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Census and impact of microplastics in the deep-sea biosphere

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1. INTRODUCTION

1.1. Deep-sea ecosystems and anthropogenic impacts

The deep sea (waters and sediments of the ocean interior beneath 200 m depth) is the largest biome of the world representing more than 65% of the Earth's surface and more than 95% of the global biosphere (Herring, 2002). It is characterized by a mean depth of 4.2 km (maximum depth greater than 11,000 m), a volume of >1.19 billion km^3 and a seabed area of ~434,386,264 km^2 (Danovaro et al. 2014; Costello et al. 2010). Despite its extension, deep sea still results the less investigated environment of the Planet since only 5% of the deep oceans have been explored in detail so far and less than 0.001% has been sampled and described in detail in terms of biodiversity (Danovaro et al. 2017).

The deep sea is characterized by extreme and apparently inhospitable conditions for life, such as darkness, high pressure (with an average hydrostatic pressure of 400 atm), extreme ranges of temperature with variations from -2°C to +450°C (in Arctic Ocean and hydrothermal vents, respectively) and limited and sporadic food supply (with only few exceptions, Danovaro et al. 2014, Danovaro et al. 2017, Sweetman et al. 2017). However, due to their stability and extension, deep-sea environments host a huge biodiversity, with high levels of species richness, both for macroscopic (animal species) and microscopic (meiofauna, prokaryotes, fungi, viruses) organisms (Danovaro et al. 2010, Ramirez Llodra et al. 2011, Corinaldesi 2015, Danovaro et al. 2015). The deep-sea life has been documented everywhere with active metabolic pathways and microbial activities from -2°C to >150°C, in sediments at 10,000 m depth and even down to 1,000 m below the seafloor (Todo et al. 2005). So far, ca. 500.000 animal species have been discovered in deep-sea ecosystems (Danovaro et al. 2010) and recent studies have highlighted that about 1.5 million species are expected to be discovered in the deep sea (Costello et al. 2017).

In the last decades, the deep-sea exploration has resulted in the discovery of a plethora of previously completely unknown habitats and ecosystems (including hydrothermal vents, cold seeps, frozen methane hydrates, cold-water coral reefs, ridges, seamounts, canyons, oxygen minimum zones, abyssal plains and hadal trenches) along with thousands of species with specific adaptations inhabiting these remote environments (Gage and Tyler 1991; Sibuet et al. 1998; Snelgrove 1999; Tyler et al. 2003; Helly and Levin 2004; Baker et al. 2004; Weaver et al. 2004; Niemann et al. 2006; Roberts et al. 2006; Teske and Sørensen 2008; De Leo et al.

2010; Jamieson et al. 2010; Rex and Etter 2010; Danovaro et al. 2014). Peculiar habitats may be characterized by the presence of ecosystem engineers, such as sponges, xenophyophore fields and deep-sea coral banks (Ramirez Llodra et al. 2010), which promote extremely high levels of biodiversity (Bongiorni et al. 2010).

Information about anthropogenic impacts and consequent responses of biological communities is still limited. The deep sea, being the most remote environment on Earth, has been for long time very far from the public consciousness, thus the initial exploitation and anthropogenic impact did not have important social implications (Ramirez Llodra et al. 2013). In this context, indeed, the deep sea was used as a favourable site for waste disposal and as a source of mineral and biological wealth over which there was no national jurisdiction, especially where land options were not politically and "ethically" convenient (Ramirez Llodra et al. 2011). Nevertheless, in the last decades the decrease in the amount of terrestrial and coastal resources has driven increased interest in the exploration and exploitation of deep-sea goods and services, favoured also by a rapid technological development (Thiel 2003; Van den Hove et al. 2007; Benn et al. 2010). As a result, human activities and impacts in the deep sea have been increasingly documented since the beginning of this century (Van Dover et al. 2014). Different and concurrent anthropogenic pressures such as oil spill, bottom trawling, drilling of hydrocarbon wells and mining (Gage et al. 2005; Continental Shelf Associates, Inc 2006; Smith et al. 2008), can negatively affect deep-sea habitats and fauna, acting also cumulatively or synergistically (Ramirez Llodra et al. 2011).

Several studies have reviewed different impacts of human activity on the deep sea (Thiel 2003; Smith et al. 2008b; Ahnert et al. 2000; Glover et al. 2003), however, to date their effects on life and ecosystems are scarcely documented in comparison with those from land areas and shallow ecosystems, making environmental management in the deep sea difficult but necessary (Ramirez-Llodra et al. 2011).

Litter and plastic pollution are among the anthropogenic impacts acting on deep-sea ecosystems, which could jeopardize deep-sea pelagic and benthic communities in the near future (Ramirez Llodra et al. 2011), however information on the severity degree of this contamination and impact on deep-sea communities is still only fragmentary.

Litter in the sea is continuing to accumulate, through illegal disposal from ships, lost/discarded fishing gears, as well as material advected from the coast and rivers discharge (Browne 2015). It has been estimated that approximately 6.4 million tonnes per year of litter are dumped into

the oceans, part of which sinks down to bathyal and abyssal depths (UNEP 2009). Although the type of litter found in the world's oceans is highly diverse, plastics are by far the most abundant material recorded (Derraik et al. 2002; Barnes et al.2009; Sheavly et al. 2007, Yarsley et al. 1945) and are expected accumulate and impact deep-sea life and ecosystems in the coming years.

1.2 Plastics in the oceans

Plastics comprise a class of synthetic organic polymers typically derived from fossil fuel feedstocks composed of long chain-like molecules with a high average molecular weight (American Chemistry Council 2015, Worm et al. 2017). Plastics global production has increased exponentially since 1950, with 311 million metric tons produced in 2014 (Plastic Europe 2015, Law et al. 2017). The increasing production and use of durable synthetic materials has led to a gradual, but significant accumulation of litter also in the marine environment (Barnes et al. 2009; Kühn et al. 2015). Indeed, owing to slow rates of degradation in the oceans (much slower than on the land), plastic can persist for years, decades or even centuries (Andrady 2011, Andrady 2015a). As a result, plastic pollution in the oceans, firstly reported in the 1970, is nowadays ubiquitous and persistent in natural systems, thus representing an environmental concern addressed by scientists in environmental engineering, ecology, toxicology, marine biology and oceanography.

In the ocean, plastics is characterized by a wide range of size, from microplastics (< 5 mm in diameter) to macroplastics (> 5 mm in diameter) (Pham et al. 2014; Cózar et al. 2014). In addition, different types of plastic polymers such as polyethylene (PE), polyamide (PA), polypropylene (PP), polyester (PES), polystyrene (PS), polyurethane (PU), polyvinylchloride (PVC), polyethylene terephthalate (PET), poly methylacrylate (PMMA), acrylic, polyoximethylene (POM), polyvinyl alcohol (PVA) and alkyd have been identified (Browne et al. 2010; Hidalgo-Ruz et al. 2012; Song et al. 2014; Enders et al. 2015).

In the last decade, scientific studies on the source, pathways, transformation, impacts of plastics on marine life and environment have rapidly increased (Andrady et al. 2011; Cole et al. 2011; Wright et al. 2013b, Browne et al. 2015).

Plastic debris has been indeed investigated across all the globe, including the open ocean (Law et al. 2010; Cózar et al. 2014; Eriksen et al. 2014), polar ice caps (Obbard et al. 2014), deep-

sea sediments (Van Cauwenberghe et al. 2013), and beaches of remote mid oceanic islands (Do Sul et al. 2013). However, most of the studies have been conducted on coastal ecosystems, especially on sandy beaches (95% of studies conducted in coastal systems, Browne et al. 2015), rather than in deep sea also due to sampling difficulties, inaccessibility and costs associated with the research in deep environments (Corinaldesi 2015).

In marine environment, plastics can be transported by a variety of processes, depending on their size, shape and density. Positively buoyant items will tend to accumulate near the sea surface, where they are transported by winds and superficial currents, whereas negatively buoyant items will sink along the water column to the underlying sediments (Van Cauwenberghe et al. 2013).

Most of the plastic polymers have a lower density than seawater (e.g. PE and PP), which make them passively floating at the ocean's surface where they are subjected to mixing within the surface boundary layer (Kukulka et al. 2012). In the oceans, several high-accumulation zones including the subtropical gyres (5.25 trillion floating plastic particles, Eriksen et al. 2014), the North Pacific and North Atlantic regions have been identified (Moore et al. 2001; Cózar et al. 2017). Model simulations have suggested that the debris accumulating in these convergence zones can remain even for several years (Pichel et al. 2007; Law et al. 2010; Howell et al. 2012). Polymers that are denser than seawater (e.g. PET and PVC) tend to settle to seafloor (Engler, 2012). When these fragments sink to the seabed, it becomes the ultimate repository for the plastics (including those that were initially buoyant; Barnes et al., 2009). Besides density, other factors can affect the behaviour of plastic in the ocean including their partial crystallinity, oxidation resistance, biodegradability, the presence of residual monomers and additives (Andrady 2017). As an example, the plastic exposure to UV radiation triggers photo-oxidative degradation, reducing average molecular weight, weakening the material until shear or tensile stresses can cause fracturing and fragmentation (Andrady 2015b). Recently, Kowalski et al. (2016) investigated how sinking velocities of plastics with diverse densities change in function of water salinity and effects of weathering processes (e.g. UV irradiation, mechanical stress).

Another important aspect of this "pollutant" is that plastic debris also acts as a sink for toxic chemicals. Plastic sorbs persistent, bioaccumulative, and toxic substances (PBTs), such as polychlorinated biphenyls (PCBs) and dioxins, from the water or sediment, that might be bioavailable and ingested by organisms, thus representing a toxic hazard to them (Engler 2012). Several studies reported that plastic can be ingested by marine organisms (Kuhn et al. 2015;

Gall et al. 2015) and laboratory studies revealed the transfer of microplastics from zooplankton to mysid shrimp (Setälä et al., 2014) and from mussels to crabs (Farrell and Nelson, 2013).

In particular, due to their small size, microplastics fall within the optimal pray range of many marine animals (Wright et al. 2013). Therefore, ingestion has the potential to have far-reaching implications at the population level, posing a threat to the conservation of the affected species and to the functioning of marine ecosystems (Reid et al. 2013).

Microplastics (MPs) can be ingested by different marine organisms from filter, suspension and detritus feeders to invertebrates, fish, turtles and species used for human consumption (Lusher 2015). In addition, plastics can contain a mix of polymers, residual monomers and chemical additives that can reach animal tissues and alter metabolic and reproductive processes (Koelmans et al. 2016; Galloway 2015). Chemicals (such as PCB, IPA) present in seawater can be absorbed by plastic particles (Ogata et al. 2009, Hirai et al. 2011, Rochman et al. 2013), with a rate and extent of accumulation depending on the plastic polymer type, physical and chemical properties (especially those resulting from weathering and biofilm formation), particle area and chemical exposure throughout the particle's drift history (Rochman 2015). Recently, a study highlighted that the surface of nanomaterials in marine environment can rapidly be coated with proteins, other biomolecules and microorganisms, resulting in the formation of an "ecocorona", which strongly influence the interaction between microplastics with organisms and ultimately their persistence, bioavailability and toxicity (Smita et al. 2012, Galloway et al. 2017).

These biological processes increase the relative size, chemical signature and density of the plastic particles, thus influencing the sedimentation coefficient in the water column, and potentially resulting in larger export to the seafloor (Galloway et al. 2017). It has also been shown that, being able to adsorb bioactive molecules, plastics can influence the ingestion rates and animal behaviour (Nasser et al. 2016; Savoca et al. 2016). The "ecocorona" concept could help to explain the high rates of ingestion of microplastics reported in several marine organisms across multiple trophic levels (Wright et al. 2013), by enhancing their attractiveness as a food item (Galloway et al. 2017).

Since microplastics have been found in shallow-water ecosystems, deposit- and detritus-feeders can be susceptible to their exposure as well as fauna such as crustacea and shellfish (Murray and Cowie, 2011; Rochman et al. 2015b; Wright et al. 2013; Green et al. 2016). Effects of microplastics on marine organisms have been explored primarily through laboratory experiments and ingestion of plastic fragments has been observed in a lot of marine animals

such as amphipods (detritivores), barnacles (filter feeders), lugworms (deposit feeders) (Thompson et al. 2004), decapod crustaceans (Murray and Cowie, 2011), bivalves, echinoderms, bryozoans (Ward and Shumway, 2004; Browne et al. 2008), copepods (Cole et al. 2013). Previous investigations indeed have shown experimentally that deposit feeders such as polychaetes readily consume plastics, which cause detrimental effects on their health and functionality (Wright et al. 2013; Green et al. 2016). Other information indicates that plastics affect reproduction in oysters (Sussarellu et al. 2016). Sea cucumbers have been also shown to selectively swallow plastics over sand particles, suggesting that holothurians could voluntarily ingest plastics (Graham and Thompson, 2009). Scleractinian corals are not exempted from this impact, indeed they can ingest different types of plastics wrapped into mesenterial tissue within the coral gut cavity (Allen et al. 2017, Hall et al. 2015).

Other investigations have revealed that marine organisms collected from the shallow water environments contain microplastics. Previous studies indeed revealed that 33% of gooseneck barnacles collected contained 1-30 items of microplastics (Goldstein and Goodwin, 2013) and that bivalves reached even a value of 100 microplastic particles per animal (Mathalon and Hill, 2014; Vandermeersch et al., 2015; Railo, 2017). Desforges et al. (2015) reported an encounter rate between microplastics and zooplankton of one microplastic particle every 34 copepods and one particle every 17 euphausiids.

Recently, the highest international organizations have addressed the problem of plastic persistence in natural environments (Law 2017; UNEP 2018; G7 2015; G7 2018). Indeed, during the G7 in 2015 marine plastic debris has been categorized as marine pollutant of international environmental, economic, public and political concern, posing threats to marine life, industry, and food security and then in 2018, G7 members commit to undertake international and/or domestic initiatives, individually or jointly, in support of a common objective to promote innovation in addressing marine plastic pollution by managing plastics more sustainably throughout the whole life-cycle. To minimize the negative impacts, several legislative/operational instruments have been developed at international, regional and national levels to prevent, reduce and manage marine litter. These instruments comprise conventions, agreements, regulations, strategies, action plans, programs and guidelines. They contain specific management measures that are either compulsory or voluntary. In this regard, the increased interest of policy makers is evidenced by the formation of focus groups, which have sprung up in many agencies. For example, programs and working groups focusing on plastic debris have been created at the National Oceanic and Atmospheric Administration (NOAA)

and the US Environmental Protection Agency (USEPA) in the United States, the Ministry of the Environment in Canada, the European Commission, the Northwest Pacific Action Plan in Asia, the Department of Environmental Affairs in South Africa and globally at the United Nations Environment Programme (UNEP) (Rochman et al. 2016). One of the most important EU initiatives regarding marine debris is the Marine Strategy Framework Directive (MSFD, EU) that in 2008, included microplastics as pollutants to be quantified. This directive establishes a framework, within which member states shall take necessary measures to achieve or maintain a good environmental status (GES) in the marine environments by 2020. Microplastics are considered specifically in the Descriptor 10 of the MSFD that establishes their baseline quantities, properties, and potential impacts of (Gago et al. 2016). However, roles and tools for controlling the impact of (micro)plastics in deep-sea ecosystems are still far from to be take into consideration, since information on their amount, polymer typology and impacts on marine life in such remote environment is still limited.

1.3 Potential environmental and biological vectors of (micro)plastics to deep-sea floor

The transport/sinking of (micro)plastics to deep-sea ecosystems and their persistence might be favoured by a number of physical-chemical and biological processes, which would increase the relevance of these contaminants in the deep sea, being they constantly introduced into marine environment. In addition, the relatively stable conditions of deep-sea ecosystems, the lack of light and very low temperatures (down to -2°C) typically found in these extreme ecosystems could delay degradation rates, thus favouring the persistence of this pollution source.

At global scale, movement, spatial distribution and accumulation of plastics have predominantly been studied by hydrodynamic models, tracks of ocean drifters and scientific campaigns throughout the world's oceans (Lebreton et al. 2012; Maximenko et al. 2012; Cózar et al. 2014; Eriksen et al. 2014).

On the ocean seafloor, the geographic distribution of debris is strongly influenced by hydrodynamics, geomorphology, prevailing winds and human factors (e.g., coastal density population, fishing grounds and shipping routes, Galgani et al. 1996; Pham et al. 2014). Previous studies, also reported that the accumulation of plastics in specific geographic areas of the deep-sea floor could change seasonally, and according to the presence of fishing areas and oceanic current convergence zones (Galgani et al. 1995). However, this information is difficult

to be interpreted because when the plastic debris has been settled in deep seafloor plastics and its residence times remain unknown.

Previous surveys conducted in benthic deep-sea ecosystems shown the presence of large plastic fragments, by dredging or by remotely operated underwater vehicle (ROV) cameras. Despite plastics can be found everywhere in deep-sea oceans, submarine canyons, deep-sea trenches and depressions, acting as preferential conduits for accumulation of organic/inorganic material, could represent the main plastic repositories (Galgani et al. 2000; Wei et al. 2012; Ramirez-Llodra et al. 2013; Pham et al. 2014; Mordecai et al. 2011; Canals et al. 2013). Plastic debris such as bottles, bags and abandoned fishing nets have been indeed observed (Watters et al. 2010; Richards and Beger, 2011; Tubau et al. 2015; Corcoran, 2015) along submarine canyons, often concentrated due to the topography or currents (Schlining et al. 2013; Tubau et al., 2015). Pham et al. (2014) hypothesized that the relative scarcity of macroplastics on shelves areas was due to their transport by currents into deep waters, particularly via submarine canyons. In addition, being less disturbed by bottom trawling than continental shelf sediments, submarine canyons may provide a good record of the history of plastics influx during the Anthropocene (Rockstrom et al. 2009; Lewis and Maslin 2015; Zalasiewicz et al. 2017).

The transfer of microplastics to these deep-sea habitats has been associated with dense shelf water cascading (DSWC), severe coastal storms, offshore convection and saline subduction (Talley 2002; Ivanov et al. 2004; Canals et al. 2006; Sanchez-Vidal et al. 2012; Durrieu de Madron et al. 2013; Stabholz et al. 2013; Fernandez-Arcaya et al. 2017). In particular, DSWC (responsible for the ventilation of deep waters, ocean circulation, ecosystem functioning and biogeochemical cycles (Aubry et al. 2018; Sanchez-Vidal et al. 2008) are involved in the transport of sedimentary material, pigments, amino acids, lignin and organochlorine compounds (Canals et al., 2006; Palanques et al., 2006, 2009; Puig et al., 2008; Pasqual et al., 2011; Salvadó et al., 2012; Salvado et al., 2013), leading to hypothesize a similar transport mechanism also for microplastics and their absorbed contaminants such as polycyclicaromatic hydrocarbons (PAHs) (Salvadó et al. 2017).

Besides environmental processes, there is evidence that also biological vectors can favour the sinking of (micro)plastics in deep sea such as the colonization of these materials by microbes and/or by macro-organisms with the formation of microbial biofilms (Zettler et al. 2013) and macro-biofouling, respectively (Fazey and Ryan, 2016). The first evidence of plastic

colonization by bacteria and diatoms goes back to 1972 (Carpenter & Smith, 1972). However, the investigation of biological colonization of plastics has been abandoned for three decades to be reconsidered only recently (Harrison et al., 2011). There is evidence that the increasing amount of plastic littered into the sea may provide a new substratum for a wide range of organisms (Oberbeckmann et al. 2014, Pauli et al. 2017). Previous studies carried out in the North Pacific Gyre reported that microbial assemblages including bacteria, diatoms, dinoflagellates, coccolithophores, and radiolarians can colonize plastics and that bacteria seem to grow with a preference for foam polystyrene polymers rather than polyethylene or polypropylene while diatoms seem to be influenced by roughness and plastic concentration. However, environmental factors influencing the distribution were unclear (Carson et al. 2013). Other information suggests that microbial communities colonizing different polymer types are influenced by seasons and geographical location (Oberbeckmann et al. 2014). Rich eukaryotic and bacterial assemblages have been found on polyethylene and polypropylene microplastics collected from the North Atlantic Ocean (Zettler et al., (2013), North Pacific (Bryant et al., 2016) and the North Sea (De Tender et al., (2015). The communities associated with these microplastics were distinct from those in the surrounding surface water and sediment, indicating that plastics provide a novel habitat for microorganisms and that species sorting occurs, particularly, during the early colonization stages (Harrison et al., 2014; Zhang et al., 2014; Bryant, 2017). Bacteria and other microorganisms settle on the surface within a few hours, building a biofilm which provides cues for the settlement of larger organisms such as algal spores and the larvae of benthic invertebrates (Zardus et al. 2008; Briand et al. 2012). In the fouling community, a total of 21 families were identified belonging to the phyla Foraminifera, Porifera, Cnidaria, Mollusca, Annelida, Nematoda and Bryozoa. At the family level, Foraminifera were the most diverse phylum (Pauli et al. 2017). Gündoğdu et al. (2017) found 17 different fouling species belonging to 6 phyla (Annelida, Arthropoda, Bryozoa, Chordata, Cnidaria, Mollusca), 7 classes and 11 orders were discovered on plastics. *Spirobranchus triqueter*, *Hydroides sp*. and *Neopycnodonte cochlear* were the most abundant species. All these studies revealed that plastic substrates can contain a very high diversity of life just like natural substrates (Gündoğdu et al. 2017).

Microbial films and/or biofouling, which rapidly develop on submerged plastics (depending also the plastics's size) are able to change their physicochemical properties, as surface hydrophobicity and buoyancy thus promoting their sinking (Lobelle and Cunliffe, 2011; Kooi et al. 2017). Another process described as responsible of plastic sinking to deep sea is marine

snow (Van Cauwenberghe et al. 2013, Cole et al. 2016; Porter et al. 2018). Marine snow is ubiquitous in the oceans and is represented by microscopic to centimetre sized aggregations of small particles, mainly detritus, prokaryotes and phytoplankton, and generally indicates high concentrations of aggregates of organic matter, which may initially originate from the extracellular polymeric substances (EPS) released by decaying organisms and/or other organic matter in marine environments (Tansel 2018). When microplastics are englobed into marine snow, being these colonized by specific microbial communities, microbial abundance and diversity of marine snow can be increased (Galloway et al. 2017). Furthermore, microbial communities associated to the marine snow may reach concentrations 10,000 times higher than in surrounding water and this enhances the release of "quorum sensors" (i.e. signalling molecules released by bacteria in response to cell abundance that control many metabolic processes including the hydrolysis of complex organic materials; Jatt et al. 2015). This could suggest that such quorum sensing regulators could favour the formation of microbial communities capable of degrading hydrocarbon polymers, thus accelerating the degradation and mineralization of the incorporated plastics (Galloway et al. 2017).

