrative perspective required by the MSFD. Importantly, beyond methodological advancement, this research integrates the monitoring component with a preliminary assessment of the potential impacts of marine litter on the investigated organisms. By coupling detection protocols with impact evaluation, the study enhances the ecological relevance of Descriptor 10 implementation and contributes to a more comprehensive understanding of litter-related pressures in deep-sea ecosystems.

One main feeling comes up what has just been discussed: although some scientific questions and needs have been addressed as far as possible for this project, just as many questions and needs have arisen. The overall results of this thesis are a drop in the ocean of knowledge that needs to be fulfilled for understanding the problem of plastic pollution. The effective applicability of the digestion protocol to other species, the amount of plastic pollution affecting coral forests at basin scale, the biological responses of octocorals to different types, concentrations and conditions of MPs throughout the life cycle, are just some of the issues requiring an answer. Further investigations will follow the studies conducted during this project to shed light on this topic.

However, gaining awareness about the issue must necessarily be accompanied by action plans to reduce waste disposal. Some authors have modeled future trends to estimate plastic waste in the upcoming decades. Lebreton and Andrady (2019) predicted that, keeping the current rates of waste generation, thus the “business-as-usual” scenario, the mismanaged plastic waste (MPW) amount could nearly triple by 2060, reaching 155–265 million metric tons annually. Furthermore, rapidly developing regions, particularly Africa and Asia, are projected to contribute disproportionately to future MPW generation due to demographic growth and expanding economies not yet supported by adequate waste management infrastructure. Under the business-as-usual scenario, the annual rate

of micro- and macroplastic entering global aquatic systems from land may increase 2.6-fold from 2016 to 2040 (Lau et al., 2020). Both models (Lau et al., 2020; Lebreton and Andrady, 2019) suggested that the implementation of currently feasible actions, including reduction in plastic consumption, increased reuse, improved collection, expanded recycling, and safer disposal systems, could reduce annual plastic pollution in the oceans. Lebreton and Andrady (2019) identified two optimistic scenarios where MPW could decrease up to 14-94 million metric tons year⁻¹, reaching levels lower than those of 2015. Lau et al. (2020) reported a potential reduction in plastic pollution for 2040 of 40% from 2016 rates. Nevertheless, even in the most ambitious intervention scenario, approximately 710 million metric tons of plastic waste would cumulatively enter aquatic and terrestrial ecosystems over the modeled period (Lau et al., 2020). Koelmans et al. (2017) calculated that approximately 99.8% of the plastic that had entered the ocean since 1950 had already been redistributed below the ocean surface layer by 2016, with an additional ~9.4 million tons settling annually. This model also suggests that surface plastic abundance could respond relatively rapidly to reductions in inputs, potentially declining within a few years under a zero-emission scenario, while deeper environments may experience long-term persistence of plastics due to slower degradation processes. Combining ecotoxicological thresholds with modeled environmental concentrations, Everaert et al. (2020) estimated that the unacceptable MP risk in surface waters may increase to 0.52% by 2050 and 1.62% by 2100 worst-case discharge scenarios. The Mediterranean Sea was identified as a current and future hotspot of MP-related ecological risk, because of its restricted circulation, high coastal population density, and intense maritime activities.

Overall, future predictions for plastic pollution highlighted the difficulty of reversing decades of linear production and consumption models. The achievement of most optimistic scenarios requires an immediate and drastic intervention. To date, despite increasing awareness and regulatory efforts that include restrictions on single-use plastics and amendments to international agreements governing plastic waste trade, a systemic and globally coordinated strategy for achieving near-zero plastic leakage has yet to be fully implemented (Lau et al., 2020). Downstream mitigation measures such as large-scale clean-up operations or ocean-based removal technologies, while increasingly promoted, address only a fraction of the problem. Another measure involves plastic

recycling which remains limited by product design complexity and polymer heterogeneity. As emphasized by Macheca et al. (2024), the idea of recycling 100% of plastics is currently unrealistic due to multi-material compositions and economic constraints. Similarly, biodegradable plastics, while promising in controlled settings, require specific environmental conditions for degradation and are often unsuitable substitutes for conventional polymers in many applications (Laface et al., 2023; Macheca et al., 2024).