1.4 Census of (micro)plastics in deep-sea floor

Marine pollution due to microplastics in deep-sea sectors has been investigated only in recent years and few quantitative estimates have been made in Arctic, Southern, Atlantic, Pacific, Indian Oceans and Mediterranean Sea.

One of the first evidences documented by using ROV videos showed the presence of plastic debris in Ionian Sea (Anastasopoulou et al. 2013). Other information indicated major litter sources in deep-sea ecosystems such as in the Fram Strait (79°N, Atlantic Ocean) at 2450 m depth (Bergmann and Klages 2012) and in the Charlie-Gibbs Fracture Zone of the Mid-Atlantic Ridge at 2500 m depth (Pham et al. 2014). Further investigations conducted on Arctic deep-sea observatory HAUSGARTEN confirmed the presence of a large reservoir of plastic debris on the seafloor (Bergmann et al. 2017).

Microplastic fragments (<5mm size) were found in all Oceanic sectors investigated while macroplastics (>5mm) such as pill packages, soles, squid hoods, long line, nylon cords, fishing nets and macrofibers only in Kuril-Kamchatka trench area (NW Pacific Ocean) to an average depth of 5,000 meters (Fischer et al. 2015). The fact that in some area macroplastics were not found/reported could be due to different mesh size used during the sample processing. In addition, different approaches have also been used to sort microplastics from deep-sea samples,

thus hampering the dimensional comparison of microplastic items found. So far, the size of the microplastics found in deep sea, ranges between 11 μm and 3 mm with the most abundant class between 11 and 25 μm (Bergmann et al. 2017). Microplastics in deep-sea ecosystems were reported as fibers (Woodall et al. 2014; Fischer et al. 2015) and cracked pieces (Van Cauwenberghe et al. 2013; Fischer et al. 2015; Bergmann et al. 2017). Defining the polymeric composition of microplastics in the deep sea is very difficult due to different approaches utilized in the available studies (stereomicroscopy vs FT-IR Spectroscopy, different hypersaline solution), which not always report the typology of plastic polymers. However, despite information is sparse the polymeric composition of plastic fibers in deep sea is mostly made of polyester followed by other polymers, including polyamides, acetate and acrylic. These plastic polymers are used in a wide range of domestic and industrial applications, including packaging, textiles and electronics (Woodall et al.2014). Instead, the composition of the microplastic fragments seems to be mainly represented by polytetrafluoroethylene (PTFE), polyethylene chlorinated, polypropylene, nitrile rubber, polyethylene, polyester, polyisoprene chlorinated, polyvinylchloride, polyurethanes/varnish/lacquer, polycaprolactone, and polycarbonate (Bergmann et al. 2017). The highest microplastics' concentration was found Arctic deep-sea with a mean abundance of 4,356 items per kg^{-1} of sediment (Bergmann et al. 2017), but to make microplastic estimates present in the deep-sea ecosystems it's equally difficult to access because of the different units of measures used in the different studies. Van Cauwenberghe et al. (2013) reported the lowest value of microplastic contamination with a mean abundance of 0.5 items/25 cm^2 from the deep Atlantic Ocean and Mediterranean Sea. The particles with a mean range size of 112.4 μm (±34.70), found in this study had distinct colour and the presence of these pigments interfered with the measurements of the plastic type, and as a result the spectra obtained were these for the pigments, not those for the plastic type. These are organic pigments with a non-natural origin, indicating the presence of anthropogenic particles in these samples. Additionally, these pigments are most commonly used in the plastics industry (Lewis, 2004), which strengthens the assumption that these particles are microplastics. Fewer MPs were also detected in the sediments from the deep NE Atlantic, Mediterranean and SW Indian Ocean with a mean abundance of 13.4 items/50 ml and a range size between 2-3 mm, although only fibers were considered in different deep-sea habitat (open slope, canyon, basin and seamount; Woodall et al. 2014). Unfortunately, due to the lack of replicated samples, differences in the methodologies utilized and in size ranges considered, it is difficult make a proper comparison among samples make conclusions on patterns observed in this deep-sea habitat. Also, one of the deepest habitat of the world such as trench areas, in particular Kuril-Kamchatka Trench (NW Pacific), were

affected by microplastic with a mean abundance of 748.20 items/m² with a higher percentage composed of fibers (75%) and a smaller percentage of cracked pieces (25%) (Fischer et al. 2015).

The limited number of available studies, the relatively wide variety of adopted sampling techniques, extraction and counting methods of microplastics do not allow a proper comparison of the data, making still difficult to obtain a global estimate of plastic contamination in deep-sea habitats. However, these studies are the proof that deep-sea habitats such as abyssal plains, open slopes, canyons, basins, seamounts and trenches can act as sinks for micro and macroplastics.

1.5 Impact of (micro)plastics on deep-sea organisms

If the number of available studies on the contamination level from plastics in deep sea is limited, analyses dealing with the impact extent on deep sea organisms are even more scarce.

Despite the technological advancements made in the last decades, the remoteness and technological challenges posed by deep-sea ecosystems hamper the development of an intense and adequate scientific research (Corinaldesi 2015), including investigations to document the impact of plastic pollution on deep-sea organisms. Indeed, such explorations are more difficult and challenging hundreds to thousands of metres beneath the sea surface than in shallow water (Taylor et al. 2016).

Until now, few studies documented the presence of plastics (macro and microplastics) in deep-sea organisms. Macroplastic debris including hard pieces of plastic (56%), fragments of plastic bags (22%), nylon fishing lines and ropes (19%) or textile fibers (3%) were found in the guts of deep-sea fish (Anastasopoulou et al. 2013) where this kind of litter represented the 86.5% of ingested debris followed by metal and wood. Ingested debris categories are related to the feeding behaviour of the examined fish: nektobenthic opportunistic feeder swallowed all debris categories, pelagic and bathypelagic feeders ingested only plastic bags, whereas the hard plastics ingestion may be related to bathybenthic feeding fishes habits. Instead, Taylor et al. (2016) provided the first evidence that also microfibers (acrylic, polypropylene, viscose and polyester) may be ingested and internalised by individuals of three major phyla (Cnidaria, Echinodermata and Arthropoda), with different feeding mechanisms. If this general observation (albeit based on very few samples) of filter-feeders having lower microplastic loads, holds true more widely, the implication is that deposit-feeding organisms may be more vulnerable to microplastic ingestion than suspension feeders. Subsequently also other authors reported the

ingestion of microplastic fibers from deep-sea Echinodermata (*Ophiomusium lymani, Hymenaster pellucidus*) and mollusca (*Colus jeffreysianus*) (Courtene-Jones et al. 2017). They showed that *Ophiomusium lymani* ingested the greatest number of polymer types (acrylic is the dominant polymer) in comparison to the other species whereas *Hymenaster pellucidus* contained the greatest overall abundance (1.582 ± 0.448 of microplastics g^{-1} ww tissue). There were significant differences between the number of microplastics ingested among species due to the differences (in terms of number of individuals sampled and with microplastics internalised, weights and feeding mode for each invertebrate species) between *O. lymani* and *H. pellucidus* and between *H. pellucidus* and *C. jeffreysianus*. The quantities of microplastics enumerated in deep-sea fauna are on the same order as those reported in wild coastal species from a range of taxa (Courtene-Jones et al. 2017). Recently, other authors investigated microplastic ingestion in deep-sea benthic fauna. Fang et al. (2018) reported for the first time, microplastic contamination in Artic benthic fauna in starfishes, shrimps, crabs, brittle stars, whelks and bivalves. The mean abundances of microplastics uptake by the benthos were lower values than those found in other regions worldwide; the highest value appeared at the northernmost site, implying that the sea ice and the cold currents represent possible transport vectors. The starfish predator *Asterias rubens* ingested the maximum quantities of MPs, suggesting that the trophic transfer of microplastics through benthic food webs may play a critical role and also in this study fibers constituted the major type of microplastics type. Instead, Carreras-Colom et al. 2018 focused their study on economically and ecologically key species in the Mediterranean Sea, the shrimp *Aristeus antennatus*. microplastics ingestion was confirmed in a wide spatial and depth (630–1870 m) range, suggesting their great dispersion. The benthophagous diet and close relationship with the sea bottom of *A. antennatus* might enhance microplastics exposure and ultimately lead to accidental ingestion. Detailed analysis of shrimps' diet revealed that individuals with microplastics had a higher presence of endobenthic prey. Microplastic fibers are probably retained for long periods due to stomach's morphology, but no negative effects on shrimp's biological conditions were observed.

Since shallow benthic assemblages such as scleractinian corals can ingest microplastics, some authors suggested that such a behaviour might also occur in deep-sea corals (Hall et al. 2015).

Recent studies also highlighted the presence of persistent organic pollutants (POPs) in fauna living in two of the deepest hadal trenches (Mariana and Kermadec Trench), hypothesizing the potential of plastic in POPs transport to these remote zones (Jamieson et al. 2017).

Despite so far information on the impact of plastics on deep sea organisms is limited, plastic load reaching our oceans is increasing, and a large portion of plastics will likely end up on or buried in the seafloor, the potential impact on deep-marine organisms is expected be pervasive (Jambeck et al. 2015).

1.6 Gaps in the assessment of (micro)plastic impact in deep-sea ecosystems

Deep-sea ecosystems have all "the requirements" to be vulnerable to (micro)plastic impact, including environmental factors potentially favouring its preservation. However, available data to draw punctual conclusions are still too much limited and still suffer from several methodological discrepancies.

One of the main problems in the assessment of the abundance and impact of microplastics rises by the improper use of the term "microplastics" and its considered size range, which is wide and undefined (<5 mm, despite it should take into account only the fragments with a size < 1000 μm). This is a crucial issue because studies carried out so far, are focused on different size ranges (e.g., 300-500 μm vs. 2-3 mm; see Table 2 as an example), which are not comparable among them (e.g., μm fragments vs. mm fragments). Moreover, typically, fragments under 20 μm in size (which represent "real microplastic") are ignored because these particles are too small to be detected under classical microscope (Filella 2015).

These discrepancies can severely affect the reliability of general estimates of microplastic abundances and their potential effects not only on deep-sea ecosystems but also in overall marine environments. Typically, sample analysis to extract (micro)plastics includes chemical digestion of organic matter and/or density separation, in which the sample is mixed with seawater (for water samples) or a high-density salt solution (for sediment samples) in which some or all of the common plastics are expected to float (Loder and Gerdts 2015). Microplastics from sediment samples are separated by using density flotation and filtration (Coppock et al. 2017; Browne et al. 2011; Fendall et al. 2009; Desforges et al. 2014; Claessens et al. 2011; Harrison et al. 2012; Nor et al. 2014; Vianello et al. 2013; Van Cauwenberghe et al. 2013-b; Lee et al. 2014; Martins et al. 2011; Nuelle et al. 2014; Fries et al. 2013; Collignon et al. 2012; Claessens et al. 2013; Dekiff et al. 2014; Sul et al. 2013; Hidalgo-Ruz et al. 2012). Indeed, since the specific density of plastic particles varies considerably from the density of sediments (2.65 gcm^{-3}), microplastics can be easily separated. Generally, to facilitate this process a salt-saturated solution such as NaCl, $ZnCl_2$, NaI, $CaCl_2$ and $Li_2O_{13}W_4$ are added to sediments (Nuelle et al. 2014; JRC 2011; Coppock et al. 2017; Imhof et al. 2012; Liebezeit et al. 2012;

Bergmann et al. 2015; Van Cauwenberghe et al. 2013; Claessens et al. 2013; Dekiff et al. 2014; Stolte et al. 2015; Masura et al. 2015). For now, there are no universally recognized protocols for plastic sampling from different marine compartments and matrices (e.g., seawater, sediments, sea ice) and or specific procedures for deep-sea samples. Several methods are available to assess plastic abundances in water column (Lee et al. 2014; Cole et al. 2011; Desforges et al. 2014) and sediments (Harrison et al. 2012; Nor et al. 2014; Vianello et al. 2013; Van Cauwenberghe et al. 2013-b); approximately each study adopts its own procedure.

One of the gaps in the assessment of microplastic abundance and impact in deep-sea sediments is the lack of data dealing with the extraction efficiencies of the protocols used to extract the fragments. Consequently, the use of different protocols applied to matrices also from similar ecosystems can result in data difficult to compare. However, generally, protocols used for deep-sea sediments (typically mud) have not been optimized for this specific matrix but for coastal sediments although their chemical-physical characteristics are very different.

The analytical techniques used to assess the surface morphology, the composition and the concentration of microplastics are different (Eriksen et al. 2013; Vianello et al. 2013; Van Cauwenberghe et al. 2013; Fries et al. 2013; Browne et al. 2011; Claessens et al. 2011; Harrison et al. 2012; Nor et al. 2014; Martins et al. 2011; Dekiff et al. 2014; Browne et al. 2010; Frias et al. 2010; Nuelle et al. 2014). Fourier transform infrared spectroscopy (FT-IR) and Raman spectroscopy are among the most commonly used methods for the identification of polymeric composition, although pyrolysis-gas chromatography with mass spectrometry has also been used to identify polymer types and organic additives (Fries et al. 2013). However, these expensive instruments, which require specialized personnel, are not always available in all laboratories therefore some studies on microplastics in deep-sea ecosystems have been based on the use of classical microscopy (stereo- or light microscopes; Fischer et al. 2015; Anastasopoulou et al. 2013). Such a method may introduce several biases, especially due to the low detection power of the method (generally up to 20 μm), non-expert operators (e.g., misidentification of plastic polymers confused with other particles) and different operators producing data (Dekiff et al. 2014).

Another problem dealing with microplastic analysis is avoiding external contamination. The quantification of the abundance of microfibres in the marine realm can easily be confounded by contamination of samples during laboratory processing; also, because some studies do not take into consideration this kind of contamination with the risk of overestimating. As a result,

some studies on microplastic pollution (Dekiff et al., 2014; Goldstein and Goodwin, 2013; Van Cauwenberghe et al., 2013) have intentionally excluded this fraction in their analyses. Woodall et al. (2015) developed a protocol, adapted from the field of forensic fibre examination, that reduces fibre abundance by 90% and enables the quick screening of fibre populations. Critical steps to avoid contamination consists of using items that are not plastic, wearing natural fibre clothes during the analysis, using clean equipment, filtering all processing fluids used during the analyses, predisposing procedural blank and processing the samples under chemical and laminar flow hood. Adoption of consistent protocols is of vital importance in order to avoid plastic contamination and overestimation and compare data between the growing number of studies in this field of study.

Several methods for the extraction of plastics, have been published, mainly to investigate the ingested or sticked microplastics by organisms (Vandermeersch et al. 2015; Cole et al. 2014; Avio et al. 2015). Most methods are based on visual inspection of stomach and gut under microscope, acid/base digestion and enzymatic digestion followed by any floatation and filtration with salt saturated solution to make the separation between organic matter and plastic easier (Mathalon and Hill 2014; Dantas et al.2012; Foekema et al. 2013; Devriese et al. 2015). Separating plastics from other organic materials becomes increasingly difficult as particle size decreases, therefore also in this case there are difficulties associated with identification of < 20 μm microplastics, which generally are neglected. In particular deep-sea organisms were dissected and examined under a binocular microscope to identify whether or not they had ingested or internalised microplastics. However, studies applied to deep-sea organisms are still limited also due to the logistic difficulties associated with their adequate sampling, especially in quantitative terms, or to conduct in situ/ex situ manipulative experiments. Experimental field and manipulative tests should also be carried out to assess the impact of microplastics on deep-sea communities and functioning. In this regard, only one paper (Cole et al. 2016) showed, through laboratory experiments, that microplastics can alter the structural integrity, density, and sinking rates of faecal pellets egested by marine zooplankton thus decreasing the sinking rate of faecal pellets that is fundamental for the export of particulate organic matter to deeper waters.

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2. OBJECTIVES

The general objectives of my PhD thesis are:

1. **Assessing the quantitative relevance of microplastics (MPs), typology, size (2-1000 µm) and distribution patterns in different benthic deep-sea ecosystems** through optimization of procedures for the extraction, identification and quantification of microplastics from deep-sea sediment samples, investigating in particular microplastics' distribution in submarine canyons of the Mediterranea Sea.

2. **Evaluating the impact of microplastics on benthic deep-sea organisms** with laboratory experiments to assess the impact of microplastics on benthic metazoans and analysis of microplastics ingested by deep-sea nematodes.

3. **Exploring deep-sea microbial assemblages colonizing and potentially degrading microplastics** studying taxonomic composition of microbial biofilm on different plastic items in the deep-sea floor and in the surrounding sediments.

3. OPTIMIZATION OF THE PROCEDURE FOR EXTRACTION AND QUANTIFICATION OF MICROPLASTICS FROM DEEP-SEA SEDIMENTS

Introduction

In the last years, several studies have focused their attention on the analysis of the presence and abundance of microplastics in different marine environments (Setälä et al. 2018; Auta et al. 2017; Van Cauwenberghe et al. 2015). Such studies have been conducted by using different methods for the separation and identification of microplastics from different environmental matrices (Hidalgo-Ruz et al. 2012; Shim et al. 2017, Rocha-Santos et al. 2015; Löder and Gerdts 2015). Only recently, studies have been applied to benthic compartment (Thompson et al. 2004). Available methods concerning the extraction of (micro)plastics from marine sediments, are depending on the size of target plastic fragments and, typically include visual separation through stero- or light microscopy (for fragments > 500 μm; Fisher et al. 2015), flotation separation (Frias et al., 2010; Van Cauwenbeghe et al. 2015), and/or acid (Claessens et al., 2013; Desforges et al., 2014), alkaline (Tanaka and Takada, 2016; Zhao et al., 2016), oxidative and/or enzyme digestion (Cole et al., 2014; Courtene-Jones et al., 2017). However, there are no universally recognized protocols to extract microplastics from marine sediments.

Separation of microplastics from sediment samples has been carried out by density flotation and filtration (Coppock et al. 2017; Browne et al. 2011; Fendall et al. 2009; Desforges et al. 2014; Claessens et al. 2011; Harrison et al. 2012; Nor et al. 2014; Vianello et al. 2013; Van Cauwenberghe et al. 2013-b; Martins et al. 2011; Nuelle et al. 2014; Fries et al. 2013; Collignon et al. 2012; Claessens et al. 2013; Dekiff et al. 2014; Sul et al. 2013; Hidalgo-Ruz et al. 2012). This approach is based on the separation of the microplastics from the sedimentary matrix exploiting their different density. since sediments have densities ranging from 1.8 – 2.20 g cm^{-3} (for mud and sand, respectively; Hamilton et al. 1976) whereas microplastics from 0.9 to 2.3 g cm^{-3} (polyethylene and polyester respectively; Hidalgo-Ruz et al. 2012).

To separate plastic fragments from sediments a salt-saturated solution, such as NaCl, $ZnCl_2$, NaI, $CaCl_2$; $Li_2O_{13}W_4$, has been also used (Nuelle et al. 2014; JRC 2011; Coppock et al. 2017; Imhof et al. 2012; Liebezeit et al. 2012; Bergmann et al. 2017; Van Cauwenberghe et al. 2013; Claessens et al. 2013; Dekiff et al. 2014; Stolte et al. 2015; Masura et al. 2015). Other studies were based on separation of microplastics (> 63 μm and < 63 μm; 4.75, 2.8 and 1 mm) from

sediments using sieves of different mesh size, which allow the classification into different size categories (Vianello et al. 2013; McDrmin and McMullen et al. 2004).

The most used methods for identifying and characterizing (micro)plastics in benthic ecosystems are based on visual detection through stereomicroscopy and Fourier Transform Infrared Spectroscopy (FTIR, Thompson et al. 2004; Coppock et al. 2017; Zhao et al. 2018). Visual detection (conducted with stero- or light microscopes) has been especially used in the pioneering studies (Dekiff et al. 2014, references therein). However, this approach is not very reliable because may introduce several types of errors, including observer biases, misidentification of particles, or under-detection of particles that are too small to be detected (Dekiff et al. 2014, Filella 2015).

Most of these studies, however, did not investigate the recovery rate of microplastics from the sediments, despite the digestion methods used might have the potential to damage the structure or physical characteristics of plastic polymers (Cole et al., 2014; Quinn et al., 2017) and the extraction of such polymers from this complex matrix could result in underestimation of its actual abundance (Claessens et al. 2013).

Fourier transform infrared spectroscopy (FT-IR) has been adopted in more than 60% of papers available in literature (Miller et al. 2017), being reliable also for the identification and chemical characterization of fragments down to a 20 μ size. Other studies were based on the use of Raman spectroscopy (Van Cauwenberghe et al., 2013), which however is specific for the identification of inorganic compounds. In addition, pyrolysis-gas chromatography with mass spectrometry has also been used to identify polymer types and organic additives (Fries et al. 2013) but this technique is destructive (Rocha-Santos et al. 2015) and has the disadvantage that particles have to be manually placed into the pyrolysis tube. Since only particles of a certain minimum size can be manipulated manually this results in a lower size limitation of particles that can be analyzed. Furthermore, the technique allows only for the analysis of one particle per run and is thus not suitable for processing large sample quantities, which are collected during sampling campaigns or routine monitoring programs (Löder and Gerdts 2015).

To date, the most recommended method is the Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), due to the simplicity of analysis and diagnostic spectral information that it provides, but studies based on this method are still very few possibly (Browne et al. 2011; Harrison et al. 2012; Nor et al. 2014).

Despite the huge number of protocols and techniques used for the extraction of microplastics from sediments (both shallow-water and deep), most of these lack of fundamental details (e.g., the time frames required to process samples, and to separate and identify microplastics from environmental samples, the amount of sediment processed), making it difficult to determine the most (cost)effective and suitable methods to be applied to sediment samples.

Studies conducted in benthic deep-sea ecosystems are very limited and the procedures used in this framework are generally those developed for shallow-water sediments (Van Cauwenberghe et al. 2013; Woodal et al. 2014; Bergmann et al. 2017) despite the deep-sea sediments have different characteristics from the coastal ones. Generally, deep-sea sediments have a different mineralogical composition, granulometry, organic matter, porosity and water content, which can influence the extraction procedures of the plastics (Hamilton et al. 1976).

Most of the available studies have used high salt saturated solutions for extracting microplastics from deep-sea sediments such as NaI, NaCl, or Ludox and Raman or FT-IR spectroscopy to identify and characterize the fragments (Van Cauwenberghe et al. 2013; Woodall et al. 2014). A specific device (i.e., Munich Plastic Sediment Separator (MPSS)) and $ZnCl_2$ as density separation coupled with FT-IR has been also utilized to extract and characterize microplastics from deep-sea arctic sediments (Bergmann et al. 2017), and only one investigation was conducted through visual identification of abyssal trenches (Fisher et al. 2015).

However, all the available studies applied to the sedimentary matrix, did not investigate the extraction efficiency of the extraction procedures of microplastics. In addition, they were generally limited to fragments not larger than 50 μm, due to the detection limit and technical features of the instruments used to characterize the polymers. However, microplastics smaller than 50 μm could have even a stronger impact of the larger fragments entering the trophic webs and affecting processes and mechanisms at the cellular level (Galloway et al. 2017). So far, only one study on microplastics in deep-sea benthic ecosystems, investigating target fragments > 500 μm and < 500 μm by using ATR-FTIR, revealed that the dominant fraction was represented by 11-25 μm fragments with a negligible fraction of items larger than 500 μm (Bergmann et al. 2017).

In the present study, we present a specific protocol for benthic deep-sea ecosystems for the extraction and quantification of microplastics with a size range between 2 and 1000 μm. Here, we determined the extraction efficiency of the microplastics from deep-sea sediments and assessed the specific recovery yield for each plastic polymer.

Materials and Methods

Working conditions and precautions during microplastics analyses

All laboratory ware used was glass or stainless steel, thoroughly rinsed with Milli-Q (filtered to 0.2 μm nitrocellulose filters) before use. All filtration steps were performed in a laminar flow cabinet and all chemicals (e.g., $ZnCl_2$, H_2O_2 and Milli-Q) were filtered through 0.2 μm nitrocellulose filters (Sartori) to remove particulate contaminants before usage. Cotton laboratory coats were generally worn to reduce contamination from synthetic textiles. To account for possible contaminations due to processing of the samples, a procedural blank was run during all steps of the protocol, dinking a filter with Milli-Q, $ZnCl_2$ and H_2O_2 to detect the possible contaminations in working solutions and laboratory airborne.

Extracting microplastics from sediments: method validation

The validation of the microplastic extraction protocol from the deep sediment was carried out using five different plastic polymers (Fig. 1) with different density: polyethylene (PE, 0.89-0.95 g cm^{-3}), polypropylene (PP, 0.85-0.92 g cm^{-3}), polystyrene (PS, 1.04-1.09 g cm^{-3}), polyethylene terephthalate (PET, 1,38 g cm^{-3}) and polyvinylchloride (PVC, 1.16-1.41 g cm^{-3}). These polymers were selected because they account for 80% of plastic production in Europe (Plastic Europe, 2016) and are the most frequently reported plastics in marine environments (Browne et al. 2010; Karapanagioti et al. 2011; Vianello et al. 2013). Microplastics considered had a range size comprised between 2 and 1000 μm.

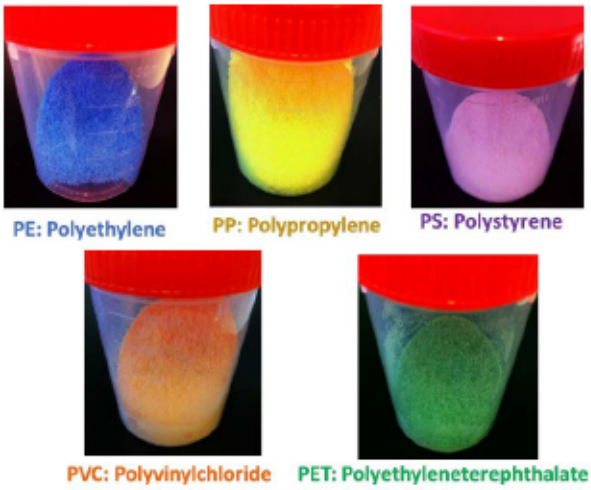


Figure 1. Plastic polymers tested for efficiency of the extraction protocol

Before evaluating the effectiveness of microplastics' extraction method from deep-sea sediment, the quantity of the polymers (PE, PP, PS, PET and PVC) to be added for each size range (500-1000 µm, 200-500 µm, 20-200 µm, 2-20 µm) was defined in order to obtain 10 mg of mixed microplastics with an equal contribution from each polymer (2 mg each).

The protocol was developed according to the methods developed by Claessens et al. 2013, Imhof et al. 2012 and Vianello et al. 2013 with some modifications. Deep-sea sediments were collected in the Gulf of Lion (Western Mediterranean Sea) at 1800 m depth. The deep-sea mud, as typically found in abyssal/hadal ecosystems (Rohal et al. 2018), was dried in oven at 50°C for 4-5 days in order to remove organic matter and plastics already present in the samples which could bias the analysis.

To assess if the partitioning of the sediment in aliquots increased the efficiency of recovery of microplastics we compared two different approaches: in the first case the procedure was applied to 250 g of sediment (bulk sediment, 1) while in the second case to five aliquots of 50 g of sediment each (sediment aliquots, 2).

Ten mg of the microplastic mixture were added to 2 replicates of dry sediment (250 g each). After mixing of the sediment and microplastics, the bulk sediment (2) was divided in 5 equal aliquots.

A solution of $ZnCl_2$ (1.6 - 1.7 kg/L) was added to the bulk sediment (1) e to different aliquots of sediments (2) which were stirred for 12 h. To increase the detachment of the microplastic from the sediment particles the samples were subjected to ultrasound treatment for three times of 1 minute each, with 30 seconds of manual shaking after each cycle (frequency: 40 KHz;

Bransonic Branson 3510). Then, sediment samples were maintained at room temperature for 3 days so that the denser particles (sediment) could settle down. Subsequently, since the plastic particles tend to float, supernatants were transferred into centrifuge tubes (17.000 xg for five minutes; Avanti J-30I) to allow the separation of the sediment particles from microplastics. The supernatants were filtered onto 20 µm filers (SEFAR NITEX) with vacuum filtration apparatus, and the filtrate was then subsequently collected and filtered onto 2 µm filters (Whatman, Nucleopore). To eliminate any organic matter trace and facilitate microplastic counting the filters were subjected to three cycles of treatment (5 min each) and subsequent vacuum filtration with 10 mL of H_2O_2 (30% ; Carlo Erba). Filters were maintained at room temperature under laminar flux hood.

Quantification and identification of microplastics from sediments

Quantification of microplastics in the range size between 20-1000 µm was obtained both for the bulk sediment (1) and sediment aliquots (2) observing the 20 µm filter under stereomicroscope at a magnification of 50× (Zeiss, Stemi 2000), counting 100 different optical fields. Then, microplastic extracted were checked using a Perkin Elmer FTIR Spectrometer Spectrum GX1 interfaced with a Perkin–Elmer Autoimage microscope and equipped with a photoconductive 0.25 mm Hg-Cd-Te (MCT) array detector, operating at liquid nitrogen temperature and covering the entire IR spectral range from 4000 to 700 cm^{-1}. The Spectrum Autoimage 5.1.0 software package (Perkin Elmer, Waltham, MA, USA) was used.

Particles collected on 2 and 20 µm filters were counted and identified both for the approached based on the bulk sediment (1) and sediment aliquots (2) by using Scanning Electron Microscopy (Zeiss SUPRA 40, magnification of 7.26 KX). Sixty optical fields were observed in three different areas of each filter (20 optical fields for each area). Since muddy sediments were very fine, the procedure based on the density separation solution was not able to completely remove them, resulting in a strong background due to sediment particles. Therefore, to speed up the analysis, an X-ray map (Bruker Quantax Z200) was made to distinguish the sediment grains (mapping silicon mineral) from the plastic particles in each filter area considered. Once the X-ray map was obtained, the non-silicon particles were subjected to the microanalysis to assess the presence of carbon suggesting presence of the plastic compounds (Fig. 2).

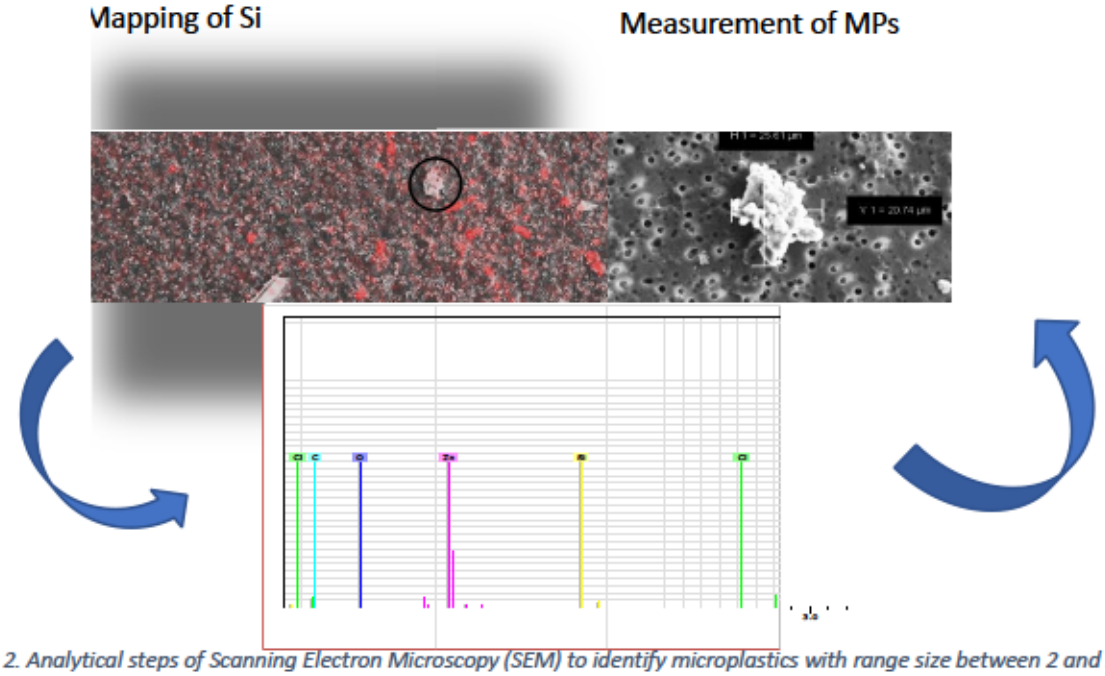


Figure 2. Analytical steps of Scanning Electron Microscopy (SEM) to identify microplastics with range size between 2 and 20 µm

Results and Discussion

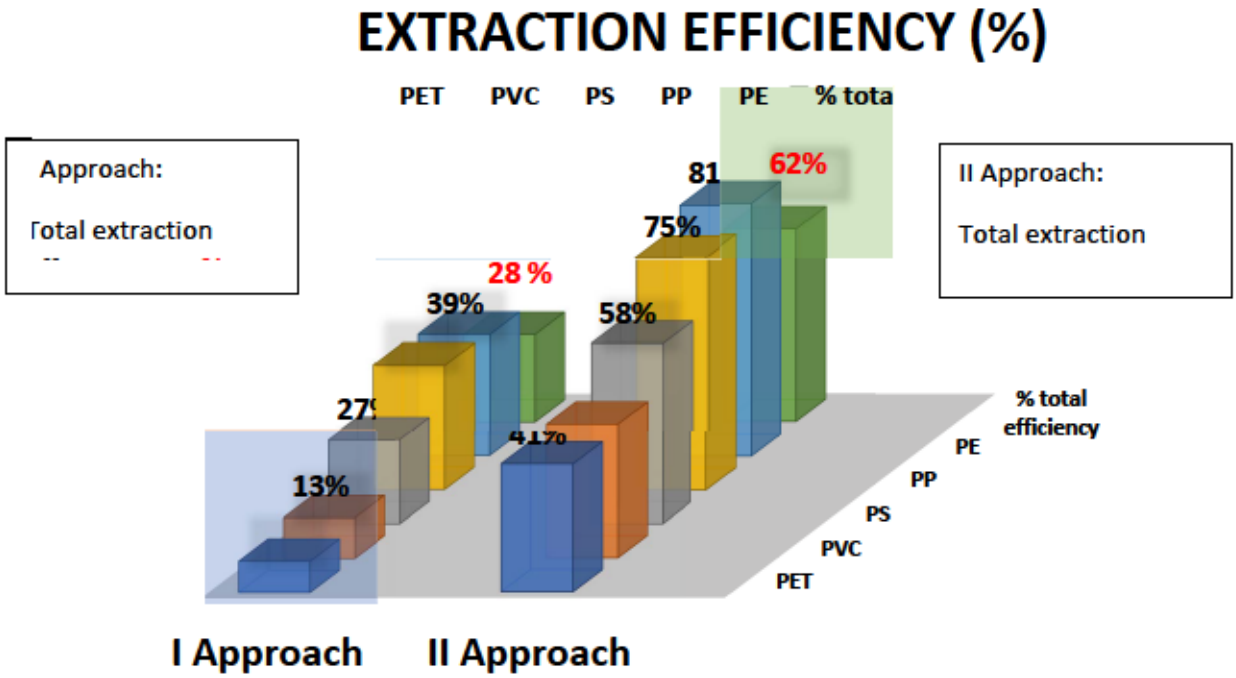
The procedure presented here was developed for extracting microplastics with a range size 2-1000 µm from deep-sea sediments according to protocols (Imohf et al. 2014, Vianello et al. 2014, Claessens et al. 2013) previously adopted for shallow-water sediments with some specific modifications. This optimized procedure is essentially based on the use of a density separation solution, Zn Cl_2, useful to recover also high-density polymers (potentially present in benthic ecosystems) and H_2O_2 to eliminate organic matter according to Imohf et al. (2014). Moreover, we also included an ultracentrifuge step for a more efficient separation of microplastics from sediments. This step was already reported also in the procedure described by Van Cauwenberghe et al. (2013) (who in turn used a protocol developed for shallow-water sediments by Classens et al. 2013) applied to abyssal sediments but we increased the times gravity (from 3500 xg to 17,000 xg) since muddy deep-sea sediments are finer than sandy ones generally found in coastal ecosystems.

Moreover, the procedure here presented was applied to bulk sediment (250 gr) and to five aliquots of 50 gr each to assess if the partitioning of the sediment in aliquots increased the efficiency of recovery of 5 microplastic polymers previously added to our samples.

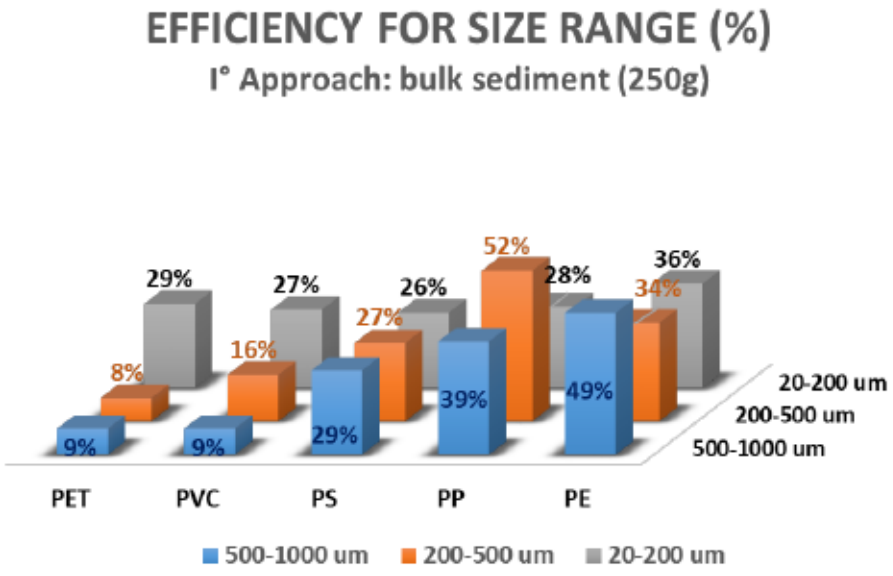
The results of the analysis revealed that the efficiency of extraction of microplastics with a size of 20-1000 µm from deep-sea sediments changed depending on the amount of sediment used. In the approach based on sediment aliquots (2) on average the recovery efficiency of

microplastics was double than in the approach based on the bulk sediment (62% vs. 28 %) indicating that the partitioning of the sediment into aliquots increases the extraction efficiency of microplastics (Fig. 3). We also observed that that efficiency changed depending on the polymer type (Fig. 3) and size range considered (Fig. 4). Indeed, in both approaches, PE, PP and PS resulted to be polymers with higher extraction efficiency than PET and PVC suggesting that a different recovery efficiency from deep-sea sediments could alter the actual estimates of these polymers in natural environments.

By using the approach (2) this efficiency even doubled for PE, PP, PS, tripled for PVC and quadrupled for PET indicating that this approach strongly improve the extraction efficiency especially for some polymers. A similar pattern was also observed when we considered the size range of microplastics. We found that the approach (2) was more efficient than the approach (1) for the extraction of microplastic belonging to all size ranges considered (20-200 µm, 200-500 µm, 500-1000 µm) but in particular for the smaller ones (Fig. 4). These results indicate that previous procedures applied to high quantity of bulk deep-sea sediments (and potentially also to shallow-water sediments) could result in biased estimates of microplastic abundance,



percentage of total extraction efficiency of method and plastic polymers



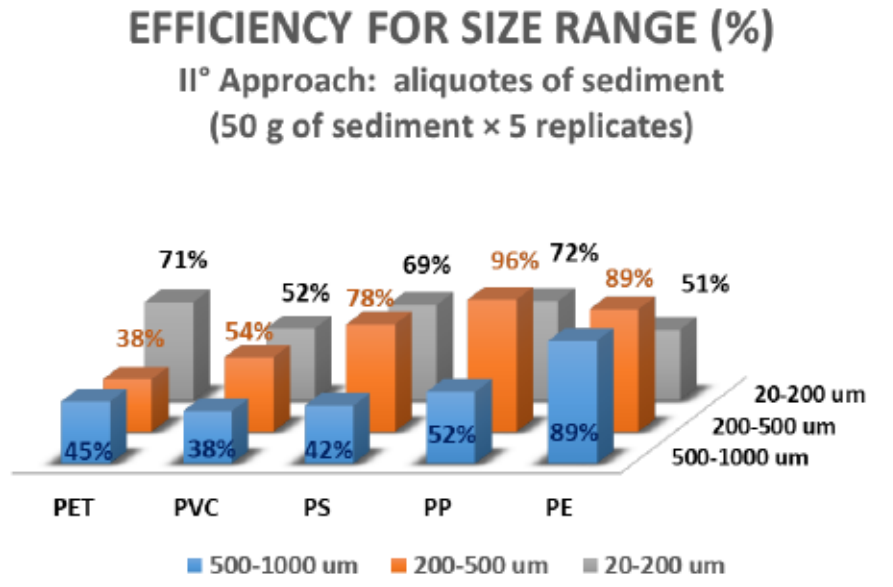


Figure 4. Recovery rates of microplastics (2-1000 µm) comparing two different approaches. Efficiency is expressed in term of percentage of size range extract for each polymer

The smallest fraction (2-20 µm) of microplastics used for determining efficiency extraction from deep-sea sediments was evaluated by Scanning Electron Microscopy since the detection limit of FTIR is 10 µm (Löder and Gerdts 2015). Therefore, we utilized for the first time this procedure to determine abundance and identify microplastics in sediment samples using the approach (2). Results obtained demonstrated that the efficiency was 60% (that is very similar to the percentage of efficiency for the fraction between 20 and 1000 µm). In this case we could not estimate the efficiency in terms of polymers extract because the microanalysis is able to identify the chemical elements (in forms of atoms) present in an object, not the chemical compound. For this reason, we could only provide an estimate of the total efficiency of the method and not for each polymer as in the case of the 20-1000 µm fraction of microplastics.

Overall, we can conclude that extraction efficiency with size range between 2 and 1000 µm for microplastics from deep-sea sediments is, on average, about 60%. This yield is even higher than the value reported for shallow-water sediments for the same microplastics size obtained by using the classic density set-up (ca. 40%, Imhof et al. 2012) despite the recovery efficiency obtained through the Munich Plastic Sediment Separator (Imhof et al. 2012) was reported to be up to 95%. However, this apparatus has high costs and, despite it has been applied to investigations in benthic abyssal ecosystems its recovery efficiency of microplastics has not been yet tested for deep-sea sediments.

Concluding the protocol optimized here for the extraction of microplastics from deep-sea sediments appears to be a good compromise for its very good recovery efficiency and relatively low costs. However, the analysis of the chemical composition and identification of microplastics, especially for smallest fragments (less than 20µm), is still laborious, time consuming and with high costs associated with laboratory instrumentations (FTIR and SEM).

Conclusions

The protocol presented here is suitable for the extraction of the most relevant plastic polymers found in marine environment especially those with a higher density, such as PVC and PET that are potentially present in deep-sea environments. This procedure allows the extraction of microplastics also with very small dimensions (lower than 20 down to 2 µm) resulting in a very high efficiency extraction from deep-sea sediments. Due to the high expected impact of the smallest fractions of microplastics for the trophic webs and cellular mechanisms/processes in the marine environment (Wright et al. 2013, Galloway et al. 2017), the protocol presented here

allows us to rigorously quantify and identify even microplastics with a size of few micrometers in benthic deep-sea ecosystems, thus representing a benchmark for investigating their distribution and impact on such ecosystems.

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4. QUANTITATIVE RELEVANCE AND IMPACT OF MICROPLASTICS IN DEEP-SEA HABITATS FROM DIFFERENT GEOGRAPHIC AREAS

Introduction

Plastics are found everywhere, from land to the oceans, from the coasts to the deep sea (Barnes et al. 2009) where it accumulates also in trenches and depressions. In the absence of changes to current waste management practices, the annual amount of plastics entering the ocean from land is predicted to rise to 250 million metric tons by 2025 (Jambeck et al. 2015). Several studies (Galgani et al. 2000; Wei et al. 2012; Ramirez- Llodra et al. 2013; Pham et al. 2014; Mordecai et al. 2011; Canals et al. 2013) highlighted that submarine canyons and trenches can act as preferential conduits for accumulation of macro and microplastics in the deep oceans. Submarine canyons, being less disturbed by bottom trawling than continental shelf sediments, may provide a good record of the history of plastics influx during the Anthropocene (i.e., the new era arisen, in which human actions have become the main driver of global environmental change; Rockstrom et al. 2009; Lewis and Maslin 2015; Zalasiewicz et al. 2017). Several surveys conducted in benthic deep-sea ecosystems showed the presence of large plastic fragments, by dredging or by using remotely operated underwater vehicle (ROV) cameras. One of the first evidences of plastics as major litter found in deep-sea ecosystems has been observed in the Fram Strait (79°N) at 2450 m depth (Bergmann and Klages 2012) and in the Charlie-Gibbs Fracture Zone of the Mid-Atlantic Ridge at 2500 m depth (Pham et al. 2014). Plastics items such as bottles, bags and abandoned fishing nets were observed (Watters et al. 2010; Richards and Beger, 2011; Tubau et al. 2015; Corcoran, 2015), often concentrated due to the topography or currents, for example along submarine canyons (Schlining et al. 2013; Tubau et al., 2015). Considering that degradation of plastics in sea water compared to a land area is much slower (Andrady 2011), plastics in marine environment could persist longer. In addition, being characterized by different biological, chemical and physical features (such as relatively stable

conditions, lack of light and low temperatures) deep-sea ecosystems could accumulate microplastics and further delay their degradation. In addition, oceanographic processes, like dense shelf water cascading, severe coastal storms, offshore convection and saline subduction (Talley 2002; Ivanov et al. 2004; Canals et al. 2006; Sanchez-Vidal et al. 2012; Durrieu de Madron et al. 2013; Stabholz et al. 2013) could favour the transfer of microplastics to deeper environments. Also, biological processes can favour the transport of microplastics to deep sea by microbial biofilm with the formation of the so-called "Plastisphere" (Zettler et al. 2013), macrobiofouling (Fazey and Ryan, 2016), marine snow (Van Cauwenberghe et al. 2013, Cole et al. 2016) or probably by ingestion of larvaceans (Katija et al. 2017).