Consequently, the most effective mitigation strategies in the long term are likely to involve upstream interventions. The waste management hierarchy prioritizes reduction and reuse as the first and most impactful steps. Limiting plastic production growth, redesigning products for durability and circularity, implementing extended producer responsibility schemes, and investing in waste management infrastructure in developing economies are considered essential components of any realistic long-term solution (Lau et al., 2020; Macheca et al., 2024; Napper and Thompson, 2020; UNEP, 2021). In this context, the transition toward a genuinely circular economy requires prioritizing reduction and reuse over recycling. Economic instruments, such as plastic taxes and deposit-return schemes, have also been proposed as effective tools to alter consumption patterns and discourage single-use applications (UNEP, 2021). However, because plastic pollution is tightly coupled with economic development and consumption patterns (Lebreton and Andrady, 2019), meaningful mitigation will require integrated global cooperation rather than isolated regional efforts. At the European level, several policy initiatives have already been established towards plastic pollution reduction within the broader framework of the European Green Deal and the Circular Economy Action Plan. These include regulatory measures aimed at banning most single-use plastics and primary microplastics in care products, pushing on recycling, and promoting sustainable product design, finally aiming to reduce plastic leakage into the environment and transition toward more circular production and consumption systems.

In short, substantial commitments to improving the global plastic system are required from businesses, governments, and the international community to solve the

ecological, social, and economic problems of plastic pollution and achieve near-zero input of plastics into the environment (Lau et al., 2020).

List of publications, posters and talks

Journal Articles:

Giova A., Pedà C., Lucido G., Pica D., Longo F., Canese S., Greco S., Romeo T., Spanò N. (submitted to Marine Pollution Bulletin). *Tracing Marine Litter and Microplastics in the Deep Ionian Sea: messages from Seabed, Deep-Sea Fish and Cold-Water Corals*.

De Vincenzis L., Battaglia P., Palummo V., Milisenda G., Canese S., Cardone F., Pica D., Salvati E., Stenico F., Consoli P., Costa V., Giova A., Greco S., Romeo T. (2025). No place to hide: Assessing habitat shifts of *Benthocometes robustus* from Cold-Water Corals to marine litter in the Mediterranean Sea. *Mar. Environ. Res.*, 10773. <https://doi.org/10.1016/j.marenvres.2025.107773>.

Giova A., Pedà C., Pica D., Greco S., Romeo T., Spanò N. (2025). Identifying a suitable digestion protocol for microplastic extraction from *Isidella elongata* (Esper, 1788): new insights for cold-water corals. *J. Hazard. Mater. Plast.*, 11:1387144. <https://doi.org/10.1016/j.hazmp.2025.100009>.

Posters:

Giova A., Pedà C., Pica D., Firmamento R., Laface F., Molino C., Scozzafava S., Greco S., Romeo T., Spanò N. (2025). Identifying a suitable digestion protocol for microplastic extraction from *Isidella elongata*. 58th European Marine Biology Symposium (EMBS). Bodo, Norway.

Talks:

“Deep-sea Lab: On board activities, ROV exploration, Video analysis” (2026). Stazione Zoologica Anton Dohrn, Messina, Italy.

“Microplastic Investigation Lab: Pollution in environment and biota” (2026). Stazione Zoologica Anton Dohrn, Messina, Italy.

“Practical Session for ROV Surveys in Deep-Sea Research: Methodologies, tools and know-how” (2025). 2nd International Summer School on the Study of Extreme Environments. Stazione Zoologica Anton Dohrn, Messina, Italy.

“Diving Deep: The Hidden World” (2023). PhD days “Science and Engineering for the Environment and the Sustainability”. University of Campania Luigi Vanvitelli. Aversa (CE), Italy.

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