There are very few studies that investigated "microplastic pollution" in remote areas such as deep-sea environment and these are not comparable due to the different target size range and typology of plastic fragments and extraction procedures (Woodall, et al, 2014, Bergmann et al. 2017, Van Cauwenberghe et al. 2013, Fischer et al. 2015). Indeed, a study conducted in Arctic Ocean focusing on microplastic fragments with size of 11-275μm was based on the use of the MicroPlastic Sediment Separator, which has been reported to have a very high extraction efficiency when applied to shallow-water sediments (Imhof et al. 2012). In this study plastic fibers were excluded from the results (Bergmann et al. 2017). Conversely, an investigation carried out in different deep-sea habitats (open slope, canyon basin and seamount) of the NE Atlantic, Mediterranean and SW Indian Ocean considered only plastic fibers of size 2-3 mm and was based on the use of two different methods involving two different density separation solutions, with possibly a diverse extraction efficiency of plastic fragments. Finally, a study conducted in the Kuril-Kamchatka Trench (NW Pacific) was focused on 300-500 μm plastic fragments extracted by using manual sorting and since no spectroscopy analysis was performed (Fischer et al. 2015), the actual identity of the fragments cannot be confirmed.

Despite the discrepancies in the available data, the studies so far conducted reveal that benthic deep-sea ecosystems from Arctic Ocean are characterized by a huge amount of microplastics (MPs; up to 1732×10^2 microplastics m^{-2}, Bergmann et al. 2017) and that the deep-sea habitats from NE Atlantic, Mediterranean and SW Indian Ocean can accumulate high fractions of plastic fibers (Woodall et al. 2014). Such an information, allow us to hypothesize that abyssal ecosystems including submarine canyons can act as a storage system for microplastics.

The aim of this study is to assess the quantitative relevance of microplastics ranging from 2 to 1000 μm, their polymer composition and distribution patterns in different benthic deep-sea

ecosystems spanning submarine canyons, open slopes and a deep-hypersaline anoxic basin present in different oceanic sectors (Arctic, NE Atlantic and Southern Oceans, Black Sea and Mediterranean Sea). In addition, to evaluate if submarine canyons can act as reservoirs of microplastic pollution we carried out a more detailed study in Ionian and Tyrrhenian canyons (Mediterranean Sea) also through the analyses of the plastic fragments ingested by nematodes, which represent the most abundant metazoans in benthic deep-sea ecosystems.

Materials and methods

Study area and sediment sampling

Deep-sea sediments analyzed during this study were collected in different geographical areas including: Arctic (Nordic Margin of Svalbard Island at depth of 1279 m), NE Atlantic (Portuguese margin in Rockall Through at depth of 1091 m), Southern (at depth of 591 m) Oceans, in Black (at 990 m depth) and Mediterranean Seas (Fig. 1 A and B). In particular, in Mediterranean Sea, sediment samples were collected in different habitats and along submarine canyons from the head to the mouth (from 84 m to 1472 m), and in a Deep Hypersaline Anoxic Basin (DAHB, La Medeè at 3027 m depth). The details of the sampling sites are shown in the Table 1.

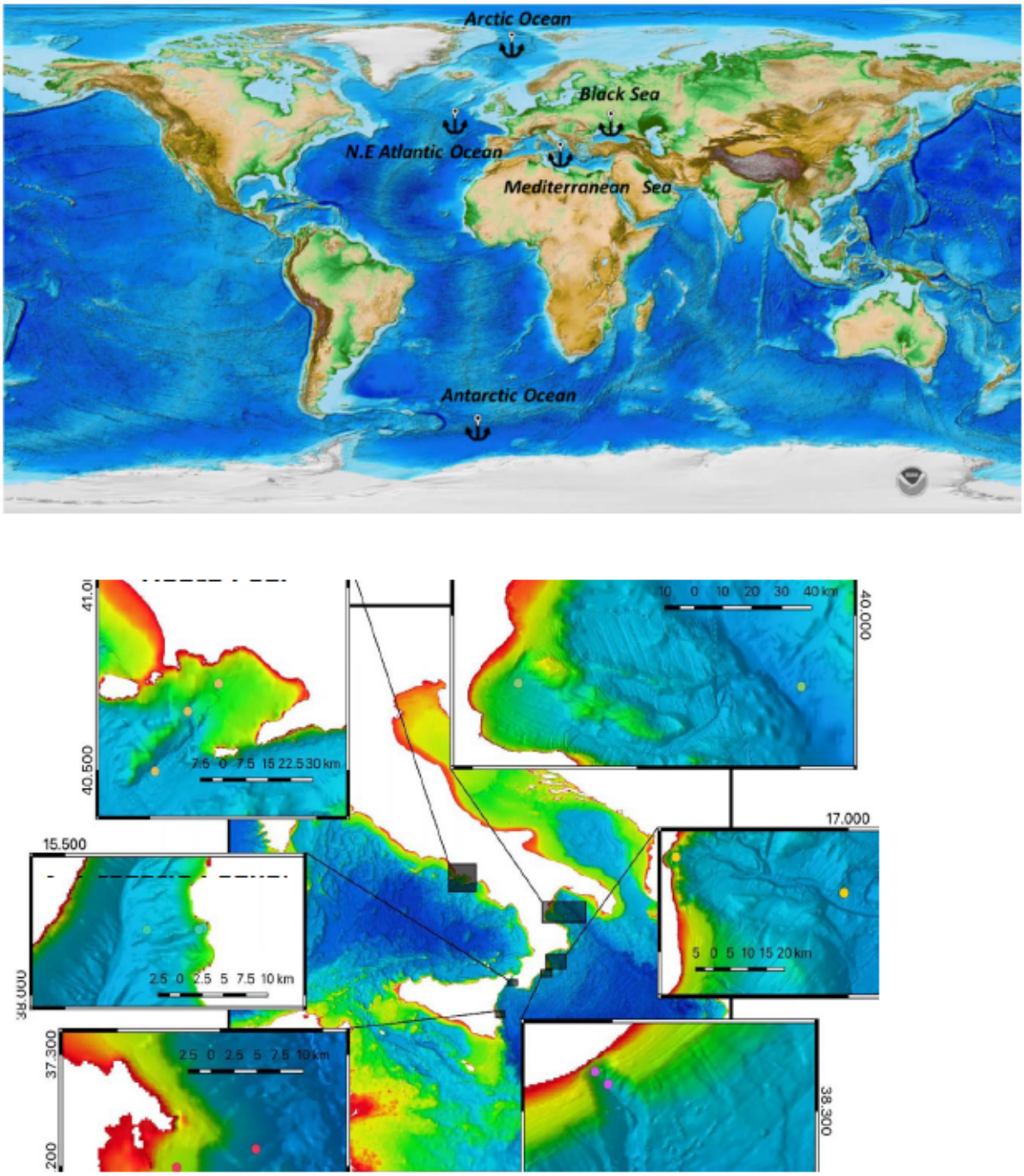


Figure 1. Sampling area (A). Sampling sites of submarine canyons in Mediterranean Sea (B)

Deep-sea undisturbed sediment cores were collected using box-core and multi-corer instruments. The sediments were collected using corers of 4 cm diameter and once recovered on board, surface sediments (the top 10 cm) were sliced and immediately frozen at -20 °C. In

laboratory, sediments were defrosted and before processing for microplastics extraction, sediments were dried in oven at 50 °C for 4-5 days. Then, for each sample, 250 g of sediment was divided in five aliquots of 50 g each.

Table 1. Details on sampling areas and stations, including, geographical coordinates, habitat typology, water depth and samping devices.

Geographic area	Coordinates	Habitat	Depth (m)	Sampling device
Svalbard Island (Nordic Margin)	79°08.00'N 06°05.57'E	Continental slope	1279	Multicorer
Rockall Through (Irish margin)	55°27.10'N 15°45.96'E	Continental slope	1091	Boxcorer
Black sea	44°10.19'N 31°26.19'E	Basin	990	Boxcorer
La Medee basin (Mediterranean Sea)	34°25.70'N 22°20.16'E	Deep-Hypersaline Anoxic Basin	3027	Boxcorer
Ross Sea (Southern Ocean)	74°00.93'S 175°02.38'E	Continental shelf	591	Mega multicorer
Dohrn Canyon (Tyrrhenian Sea)	40°39.73'N 14°06.83'E	Canyon	220 552 1024	Boxcorer
Squillace Canyon (Ionian Sea)	38°42.95'N 16°34.03'E	Canyon	450 1137	Boxcorer
Amendolara Canyon (Ionian Sea)	39° 48.62'N 16°38.36'E	Canyon	374 1472	Boxcorer
Augusta Canyon (Ionian Sea)	37°11.96'N 15°15.46'E	Canyon	84 1177	Boxcorer
S.Gregorio Canyon (Messina Strait)	38°03.78'N 15°38.51' E	Canyon	221 304 910	Boxcorer
Caulonia Canyon (Ionian Sea)	38°20.21'N 16°28.93'E	Canyon	207 478 1253	Boxcorer

Working conditions and precautions during microplastics analyses

All laboratory ware and containers used were glass or stainless steel, thoroughly rinsed with Milli-Q (filtered to 0.2 µm nitrocellulose filters) before use. All filtration steps were performed in a laminar flow cabinet and all chemicals (e.g., $ZnCl_2$, H_2O_2 and Milli-Q) were filtered through 0.2 µm nitrocellulose filters (Sartori) to remove particulate contaminants before usage. Cotton laboratory coats were generally worn to reduce contamination from synthetic textiles. To account for possible contaminations due to processing of the samples, a procedural blank was run during all steps of the protocol, dinking a filter with Milli-Q, $ZnCl_2$ and H_2O_2 to detect the possible contaminations in working solutions and laboratory airborne.

Microplastic extraction

Microplastics extraction was carried out according to a procedure based on different methods (Claessens et al. 2013, Imhof et al. 2012 and Vianello et al. 2013) with some modifications and optimized for deep-sea sediments (see Chapter 3). $ZnCl_2$ was added (1.6 - 1.7 kg/L) to the sediments (50 g for five aliquotes), as density separation solution and it was stirred for 12 h to allow sample homogenization with the solution. After 12 h, stirring was stopped and sediment samples were subjected to ultrasound treatment for three times of 1 minute each, with 30 seconds of manual shaking after each cycle (frequency: 40 KHz; Bransonic Branson 3510). Then, sediment samples were maintained at room temperature for 3 days so that the denser particles (sediment) could settle down. Subsequently, since the plastic particles tend to float, supernatants were transferred into centrifuge tubes (17.000 xg for five minutes; Avanti J-30I) to allow the separation of the sediment particles from microplastics. The supernatants were filtered onto 20 µm filers (SEFAR NITEX) with vacuum filtration apparatus, and the filtrate was then subsequently collected and filtered onto 2 µm filters (Whatman, Nucleopore. To eliminate any organic matter trace and facilitate microplastic counting the filters were subjected to three cycles of treatment (5 min each) and subsequent vacuum filtration with 10 mL of H_2O_2 (30%, Carlo Erba). Filters were maintained at room temperature under laminar flux hood.

Before chemical identification, particles collected in both filters were counted and divided within five range size classes: > 1mm, 500-1000 µm, 200-500 µm, 200-20 µm and 2-20 µm. The plastics items between 20 and 1000 µm and >1 mm were processed through infrared

spectroscopy (FT-IR) whereas the particles with range size between 2 and 20 µm were analysed with Scanning Electron Microscopy (SEM, Fig. 2).

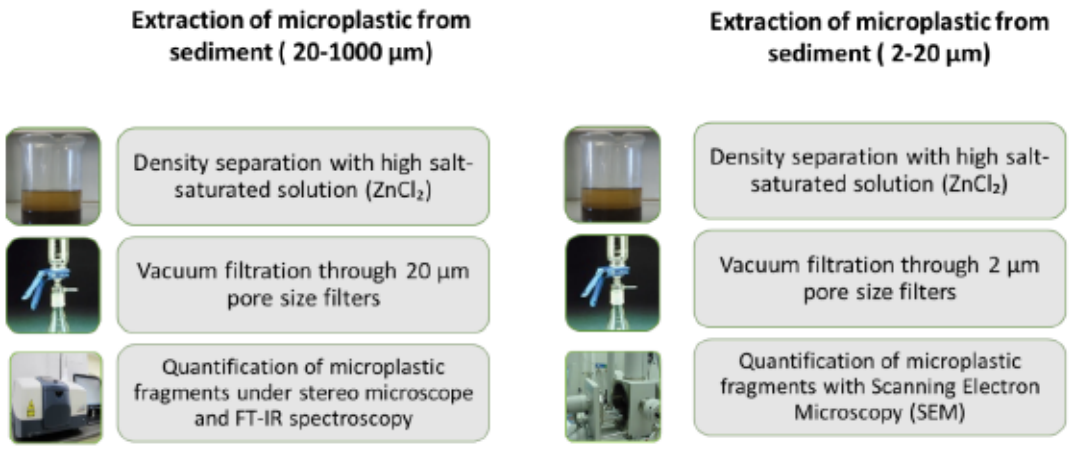


Figure 2. Extraction method for the different microplastic fractions (20-1000 µm and 2-20 µm)

Microplastic identification

FTIR spectroscopy (range size > 20 µm)

Once all the supernatant was filtered, counting and measurements of microplastic in the range size between 20-1000 µm was obtained observing the filter under stereomicroscope at a magnification of 50 X (Zeiss, Stemi 2000). Then, all particles were submitted to infrared spectroscopy (ATR/ FT-IR) for polymers identification (Fig. 3). FT-IR measurements were carried out using a Perkin Elmer FTIR Spectrometer Spectrum GX1 interfaced with a Perkin–Elmer Autoimage microscope and equipped with a photoconductive 0.25 mm Hg-Cd-Te (MCT) array detector, operating at liquid nitrogen temperature and covering the entire IR spectral range from 4000 to 700 cm^{-1}. The Spectrum Autoimage 5.1.0 software package (Perkin Elmer, Waltham, MA, USA) was used. On each sample, deposed on CaF_2 windows, both the microphotograph, obtained by means of a microscope television camera, and the corresponding average spectrum were collected. Spectra were acquired in transmission mode, at room temperature, with a spectral resolution of 4 cm^{-1} and a spatial resolution from 30 x 30 to 50 x 50 μm^2. Each spectrum was the result of 64 scans. Background spectra were acquired on clean portions of CaF_2 windows and ratioed against the sample spectrum. For data handling was used software packages Spectrum 5.3.1 (Perkin Elmer).

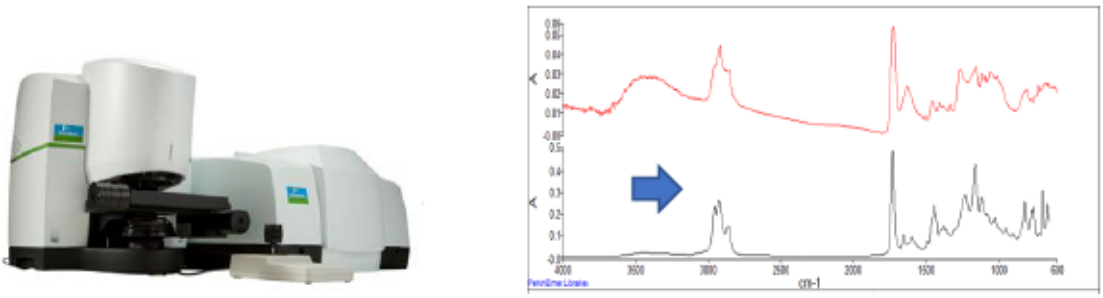


Figure 3. Identification of microplastics (20-1000 µm) by FT-IR analyses

SEM analyses (range size < 20 µm)

Filters with particles with a size < 20 µm were coated with gold using a sputter coater (Emitech K550). Then particles were counted and measured with Scanning Electron Microscopy (Zeiss SUPRA 40) at magnification of 7.26 KX. Identification of microparticles was carried out with microanalysis system (Bruker Quantax Z200) that allows precise qualitative analysis of very small components and coating analysis through X-Ray.

Sixty optical fields were observed in three different areas of each filter (20 optical fields for each area). Since muddy sediments were very fine, the procedure based on the density separation solution was not able to completely remove them, resulting in a strong background due to sediment particles. Therefore, to speed up the analysis, an X-ray map (Bruker Quantax Z200) was made to distinguish the sediment grains (mapping silicon mineral) from the plastic particles in each filter area considered. Once the X-ray map was obtained, the non-silicon particles were subjected to the microanalysis to assess the presence of carbon suggesting presence of the plastic compounds (Fig. 4).

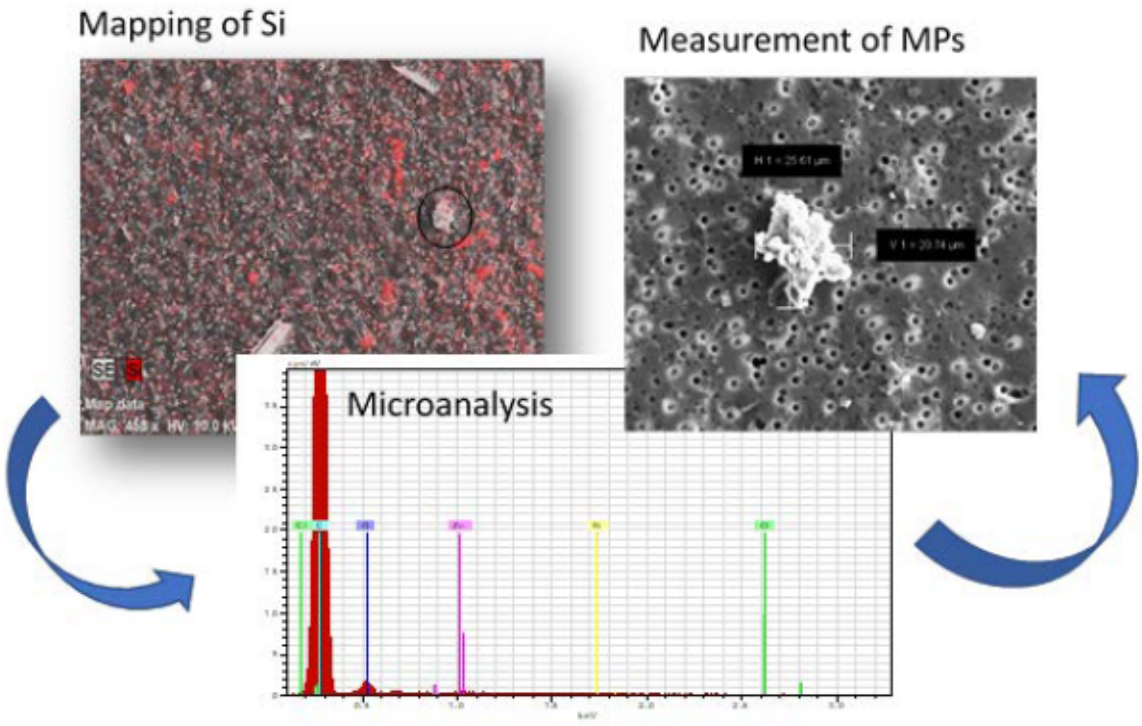


Figure 4. Analytical steps of the Scanning Electron Microscopy (SEM)-based procedure to identify microplastics with range size between 2 and 20 µm.

Meiofaunal and biodiversity analysis

Analysis of meiofauna component, in particular nematodes, was carried out in the Mediterranean canyon (Canyon Dohrn) according to Danovaro et al. 2009. For meiofauna extraction, cores were immediately frozen at -20 °C. Then, they were sectioned into different layers (0–1, 1–2, 2–5, 5–10, 10-15 cm) and sediment samples were passed through a 500 µm mesh, and a 20 µm mesh to retain the smallest organisms. The fraction remaining on the latter sieve was resuspended and centrifuged three times with Ludox HS40 (density 1.31 g cm^{-3}), as described by Heip et al. (1985). Samples were preserved in a buffered 70% ethanol solution and stained with Rose Bengal (0.5 g l^{-1}). Than all the meiobenthic animals were counted and classified taxonomically under a stereomicroscope 40 or 80X (Zeiss, Stemi 2000). The analyses were performed using three replicates (from independent deployments) from each sampling station.

For the analysis of nematode diversity 100 individuals, from the first cm of sediment, were randomly selected from three independent replicates at each deep-sea site. The organisms were mounted on slides and identified at least to species level according to Platt and Warwick (1983, 1988) and Warwick et al. (1998) nematodes have been recovered for enzymatic digestion. The

trophic composition was defined according to Wieser (1953). Nematodes were divided into four original groups as follows: (1A) no buccal cavity or a fine tubular one-selective (bacterial) feeders; (1B) large but unarmed buccal cavity-non-selective deposit feeders; (2A) buccal-cavity with scraping tooth or teeth-epistrate or epigrowth (diatom) feeders; (2B) buccal cavity with large jaws-predators/omnivores.

Extraction of microplastics from meiofauna (nematodes)

To evaluate possible microplastics ingestion in meiofauna communities, nematodes were undergone to enzymatic digestion protocol's (Cole et al. 2014 modified). Nematodes were extracted from sediments collected in Canyon Dohrn at different depths (220 m, 552 m and 1024) and on the basis of buccal cavity observed with microscope at 100x magnification (Zeiss, Axioskop 2 MOT) the nonselective deposit feeders nematodes were chosen to evaluate microplastics ingestion. One hundred nematodes were sorted under stereomicroscope (Zeiss, Stemi 2000, 40X) and processed according to the modified enzymatic digestion protocol: individuals were dried in oven at 60°C for 12 h. After 12 h, 5 ml of homogenizing solution was added to the sample and incubated at 50°C for 15 minutes. Proteinase K (41 U/mg) was added and the sample was incubated at 50°C for 2 hours. Then, sample were homogenized and incubated again at 60° C for 20 minutes, after that samples were sonicated three times. After digestion the microplastic-containing suspensions were immediately filtered on 2 µm filter with vacuum filtration and coated with gold using a sputter coater (Emitech K550) in order to allow Scanning Electron Microscopy analysis (Zeiss SUPRA 40, 7.26 KX magnification). Identification of microparticles was carried out with microanalysis system (Bruker Quantax Z200) that allows precise qualitative analysis of very small components and coating, helping to identify particles containing carbon atoms that are present in plastic.

Results

Microplastics' extraction and identification in deep-sea sediments from different geographic areas

Microplastics were detected in all deep-sea sediment samples in different geographic areas at depths > 500 m. The highest concentration of microplastics with size in the range 20-1000 µm was found in the NE Atlantic Ocean (554 items/m^2) while the lowest one in the Caulonia Canyon (101 items/m^2), in the Mediterranea Sea. On average, microplastic concentrations in deep-sea ecosystems was ca. 290 items/m^2. High values were also found in the Southern Ocean (403 items/m^2, Fig. 5).

The highest concentration of microplastics with size range 2-20 µm, based on a conservative estimate (i.e., based only on the number of microplastics actually detected in a minor portion of the filter analysed by SEM) was found in the Black Sea and Squillace canyon (353 and 302 items/m^2). In the DHAB, Atlantic Ocean and the deeper zone of Canyon Dohrn no microplastics in the range size between 2 and 20 µm were found. On average, microplastic concentrations (2-20 µm) in deep-sea ecosystems was ca. 130 items/m^2.

Assuming a homogeneous distribution of the microplastics on the filter analysed by SEM, we estimated we obtained the highest concentration was observed in the Black Sea and Squillace canyons (4.54 x10^6 and 3.89 x10^6 respectively).

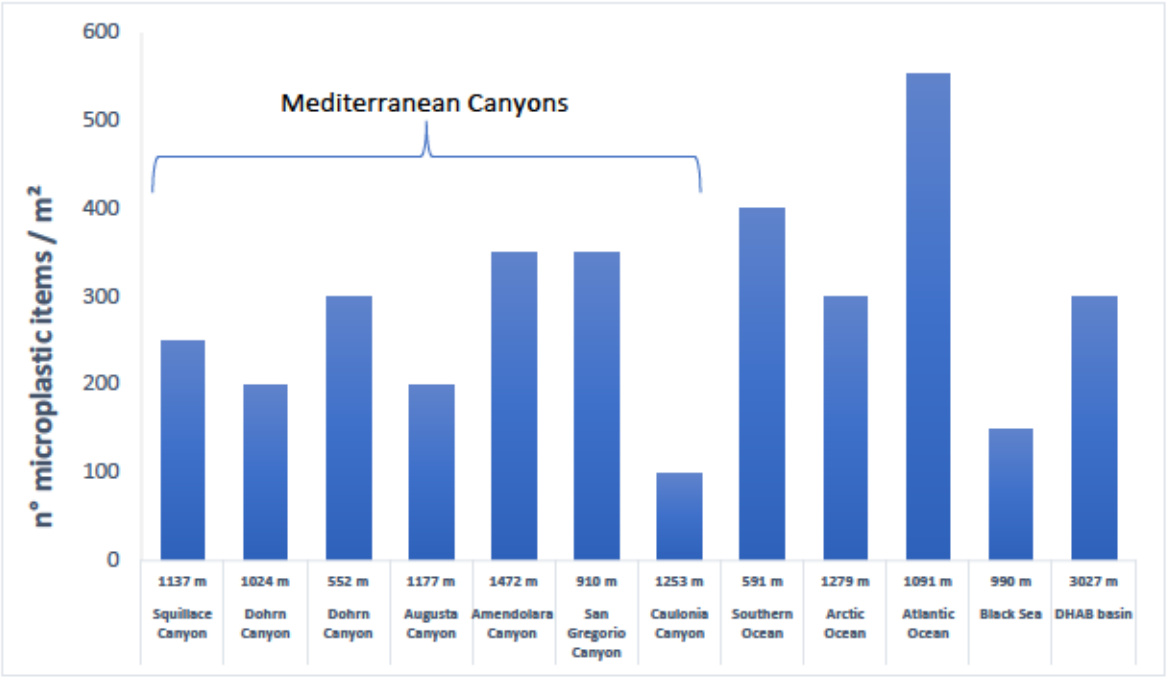


Figure 5. Microplastics abundances in deep-sea sediments at depth >500 m in different geographic areas

Different size classes (20-1000 µm) of microplastics were found in deep-sea sediments. Except for Squillace canyon and Arctic Ocean, in the other areas investigated more than 50% of microplastics had a size range between 20-500 µm. In particular, the sediments of Augusta and Caulonia canyons and Black Sea contain only plastic fragments with size between 20 and 500 µm while Atlantic Ocean was characterized by larger microplastics (500-1000 µm and >1000 µm) (Fig. 6).

The amount of microplastics between 2 and 20 µm (based on a conservative estimate) showed that this fraction can represent in some areas, such as Caulonia canyon and Black sea, ca. 70%. Instead, assuming a homogeneous distribution of the microplastics on the filter analysed by SEM, this fraction could represent a percentage between 67 and 100% except for the deepest part of canton Dohrn, Atlantic and DHAB basin where microplastics between 2 and 20 µ no microplastics in the range size between 2 and 20 µm were found.

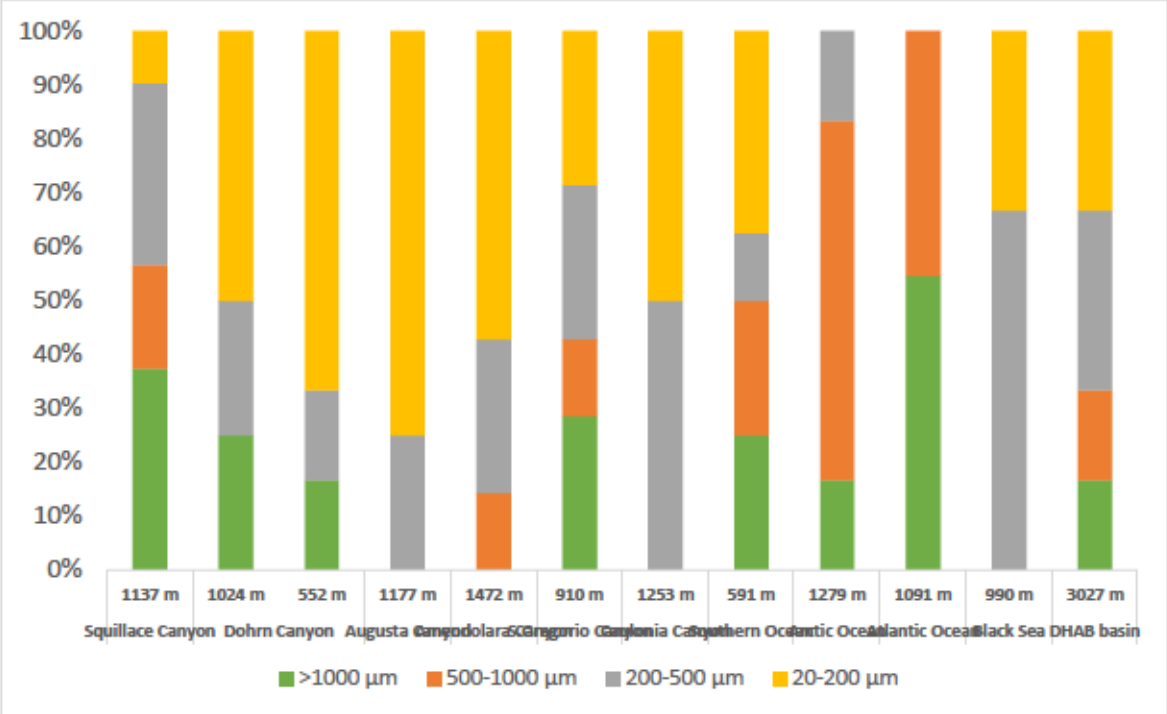


Figure 6. Microplastic size distribution in deep-sea sediments at depth >500 m in different geographic areas

Twenty different polymers' types were identified in total from all sediment samples. The polymer composition was very different among the habitats investigated with the major variety

of polymers in the sediments of Mediterranean Sea (Dohrn, Augusta and S. Gregorio canyons and DHAB basin) and Southern Ocean (Fig. 7). In particular, polymeric composition of MPs in Mediterranean canyons was characterized by a minimum of two polymers in the Squillace and Caulonia canyons (i.e., polystyrene, nylon and nylon, polyvinyl chloride, respectively) and a maximum of six polymers in S. Gregorio canyon (polystyrene, polyester, polytetrafluoroethylene, polyurethane, polyvinyl chloride, SIS (styrene-isoprene-rubber)) for a total of 10 polymers (and of these polymers, 5 were present exclusively in almost all the Mediterranean Sea canyons (polystyrene, polyacryamide, polytetrafluoroethylene, polyvinyl chloride and SIS); the sediments of the Dohrn and S. Gregorio canyons contained two exclusive polymers (Polyacryamide and SIS respectively). DHAB sediments (Deep-hypersaline anoxic basin) were characterized by 5 polymers (polyamide, polyethylene, SEBS (Styrene-ethylene-butylene-styrene, PLA (polylactic acid) and sylobloc), three of which were exclusive of this site (SEBS, PLA and Sylobloc). Black Sea sediments were characterized by only two types of polymers such as polyethylene and EPDM (Ethylene-Propylene Diene Monomer), which was present only in this site. Southern Ocean showed a wide microplastics' composition with 6 polymers (Nylon, Polyestere, Polyurethane, Polyamide, Polyethylene and PMMA (polymethylmethacrylate)), but only one of them was exclusive of this area (i.e, PMMA). We did not find polymers shared from all the different geographic areas but in terms of abundances nylon was the most detected polymer in deep-sea sediments. Images of some microplastics found within sediments are reported in Fig. 8.

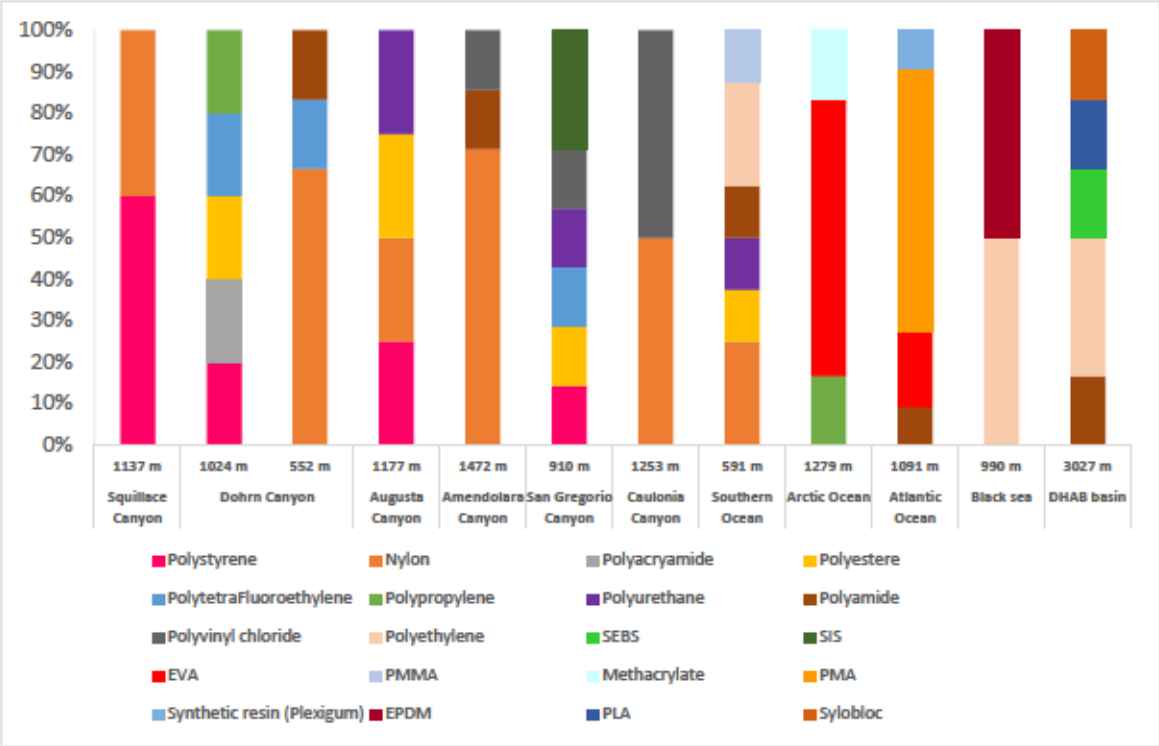
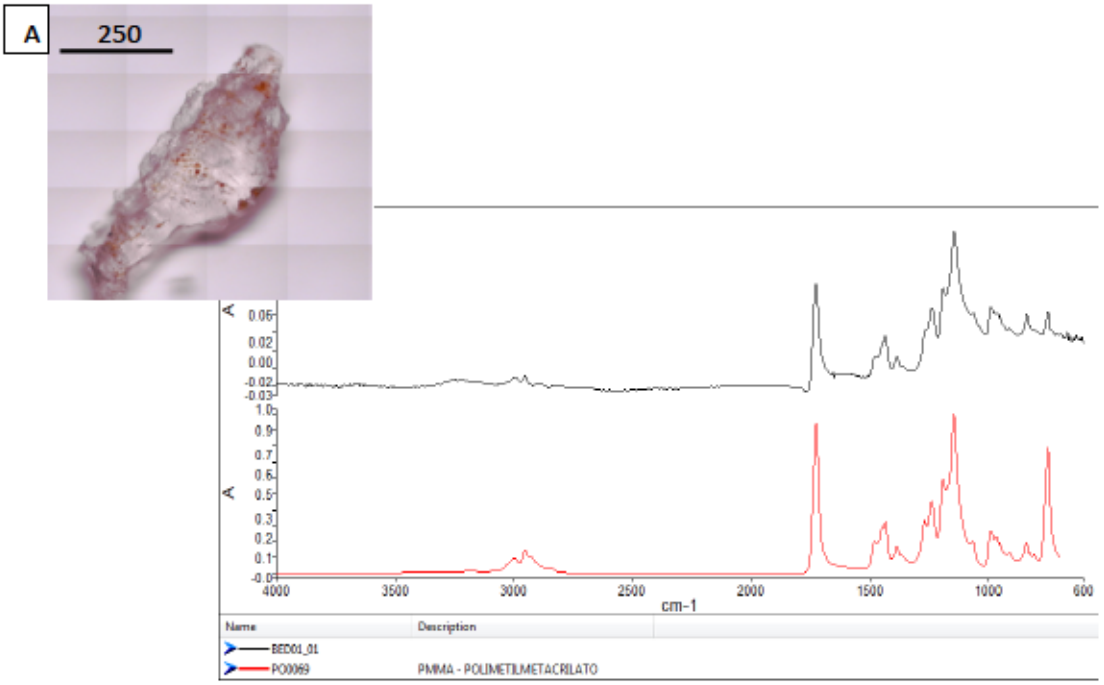


Figure 7. Polymer's composition in deep -sea sediment at depth > 500 m in different geographic areas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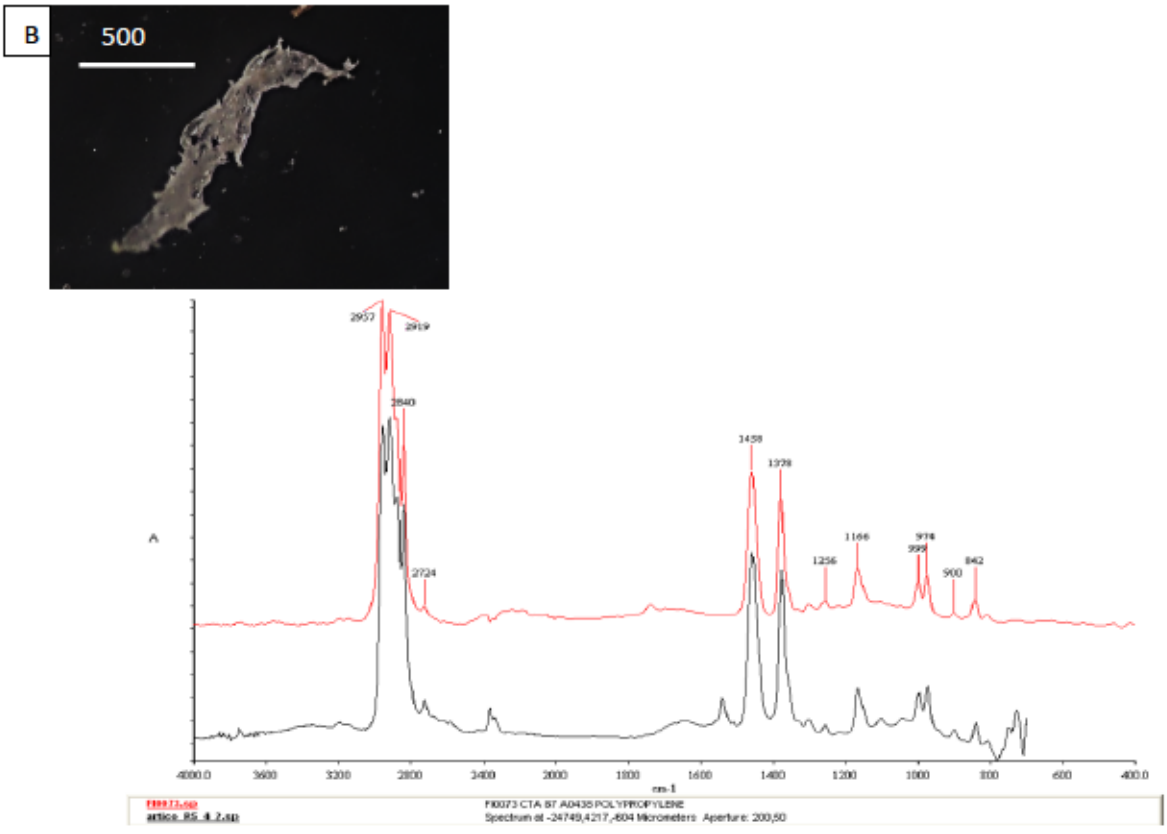
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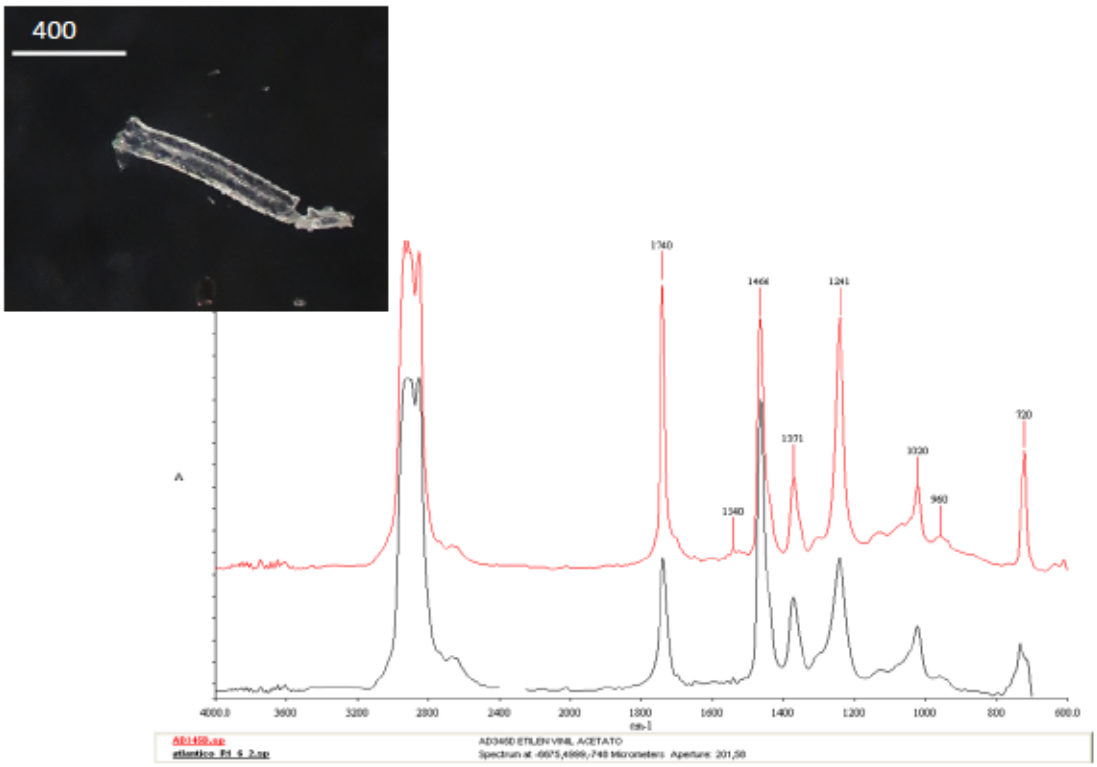
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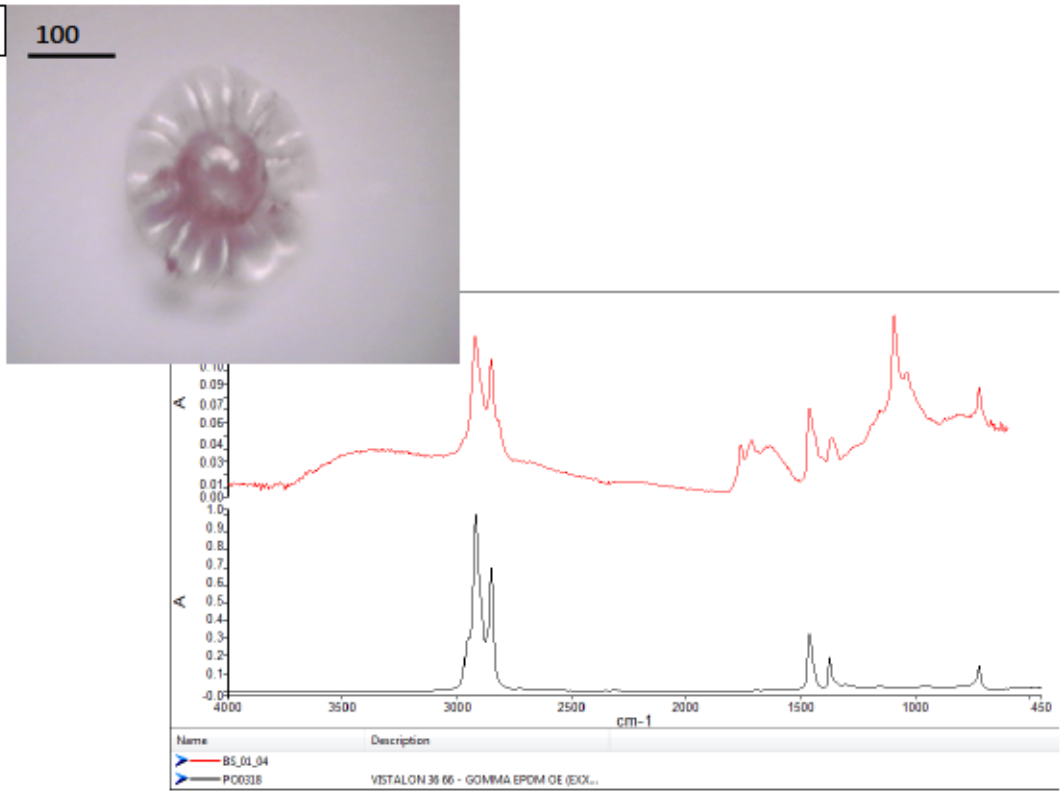
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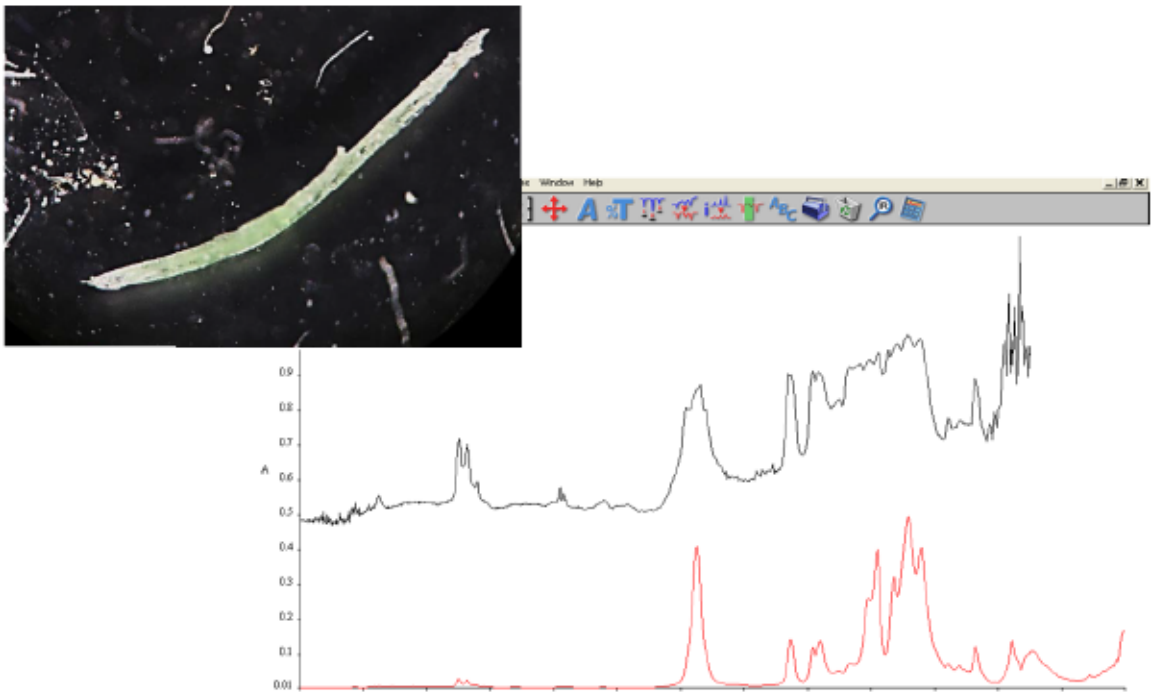


Figure 8. FT-IR Images of microplastics in deep-sea sediments found in A) Southern Ocean, B) Arctic Ocean, C) Atlantic Ocean, D) Black sea and E) DHAB basin

Microplastic extraction and identification on deep-sea canyons

A detailed study was conducted in Mediterranean canyons to investigate if these habitats could be a sink for microplastics pollution. The highest concentration of microplastics with size ranghe 20-1000 µm was found in San Gregorio canyon with 907 items/m^2 at depth of 304 m. In the Squillace, Augusta and Amendolara canyons, microplastics were more concentrated in the deepest zone of the canyon (252, 202 and 353 items/m^2 respectively), while in Dohrn and S. Gregorio canyons, microplastics' abundance was higher in the middle zone (302 and 907 items/m^2, respectively). Finally, in Caulonia Canyon the head of canyon was characterized by a higher microplastic abundance (202 items/m^2) than in the deepest zones (Fig. 9).

The microplastics in the range 2-20 µm were more abundant in Caulonia (478m) and S. Gregorio canyon (221m) with 958 - 1.23 x10^7 and 857-1.10 x10^7 items/m^2 (considering the more conservative estimate and the higher estimate based on the assumption that microplastics are homogeneusly distributed on the filter). In the Squillace, Augusta and Amendolara canyons, microplastics were more concentrated in the deepest zone of the canyon (302 - 3.89 x10^6, 50 – 6.49 x10^5 and 50 - 6.49 x10^5 items/m^2 respectively), while in Dohrn and Caulonia canyons, microplastics' abundance was higher in the middle zone (202 – 2.59 x10^6 and 958 – 1.23 x10^7

items/m^2, respectively). Finally, in S. Gregorio Canyon the head of canyon was characterized by a higher microplastic abundance (857-1.10 x10^7 items/m^2) than in the deepest zones.

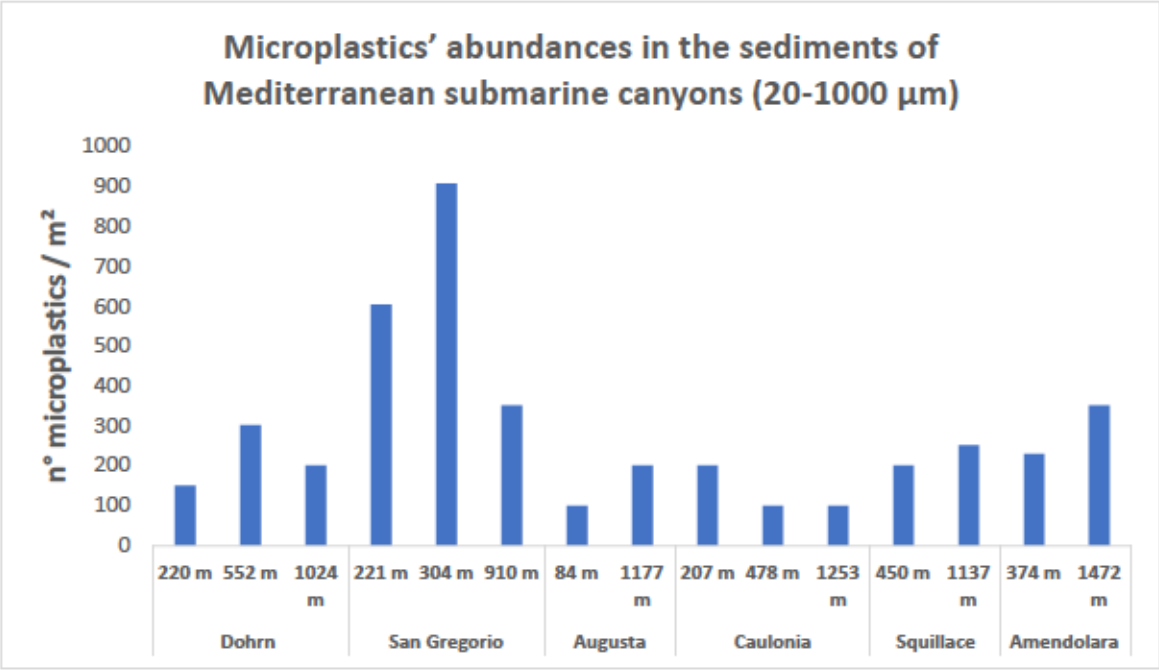


Figure 9. Microplastics abundances in Mediterranean canyon sediments

Different classes of microplastics were found in the different submarine canyons of the Mediterranean Sea. More than 50% of microplastics fragments showed a size between 20 and 500 µm in all the canyons except for the Squillace canyon, where we found a different distribution pattern of size ranges. Also, the large plastic fragments (500 -1000 µm and > 1000 µm) were found in all the canyons even if to a lesser extent, with the exception of Squillace Canyon where this fraction contributed for ca. 60-75% (Fig. 10).

The amount of microplastics between 2 and 20 µm (based on a conservative estimate) showed that this fraction can represent in some areas, such as Caulonia canyon at 478m and 1253m, 90 and 71% respectively. Instead, assuming a homogeneous distribution of the microplastics on the filter analysed by SEM, this fraction could represent a percentage between 50 and 100 %.

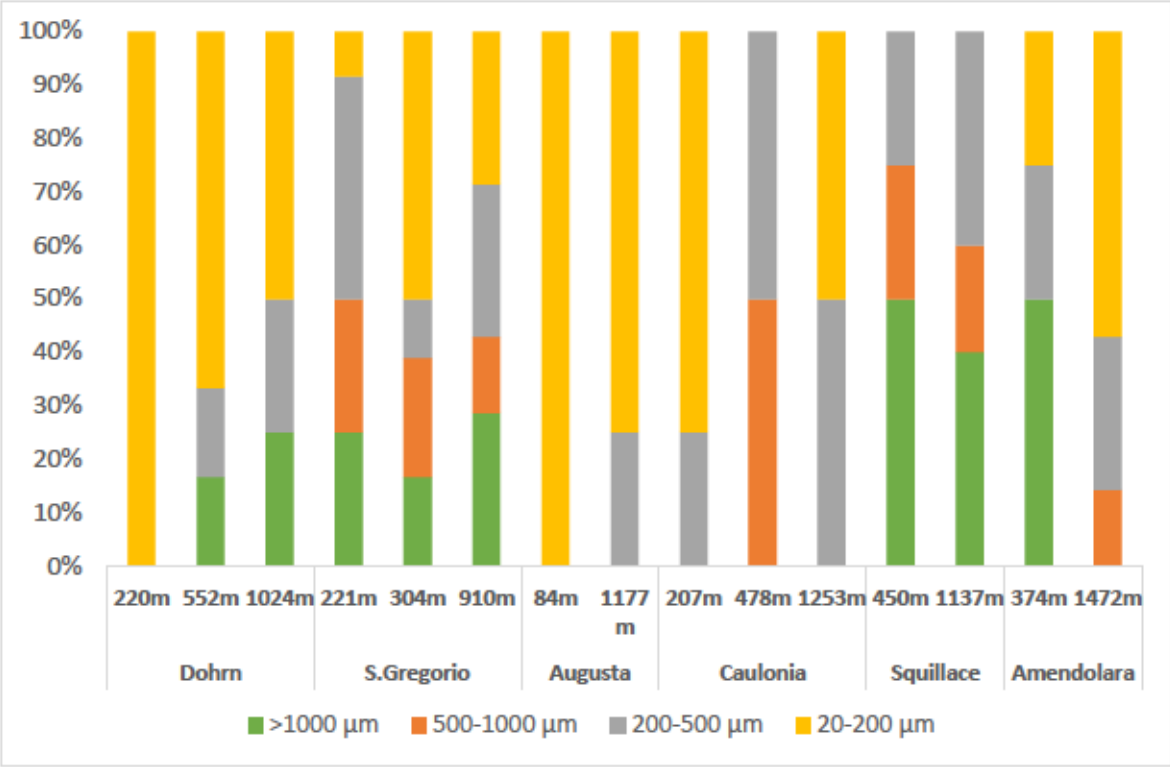


Figure 10. Microplastic classes size distribution in Mediterranean canyon sediments

Overall, twenty different polymer types were identified in all the Mediterranean canyons (Fig. 11 and 12). S. Gregorio Canyon contained the major variety of polymers; six polymers among these, were exclusive of San Gregorio Canyon (SIS (styrene-isoprene-rubber), epoxy resin, EVA (Ethylene-vinyl acetate), phthalic plasticizer, KEVLAR and copolymer PE+EVA). One exclusive polymer was found also in the Amendolara, Augusta and Dohrn canyons (Acrylic resin, SEBS and Polyacryamide respectively). Only two polymers were shared among all the canyons: nylon and polypropylene. These polymers followed by polyester were the most detected polymers in deep-sea canyons.

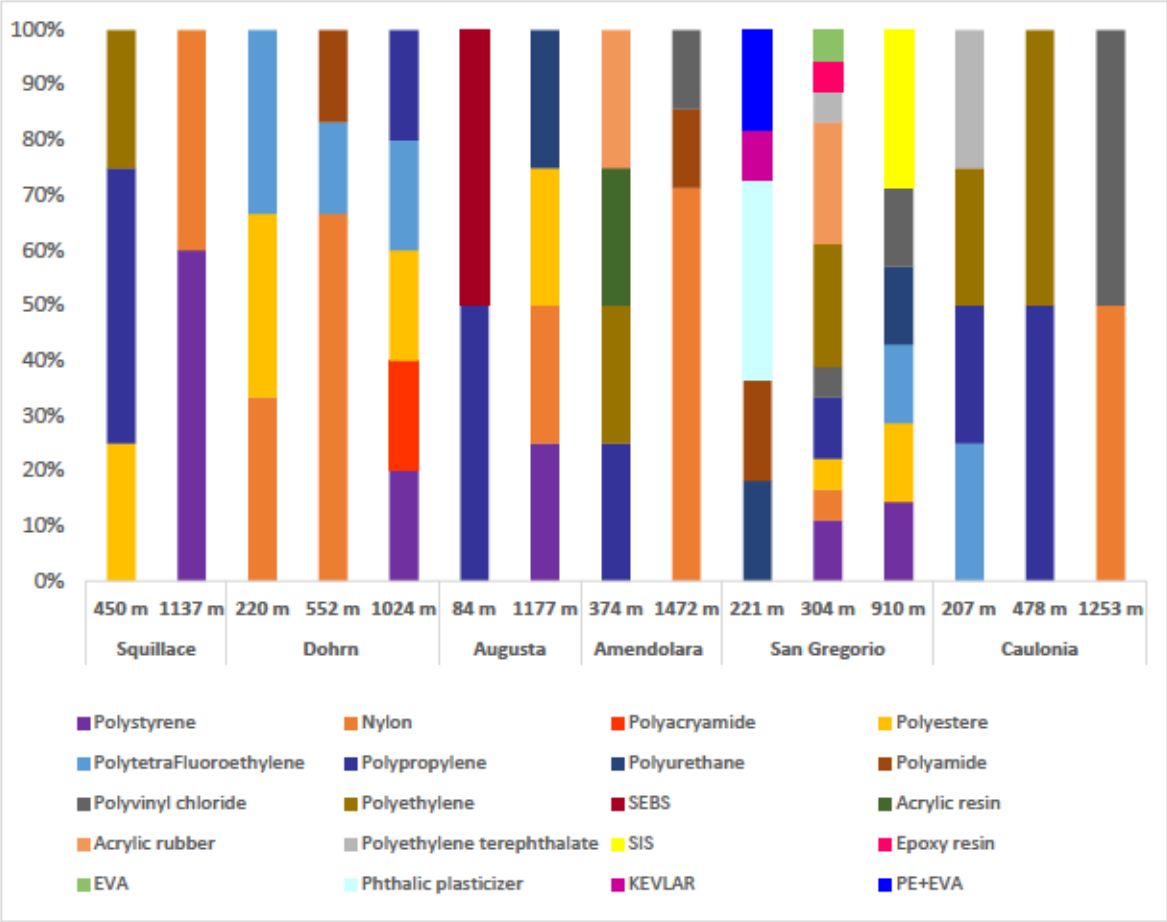
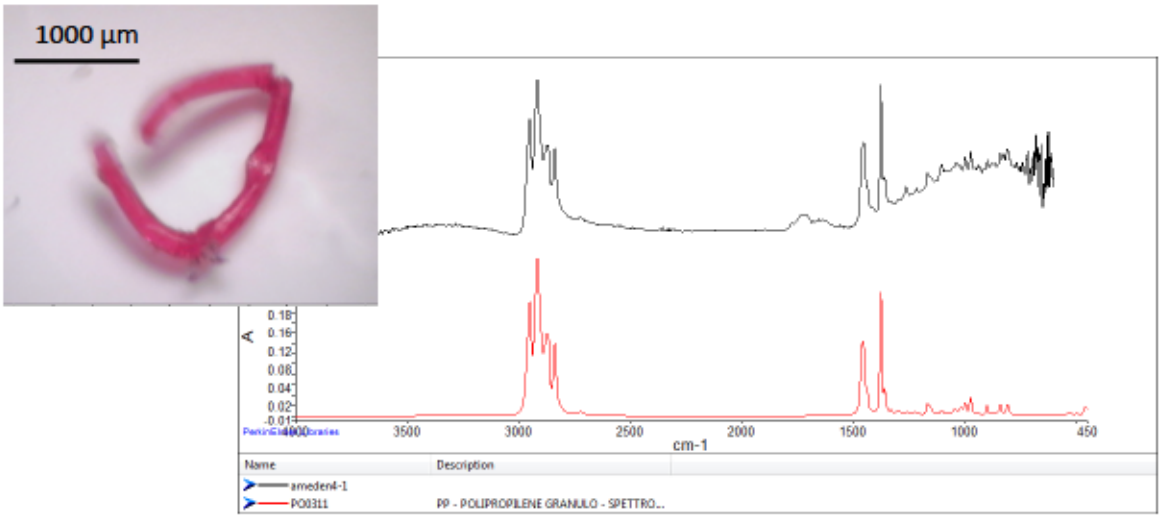


Figure 11. Polymer's composition in canyon sediments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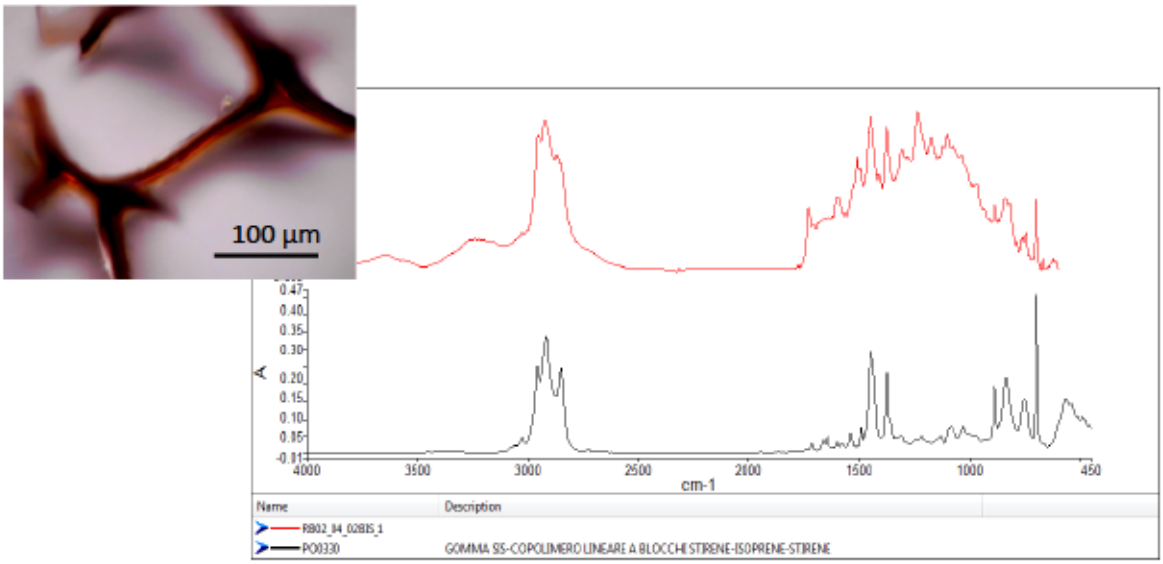
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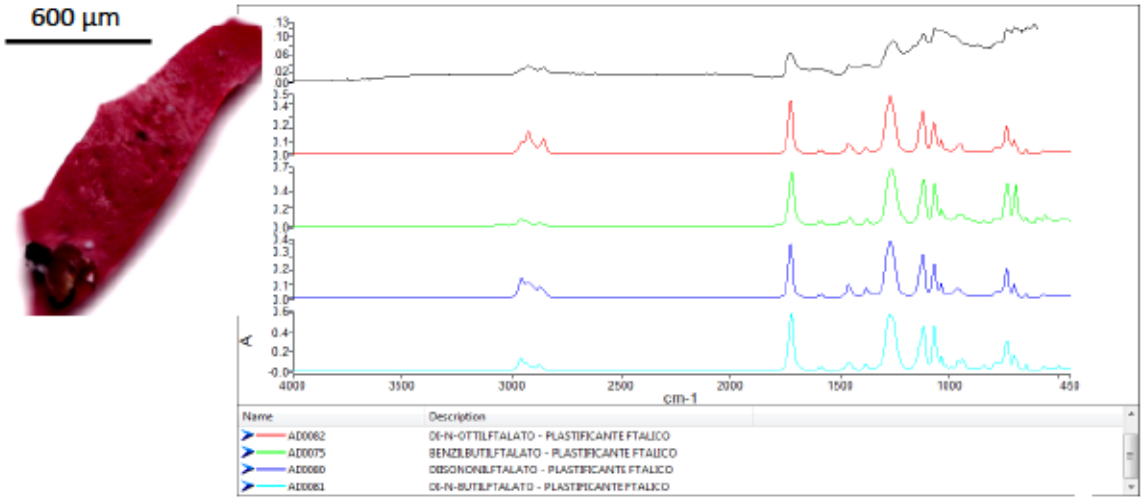
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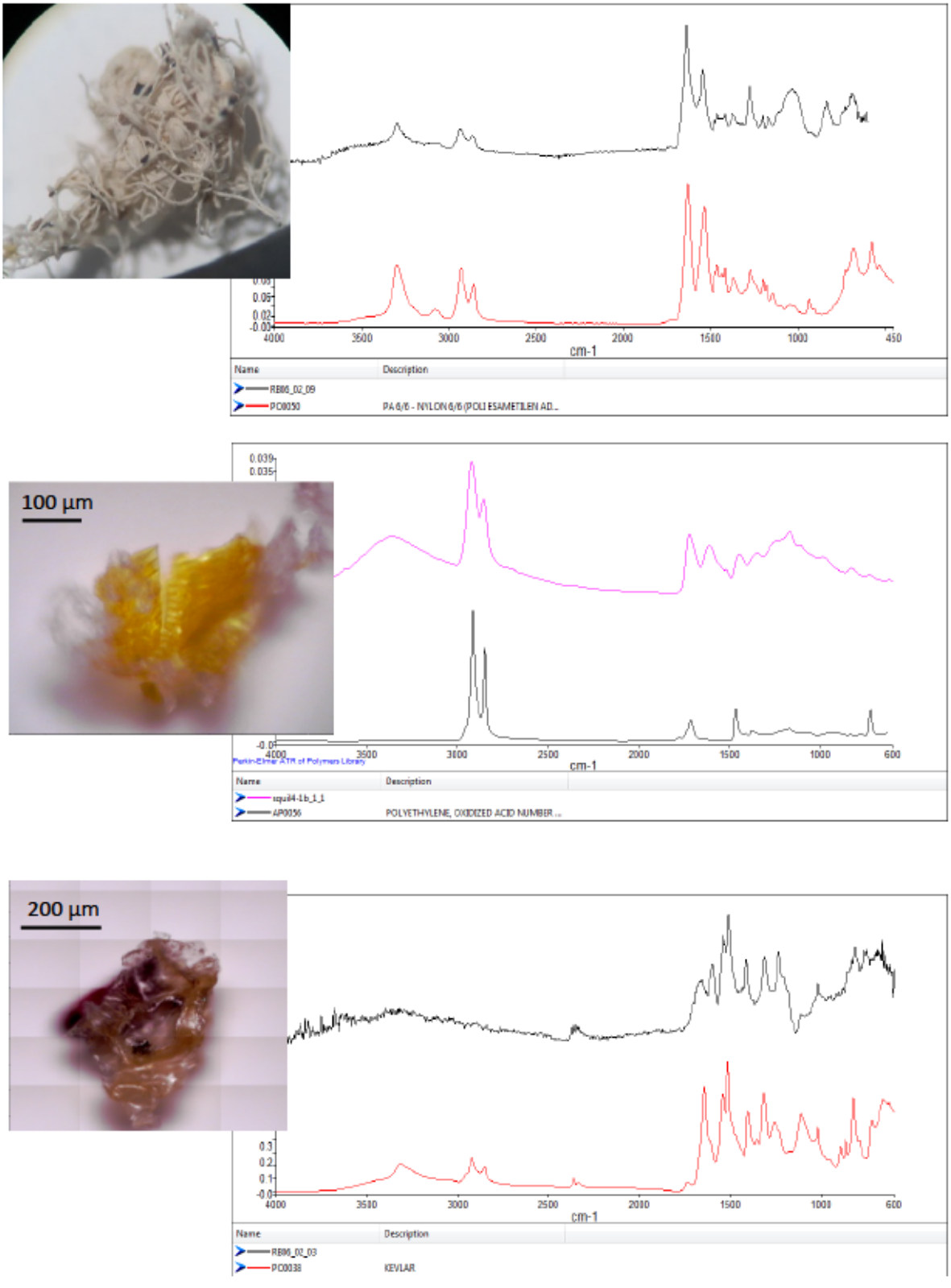


Figure 12. FT-IR images and spectra of microplastics found in Mediterranean canyons

This high variability in polymer composition along the canyons was also revealed by the Bray Curtis analysis that showed an unclear pattern of similarity among the canyons investigated and

inside the same canyon (by comparing head, middle and mouth zone); in particular the polymer composition in the sediments of the head of S. Gregorio Canyon clustered apart from the polymer compositions observed in the others zones of the canyons with a percentage of similarity less than 20% (Fig. 13).

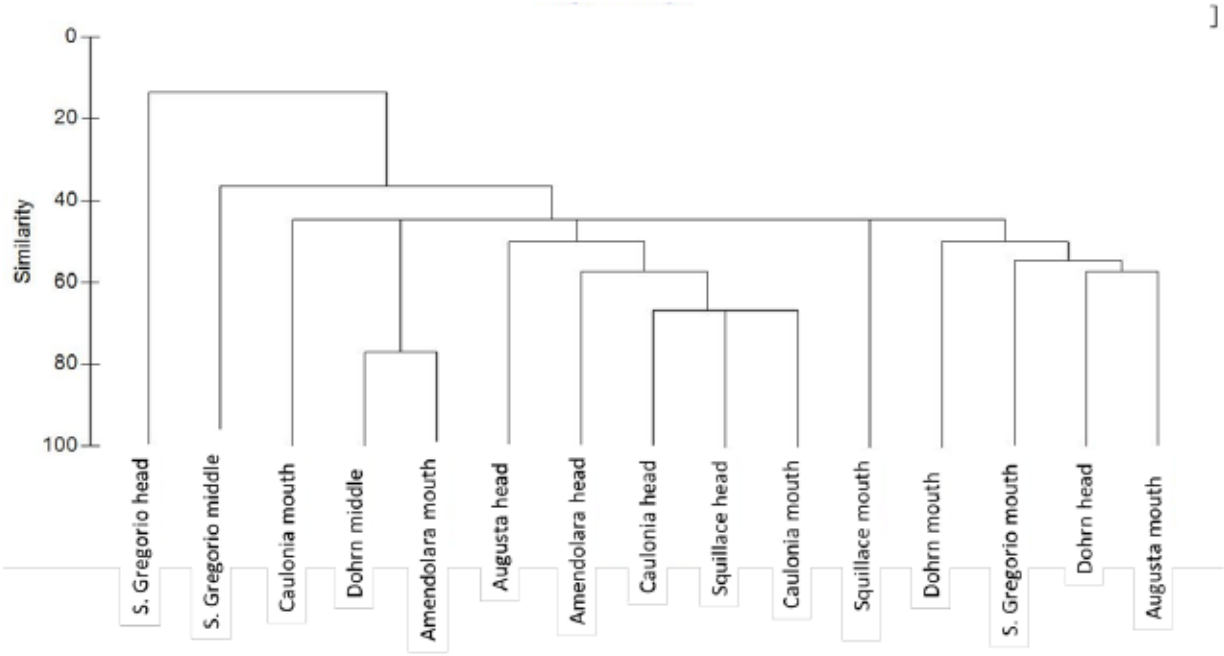
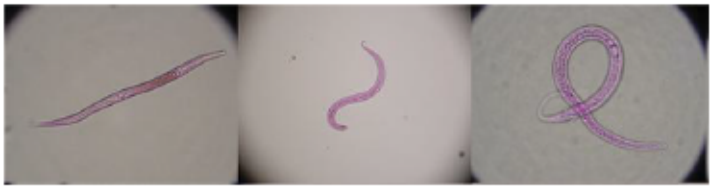


Figure 13. Bray-Curtis analysis in Mediterranean canyons. The term "head" indicates the less deep zone of the canyon, "middle" the middle zone of the canyon and "mouth" the deepest zone of the canyon.

Extraction of microplastics from Meiofauna (Nematodes)

We found 23 microplastics ingested by 100 nematodes at 220 m in Dohrn Canyon, 51 microplastics at 552 m depth and no microplastics at 1024 m depth. The size range of the ingested microplastics varied between 2 and 20 µm. A similar pattern was observed when data of nematodes' ingestion and microplastics' abundance in canyon sediments were compared. In particular, we did not find any microplastics ingested in the deepest zone of canyon Dohrn characterized by the lowest microplastic abundance (Fig. 14). In Figure 15 were reported some images of microplastics ingested by nematodes with its relative X-ray maps using SEM.



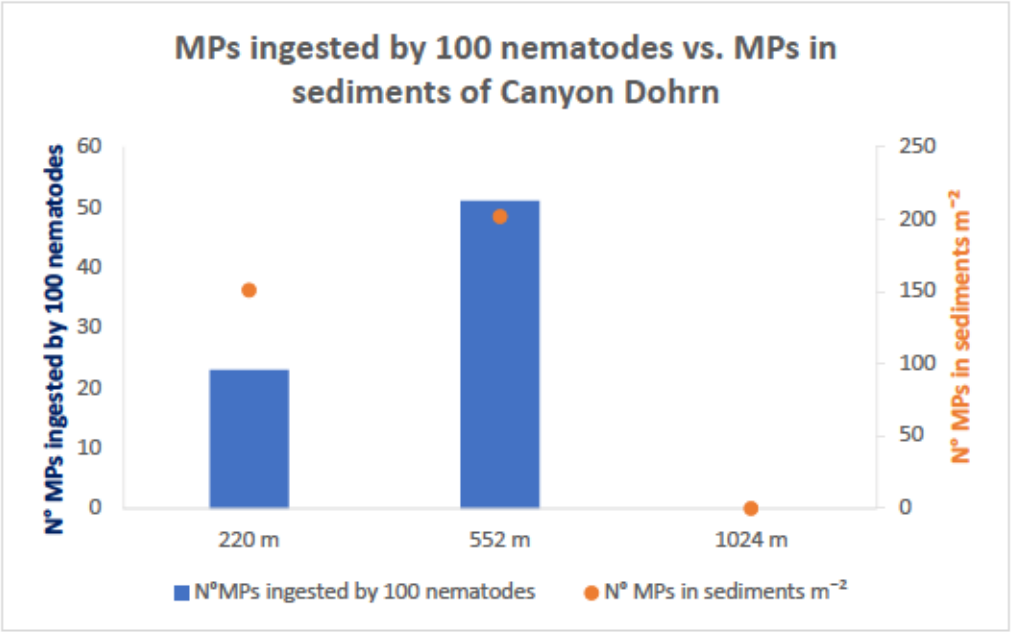
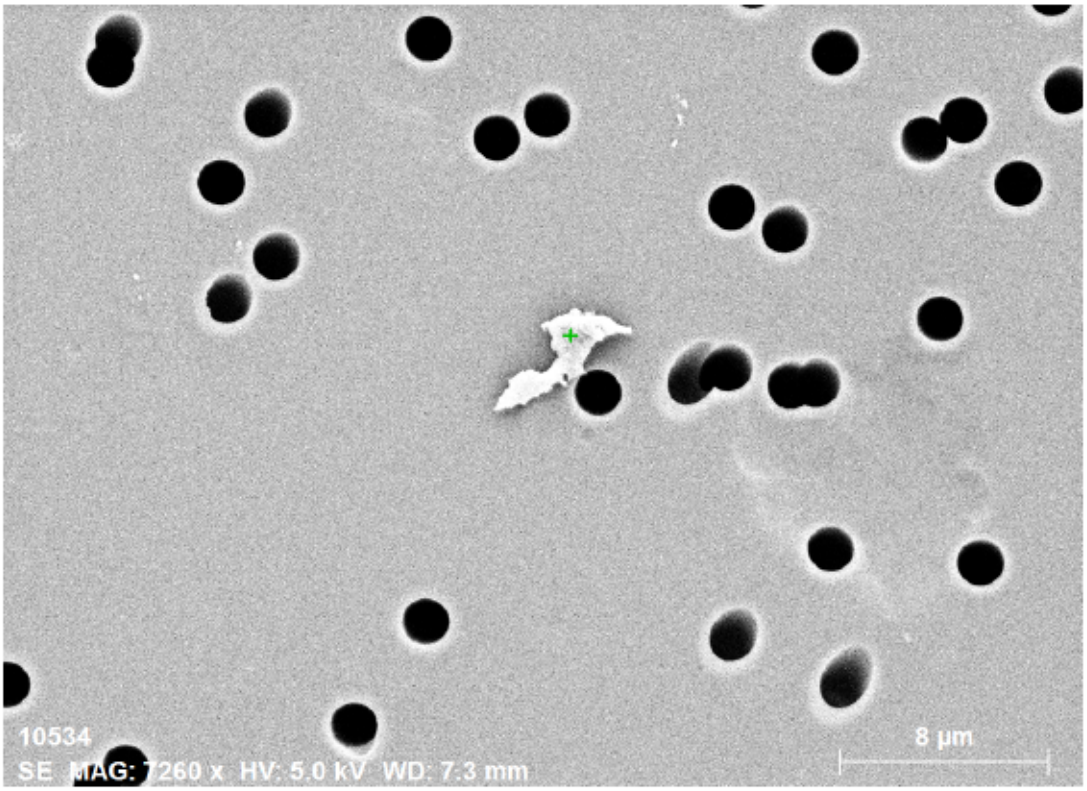


Figure 14. Microplastics ingestion in nematodes extracted from the sediments of the canyon Dohrn and relative microplastics' abundances in the sediments.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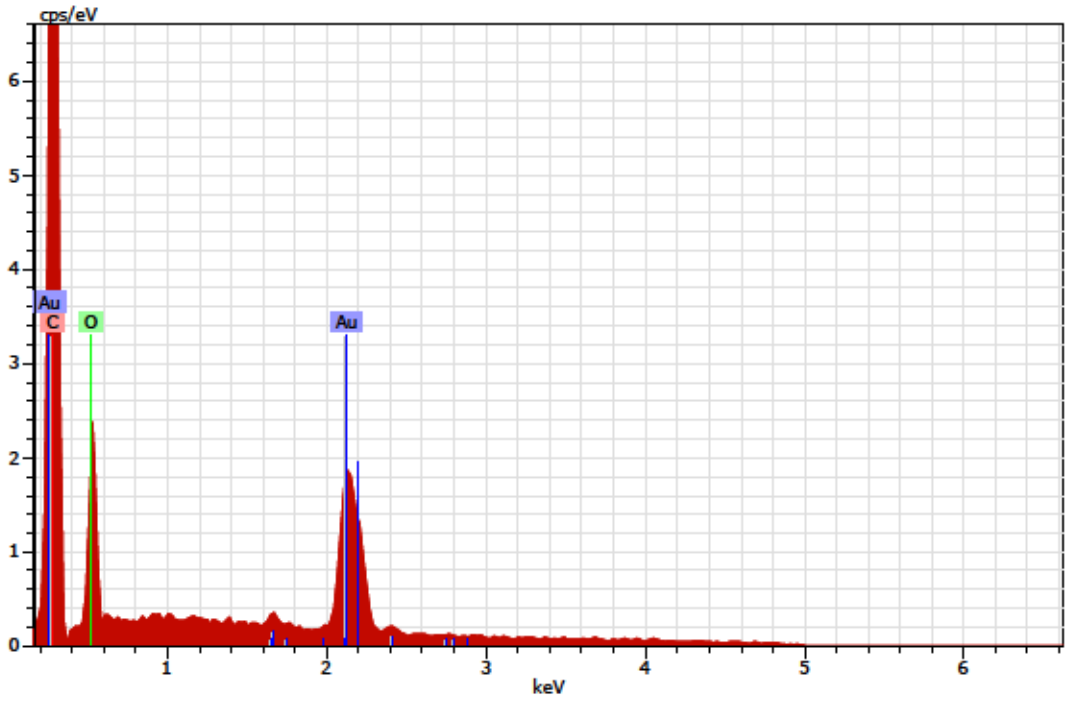
8 µm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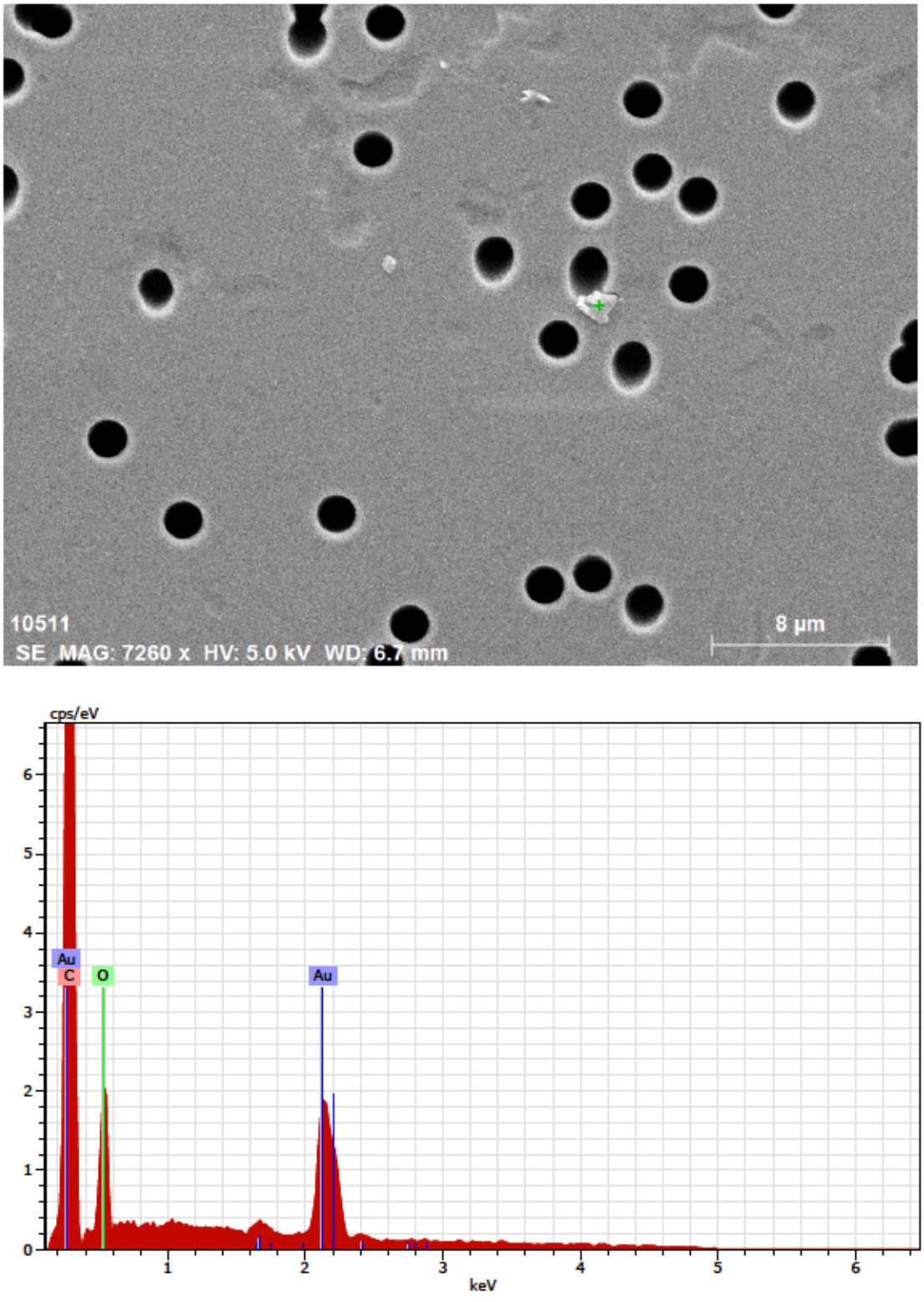

Figure 15. Scanning electron microscopy images of microplastics with its relative X-ray spectra

Discussion

This study provides new data on microplastics' contamination in deep-sea sediments from different geographical areas, including submarine canyons of the Mediterranean Sea.

Results reported for deep-sea samples (> 500 m depth) reveal that ca. 27-75 % of the microplastics are represented by polymers with a 2-20 μm size, highlighting that microplastics with smaller size than 20 μm could represent the dominant fraction of the microplastics in the deep-sea floor.

Among the deep-sea samples analysed those from NE Atlantic and Southern Oceans were characterized by the highest abundance of microplastics with size 20-1000 μm compared to the other investigated areas whereas Black Sea and Caulonia canyon (Ionian Sea) sediments contained the lowest values. Conversely, considering the smaller fraction (2-20 um) of microplastics, Squillace Canyon and Black Sea sediments showed the highest contamination (on overage, ca.$4x10^6$ particles).

Comparisons with bibliographic data is not always possible due to the different extraction procedures, target size range and typology of plastic fragments considered in the literature studies (Woodall et al. 2014; Fischer et al. 2015). However, considering our highest estimates, microplastics concentrations seem to be much higher than values found in Arctic sediments obtained by using a method developed for river sediments (from 3200 to 173.200 items/m^2; Bergamann et al. 2017), especially when the smaller microplastic fraction was considered.

Our procedure specific for deep-sea sediments resulted in a yield of 60%, therefore we applied a correction factor to microplastic abundances to obtain more actual data. Also not considering the correction factor, the discrepancy among our data from Arctic sediments and those reported by Bergmann et al. (2017) resulted to be evident. This discrepancy is difficult to be explained, however the different procedures utilized, and the sample variability could justify it.

Another crucial issue in the identification and determination of microplastic particles smaller than 20 μm is hamperd by technological limitations. Indeed, Raman spectoscopy is very expensive and its application to replicated samples for ecological investigations is very complicated. In addition, Raman spectroscopy is based on fluorescence so this can produce artefacts and can interfere with sample analyzes if they are not processed in the right way (i.e elimination of organic matter attached to particles). In this study we used SEM and microanalysis to assess the smallest plastic particles, but this protocol couldn't be applied on

all samples because it's time consuming therefore we had to make estimates consifering a homogenous distribution on the sample.

If our estimates will be confirmed, the contamination in deep-sea floors especially due to smaller microplastics could be huge.

Microplastic abundances determined in the present study (20-1000 μm) are more similar to the estimates previously reported for NE Atlantic Ocean (ca. 400 items/m^2; Van Cauwenberghe et al. 2013). Despite the high levels of pollution of the Black Sea, we found that the number of microplastics in these sediments was relatively low when the fraction between 20 and 1000 μm were considered. Conversely, microplastics abundance in DHAB ecosystems was unexpectedly high, despite these samples are separated from the overlying waters through brine layers (i.e., originating from the dissolution of ancient subterranean salt deposits from the Miocene period) that should represent a physical barrier also for microplastics. These findings suggest that the distribution patterns of microplastics in deep-sea ecosystems must be still elucidated due to the wide range of factors influencing their transport and accumulation.

Microplastics extracted from deep-sea sediments were mostly characterized by a range size 2-500 μm, whereas the largest fragments contributed for a lesser extent (ca. 30%) except for NE Atlantic Ocean.

Nylon and polymethyl acrylate (PMA) were the most abundant polymers in deep-sea sediments comparing different geographic areas and habitats. Nylon followed by polypropylene and polyester were also the most abundant polymers in Mediterranean sediments. Nylon, PMA and polyester have a density (from 1.15 g cm^{-3} and 1.39 g cm^{-3}) exceeding seawater density (1.02 – 1.03 g cm^{-3}), therefore the direct sinking to seafloor can explain their accumulation in deep-sea floor. Conversely, polypropylene has a lower density (0.65 g cm^{-3}) than seawater, thus a combination of processes including eddies and wind mixing, biofouling, incorporation in sinking aggregates and vertical transport with biota, by modifying its buoyancy, might justify their transport to the seafloor.

Polypropylene is one of the most widely demanded plastic type in Europe (PlasticsEurope 2016) and widely used for packaging and fishing gear (Andrady et al. 2015). Only few studies analysed the composition of the polymers identified in deep sea (Bergmann et al. 2017; Woodall et al. 2014). Our results partially match with the findings from these previous investigations, which reported polyester as the dominant polymer in NE Atlantic, Mediterranean, SW Indian and subarctic sediments (Woodall et al. 2014).

Surprisingly, PLA bioplastic was found in Deep-sea Hyperaline basin at depth of 3027 m and it is the first record of this type of plastic in deep-sea sediments. These polymers, being bioplastics, should degrade quickly. We hypothesize that hypersaline and anoxic conditions of DHAB could preserve the relatively high abundance of polymers and the degradable plastics found this extreme ecosystem.

To better elucidate the preferential accumulation of microplastics in peculiar topographic structures, such as submarine canyons, we analysed the distribution and polymer composition of microplastics in six Mediterranean canyons from Ionian and Tyrrhenian Seas. The sediments of San Gregorio Canyon, especially in the middle part, contained the highest abundances of microplastics while in the other canyons microplastic abundances varied within a narrow range and were comparable with the values observed in other habitats from Mediterranean Sea, NE Atlantic, Southern, Arctic Oceans and Black Sea.

The "canyon effect" (i.e., accumulation of microplastics in the mouth of the canyons) was observed only in three canyons (Squillace, Augusta and Amendolara) whereas in the other canyons the highest abundances of microplastics were found in the middle part or in the head of the canyons (Dohrn San Gregorio and Caulonia canyons, respectively).

We could not identify any significant relationship with water depth and any clear pattern with the distance from the coast line. Previous investigations in deep-sea benthic ecosystems, reported positive correlations between microplastics abundances and chlorophyll a suggesting a vertical export via incorporation in sinking algal aggregates (Bergamann et al. 2017). In the present work, we did not analysed the influence of biotic factors in driving the distribution of microplastics, however the lack of clear bathymetric patterns and coast-open sea gradients, especially in Mediterranean canyons, suggests that a wide range of factors might influence microplastics distribution such as morphology of the canyon, incline of the continental slope, proximity of the head of the canyon to the rivers and oceanic circulation and deep-sea currents. Further studies are needed to investigate the contribution of biotic and abiotic factors in the distribution patterns of microplastics.

Accordingly, the polymer composition did not show a clear pattern with these gradients, however we observed that the number of polymer classes was significantly related ($y= 0{,}0077x + 1{,}4951$, $R= 0.825$) to microplastic abundances. Additionally, we found that the quantitative relevance of microplastics was related to the ingested fragments from nematodes.

For the first time we demonstrated that microplastics can be swallowed by the most abundant metazoan phylum (80-90% of total faunal abundance; Vincx et al. 1994, Lambshead and Schalk 2001, Lambshead 2004) in deep-sea sediments: Nematoda. Nematodes, are also the most diverse metazoans of all oceans with a key role for the functioning of deep-sea food webs (Danovaro et al. 2008). If confirmed that microplastics can impact nematode assemblages, this could have relevant implications also in maintaining biodiversity and functions in deep-sea benthic ecosystems.

Conclusions

This study provides new insights in the distribution of microplastics in deep-sea environments, making an important contribution to the scarce bibliography present for this topic. Our findings revealed that microplastics is widely present in every type of deep-sea habitats, and that canyons can act as conduits for microplastics facilitating the accumulation of these contaminants, but this is not the rule. Among our samples, NE Atlantic sediments were the most contaminated area analzying the microplastics fraction between 20-1000 μm and Black Sea when fraction between 2 and 20 μm was examined, but the Southern Ocean and S. Gregorio sediments presented the wide range of plastic polymers. The microplastics' distribution in deep-sea sediments is more complex than expected and oceanographic processes and topographic characteristics should be taken into account for a better understanding of the dynamics of these contaminants in these ecosystems. The ingestion of microplastics from nematode such as from other deep-sea benthic communities, as previously reported in literature, further highlight the need to better comprehend the dynamics, interactions and interference of microplastics with deep-sea marine organisms and their functions for improving the management of this anthropogenic impact in the largest ecosystem on Earth.

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5. IMPACT OF MICROPLASTICS ON DEEP-SEA CORALS (*Corallium rubrum*): A MESOCOSM EXPERIMENT

Introduction

Marine plastic pollution is a major environmental issue and the impact on marine life is a growing concern worldwide (Paul-Pont et al. 2018). Ingestion of plastics by marine organisms has been reported as the most serious among the biological effects of plastic pollution (Kuhn et al. 2015; Gall et al. 2015). This impact has the potential to have far-reaching implications at the population level, representing a threat to the conservation of the affected species (Reid et al. 2013) and to the functioning of marine ecosystems (Katija et al. 2017; Cole et al. 2016; Cole et al. 2015). Especially, microplastics can severely impact marine life as due to their small size they fall within the optimal pray range for many marine animals (Wright et al. 2013).

So far, most of the available information dealing with the responses of marine organisms to plastics contamination is focused on shallow-water and coastal ecosystems (Setälä et al. 2018; Lamb et al. 2018; Rochman et al. 2015; Sussarellu et al. 2016). Conversely, the impact of plastic debris on deep-sea organisms has been less investigated (Carreras-Colom et al. 2018; Courtene-Jones et al. 2017), potentially due to the logistic difficulties associated with the remoteness of abyssal-hadal ecosystems (Taylor et al. 2016; Corinaldesi 2015). Studies, indeed, investigating ingestion of microplastics by deep-sea organisms, including invertebrates, and their impact level are still very limited (Anastasopoulou et al. 2013, Lusher, 2015, Taylor et al. 2016, Courtene-Jones et al. 2017).

Since the plastic waste reaching our oceans is increasing, and a large portion of this will likely end up on deep-sea floor or buried within sediments, the potential impact on deep-marine organisms is expected to be pervasive (Jambeck et al. 2015).

Invertebrates like the red corals can provide important information about the extent of microplastic impact on deep-sea organisms because they are filter feeders and each polyp can remove up to 2.4 prey per day (Tsounis et al. 2005). Despite these organisms rely on chemoreception to capture prey and their feeding responses are stimulated by prey (Lenhoff and Heagy, 1977), available information on tropical corals reveals that they mistake microplastics for prey and can ingest up to ~50 μg plastic cm^{-2} h^{-1}, similarly to the consumption of plankton and *Artemia nauplii* in experimental feeding assays (Hall et al. 2015). Other recent

investigations, confirmed that plastic debris can harm reef-building corals also increasing their susceptibility to microbial disease (Lamb et al. 2018).

The red corals, such as *Corallium rubrum* (Linnaeus, 1758), are long-lived, slow-growing species, distributed throughout the Mediterranean Sea and adjacent Atlantic coasts, where can be found between 10 and 800 m depth but more commonly between 30 and 200 m (Rossi et al. 2008, Follesa et al. 2013). *Corallium rubrum* is a key species in the maintenance of the structural complexity of the Mediterranean habitats, and of their biodiversity and ecosystem functioning (Levin et al. 2001, Danovaro et al. 2008). Declines in the abundance of long-living and habitat-forming species can therefore lead to a rapid fragmentation in community structure, species and habitat loss, thus affecting ecosystem functioning (Hughes et al. 1994).

Due to the progressive impoverishment of *Corallium rubrum* populations during the last two decades caused by over-exploitation, especially for its relevance in the jewellery market worldwide (Tsounis et al. 2007), and temperature driven mass mortality events, this species has been defined as endangered and included in the IUCN red list of Anthozoans in the Mediterranean and subjected to national legislations to regulate Red Coral fishing (Otero et al. 2017, IUCN).

Considering the evidence of the global impact of plastics on marine ecosystems, in the present study we tested the hypothesis that microplastics can severely threat the functions of red corals, thus contributing to reduce its abundance and biomass. In particular, we performed a mesocosm experiment exposing *Corallium rubrum* colonies to different concentrations of the most common plastic polymers in marine environment, and assessed their responses in terms of feeding rate, stress (gene expression responses and mucus production), physical damage (% of damaged tissue) and changes in terms of abundance and composition of microbial assemblages associated with the corals.

We also hypothesize that red corals can be impacted indirectly through the ingestion of prey, which in turn incorporates microplastics, with further implications on deep-sea trophic webs.

Materials and Methods

Corals' sampling and experimental setup

Coral specimens were collected at the Marine Protected Area of Portofino (Ligurian Sea, Mediterranean Sea). After recovery, the coral specimens were brought to the laboratory and maintained in a tank (30 L) at in situ temperature (13 ± 0.8 °C) and subjected to continuous seawater flux filtered onto 0.7 µm by using two submersible pumps (Euronatale, 203 V, 50 Hz, 4 Watt). For the experimental study, the fragments of *C. rubrum* were transferred to 9 glass tanks (12 L; 34.5 cm long × 23.5 cm wide × 24.5 cm high) in a temperature-controlled container, in darkness conditions, where colonies were maintained for three days to allow acclimation before the start of the experiment. Forty-five fragments containing approximately the same number of polyps (on average 238 coral polyps for each tank), were equally distributed in the glass tanks.

The glass tanks were equipped with an aeration system for each tank, which allowed us to create laminar current in seawater in order to provide a homogeneous distribution of microplastics. We tested the effect of a mixture of microplastics (MPs) using the polymers most commonly found in marine environment (i.e., polypropylene, polyethylene, polystyrene, polyvinylchloride and polyethylene terephthalate, Paul-Pont et al. 2018). Three different concentrations of microplastics (100 items L^{-1}, 500 items L^{-1} and 1000 items L^{-1}) were added to the tanks containing red coral fragments in order to evaluate the potential effects due to increasing microplastic concentrations whereas further fragments were not exposed to microplastics. As an additional control, we also used three glass tanks filled with seawater and microplastics but without red corals.

The experiment was carried out for 2 weeks and it was stopped when the corals exposed to plastics (especially to 1000 items L^{-1}) did not show signs of vitality (i.e. the corals did not feed, and the polyps were not active anymore).

Mucus production, and determination of microplastics and prokaryotes incorporated in mucus

To evaluate the first symptoms of coral stress, a photographic report was conducted daily. Microplastic abundance trapped in mucus was estimated using an enzymatic digestion protocol's (Cole et al. 2014) with some modifications. Mucus produced by corals exposed to higher microplastics concentrations, was dried in oven at 60°C for 12 h. After 12 h, five ml of

homogenizing solution was added to the samples and incubated at 50°C for 15 minutes. Proteinase K (41 U/mg) was added and the samples were incubated at 50 °C for 2 hours. Then, we homogenized samples and we incubated again at 60° C for 20 minutes, after that samples were sonicated three times. After digestion the microplastic-containing suspension was filtered on 0.2 µm filter in a vacuum filtration system. Filter was analysed at stereomicroscope at 50X of magnification (Zeiss Stemi 2000).

Prokaryotic abundances in mucus were analyzed by epifluorescence microscopy after the extraction of prokaryotic cells from the mucus using pyrophosphate (final concentration, 5 mM) and ultrasound treatment (three 1-min treatments using a Branson Sonifier 2200; 60W) (Danovaro, 2009). Samples were then diluted from 50- to 100-fold with sterile water (filtered through 0.02-µm-pore-size filters) and filtered onto 0.2 µm pore size filters (Anodisc filters (black-stained polycarbonate)). The filters were stained using SYBR Green I (10000× in anhydrous dimethyl sulfoxide, Molecular Probes-Invitrogen) diluted 1:20 in pre-filtered TE buffer (pH 7.5) and incubated in the dark for 20 min and mounted on glass slides with a drop (20 µm) of antifade solution (50% phosphate buffer (6.7 mmol L-1; pH 7.8) and 50% glycerol containing 0.5% ascorbic acid (Noble and Fuhrman, 1998)) onto glass slide and mount the filter on the drop. Prokaryotic counts were performed under epifluorescence microscopy (magnification, 1000X; Zeiss filter set #09, 488009-9901-000, excitation BP 450–490 nm, beam splitter FT 515, emission LP 520), by examining at least 20 fields per slide and counting at least 400 viral particles per filter.

RNA extraction and cDNA synthesis

Each coral branch was sampled in sterile conditions to avoid any contamination and washed twice with 5 ml of sterile artificial seawater pre-filtered through 0.2-µm-pore-size filters (FSW; Whatman Anodisc, GE Healthcare, Buckinghamshire, UK) and then frozen in liquid nitrogen and immediately kept at -80 °C. Experiments were conducted in triplicate using corals collected from three different water tanks. Total RNA was extracted from 20 mg of *C. rubrum* epidermis using Quick-RNA™ MiniPrep (Zymo research, Freiburg, Germany) according to the manufacturer's instructions. Once scraped, coral tissues were placed in new 2 ml sterile tubes and washed three times with phosphate buffered saline (PBS 1X PH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Samples were centrifuged at 1800 rcf for 10 min in Eppendorf® 5810r refrigerated centrifuge using a swing-out rotor at 4◦ C, and after removing the supernatant, were homogenized for 5 mins with a RNase free sterile glass stick in RNA lysis

buffer. Contaminating DNA was degraded by treating each sample with a DNase enzyme dissolved in RNase-free water included in the kit according to the manufacturer's instructions. The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies, Wilmington, Delaware). The integrity of RNA was evaluated by agarose gel electrophoresis. Intact rRNA subunits (28S and 18S) were observed on the gel indicating minimal degradation of the RNA. For each sample, 150 ng of total RNA extracted was retrotranscribed with an iScriptTM cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. Synthesized cDNA was used in real-time qPCR experiments with a dilution of 1:2. To evaluate the efficiency of cDNA synthesis, a PCR was performed with primers of the reference gene, cytochrome oxidase I (COI). The reaction was performed on the Veriti™ 96-Well Thermal Cycler (Applied Biosystem, Monza, Italy) in a final volume of 25 µl with 5 µl of 5X PCR reaction buffer (Bioline, Luckenwalde, Germany), 1µl of 5U/µl of MyTaq™ HS DNA Polymerase (Bioline, Luckenwalde, Germany), 10 µM of each oligo, template cDNA and nuclease free water. The PCR program consisted of a denaturation step at 95 °C for 1 min, 35 cycles at 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s and a final extension step at 72 °C for 10 min.

Gene expression by real-time qPCR

For all real-time qPCR experiments, the data from each cDNA sample were normalized using COI as the endogenous control level according to Haguenauer et al. (2013), whose level remained relatively constant between control and stress conditions. The stability of the housekeeping gene in real-time PCR was checked with Bestkeeper software by using repeated pair-wise correlation analysis (Pfaff 2004). The expression level of five genes, previously analysed by Haguenauer et al. (2013) in *C. rubrum* in response to heat stress, were followed by real-time qPCR.

Whereas, for the *cytb* (Cytochrome b) gene, target-specific primer pairs were designed with primer 3 (http://primer3.ut.ee; Untergasser et al., 2012) on the basis nucleotide sequences retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) for *C. rubrum* (Table 1). The specificity of amplification reactions was verified by melting curve analysis. The efficiency (E) of each primer pair was calculated according to standard methods curves according to the equation: E=10-1/slope. Five serial dilutions were set up to determine Ct values

and reaction efficiencies for all primer pairs: 1:1, 1:5, 1:10, 1:50 and 1:100. Standard curves were generated Ct values versus the logarithm of each dilution factor. PCR efficiencies were calculated for control and target genes and ranged from 1.8 to 2.

Table 1. Gene name, acronym, accession numbers, primer sequences (5'=>3') and lengths of PCR amplified fragments are reported for the reference gene and the genes analyzed. For the genes that have a reference, the lengths of PCR fragments were not reported.

Gene	Acc. Number	Primer	Sequence (5'=>3')	PCR fragment (bp)
Cytochrome oxidase I *(COI)**	NC_022864.1	Cr_COI_For	GTTCCCACCGGGATTAAGAT	
		Cr_COI_Rev	CTCCGGTTAAACCACCAATG	
Heat shock protein 70 *(hsp70)**		Cr_HSP70_For	TCGACCCAAAGTTGAAGTCC	
		Cr_HSP70_Rev	TGCATCTTTTGTCGCTTGAC	
Heat shock protein 60 *(hsp60)**		Cr_HSP60_For	TATCGCCAAGGAAGGTTTTG	
		Cr_HSP60_Rev	TCTTCCGGGGTTGTTACTTG	
Manganese superoxide dismutase *(MnSOD)**		Cr_MnSOD_For	ATGGGGATGGCTTGGTTATT	
		Cr_MnSOD_Rev	ATACCGAACAAAGGCACCAG	
Mismatch repair protein *(mtMutS)**		Cr_MtMus_For	ATAAGCCGGATGTTCCTAGTGTA	
		Cr_MtMus_Rev	CCATTTGAAGCAAGGATCTTTTA	
Elongation factor-1 *(EF1)**		Cr_EF1_For	CTCCATCTGCCATTTCCACT	
		Cr_EF1_Rev	GCTGCTTTTGGTGGATCATT	
Cytochrome b *(cytb)*	NC_022864.1	Cr_Cytb_F2	TGGGAGCTAGTATCTTGGTGC	159
		Cr_Cytb_R2	GGTTCCTCTACCGGGTTAGC	

Genes encoding for heat shock protein 70 (*hsp70*), heat shock protein 60 (*hsp60*), manganese superoxide dismutase (*MnSOD*), mismatch repair protein (*mtMutS*), elongation factor-1 (*EF1*), cytochrome b (*cytb*), were selected for their fundamental role in response to various types of stress within cells (Table 2).

Table 2. Function of the genes analyzed in the present study

Gene Name	Acronym	Function	Reference
Heat shock protein 70	*hsp70*	chaperonins involved in folding new polipeptide chains	Hartl (1996)
Heat shock protein 60	*hsp60*		
Manganese superoxide dismutase	*MnSOD*	antioxidant activity, scavenger of free radicals	Bresciani et al., 2015
Mismatch repair protein	*mtMutS*	DNA repair	Bilewitch and Degnan (2011)
Elongation factor-1	*EF1*	delivery of aminoacylated tRNA to the elongating ribosomes during protein synthesis	Sasikumar et al., 2013
Cytochrome b	*cytb*	component of the electron transport system	Larosa and Remacle (2018)

The expression level of these genes was followed by real-time qPCR. Diluted cDNA was used as a template in a reaction containing a final concentration of 0.4 µM for each primer and 5 µl of 2X SensiFAST™ SYBR® & Fluorescein mix (Bioline, Luckenwalde, Germany) in a total volume of 10 µl for each well. PCR amplifications were performed in a CFX Connect™ Real-Time PCR detection system (Biorad, Milan, Italy) thermal cycler using the following thermal profile: 95 °C for 2 min, 1 cycle for cDNA denaturation; 40 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 20 sec for amplification; 1 cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all real-time qPCR reactions were performed in triplicate. Fluorescence was measured using CFX Manager™ software (Biorad, Milan, Italy). All genes tested by qPCR in this study was amplified with primers purchased from Life Technologies/Thermo Fisher Scientific (Milan, Italy)

The fold change in target gene mRNA expression of corals exposed to microplastics compared with the control was calculated using the $2^{-\Delta Ct}$ equation (Schmittgen and Livak, 2008).

Scanning Electron Microscopy (SEM)

Storage and preparation of samples for SEM analysis were performed according to different protocols (Goff et al. 2017; Luna et al. 2007). One coral nubbin from each mesocosm was stored in 0.7 µm pre-filtered sea water with 4% formalin. After 24 h, samples were washed with 0.7 µm pre-filtered sea water and dehydrated for 3 h in 20% ethanol. After 3 h they were washed in the same way and dehydrated in ethanol 50%. After 3 h, samples were stored in ethanol 70%. Samples were stored at +4°C. We dried samples using different gradients of ethanol solutions (70-80%,80-90%, 90-95%, 95-99%) in a couple of days (Luna et al., 2007). After that, we prepared samples using HMDS (Hazrin-Chong et al. 2012; Hexamethyldisilazane, Aldrich 440191). Dehydrated samples were mounted on aluminium stubs using Leit-C glue (conductive

carbon cement, Neubauer Chemikalien) and sputter-coated with gold. Samples were examined with a scanning electron microscope Zeiss SUPRA 40). In addition, using PhotoQuad v1.4 software, SEM photos at 200X of magnification have been used for determining tissue damages in three different time intervals during the experiment: at the start of experiment (T0), after 7 days (T 1/2) and at the end of experiment (T final). PhotoQuad v1.4 is a custom software for advanced image processing of 2D photographic quadrat samples, dedicated to ecological applications (Trygonis and Sini 2012). The percentage of tissue damage was provided with the software PhotoQuad v1.4.

Corallium rubrum's feeding rates

To assess the impact of microplastics on coral activity we measured the feeding rate (clearance) of the polyps according to previous protocols (Tsounis et al. 2010; Gori et al. 2015; Lewis et al. 1976). Artemia salina nauplii were obtained incubating 1 g of cysts (Ocean Nutrition) in 1 L of seawater filtered onto 0.2 µm filter in a separatory funnel, two days before the analysis of feeding rate (i.e., clearance rate). The day of the hatching, naupli were counted and maintained in vials to obtain the concentration of 1000 nauplii L^{-1}.

Clearance rate was measured in 1L beakers (a beaker for each treatment and control) containing nubbins and seawater taken from the tanks. The experiments started at t0 with the addition of alive *A. salina* nauplii (at a concentration of approximately 1000 nauplii L^{-1}). To avoid stress, corals were positioned in the bakers keeping them completely submersed. After the start of the experiment (~30 s after), three aliquots of 10 ml seawater were collected after 2 and 4 hours during the first week of exposure (day 2) and during the second week of exposure (day 10) and preserved in pure buffered formalin (4%). Samples were counted in Delfuss cuvettes. Three tanks containing only seawater and nauplii (without corals) were also used to evaluate the possible removal of nauplii due to their sinking to the beaker bottom.

Microplastics ingestion from Corallium rubrum

Microplastic abundance retained in the coral polyps was evaluated by dissolving polyp and skeleton by using an acid/base digestion protocol's (Allen et al. 2017) with some modifications. The solution obtained was then filtered. We soaked colonies in 5 ml of 4.5% sodium hypochlorite (NaClO) for 24 h, then dissolved the skeletons in 5 ml of 37% HCl for 30 min. Particulate material was retained on a 0.2 µm filter in a vacuum filtration system and then analysed under a stereomicroscope at 50× magnification (Zeiss Stemi 2000). To evaluate possible acid/base damage to plastic polymer's used in the experiment (polypropylene, polyethylene, polystyrene, polyvinylchloride and polyethylene terephthalate) we exposed polymers at the same volume and concentration of NaClO and HCl during digestion.

Effects of microplastics on Artemia salina

Additional tests were performed, contextually to the experiments to determine *C. rubrum* feeding rates, in order to assess the possible impact of microplastics on *Artemia salina* as vector of microplastics for the corals.

Ingestion of microplastics from *A. salina* was evaluated using an enzymatic digestion protocol's (Cole et al. 2014) with some modifications. The individuals exposed to microplastics were collected at the same intervals considered for determining feeding rates of *C. rubrum*. Then, they were sorted under stereomicroscope, observed for their gut content and photographed. Finally, nauplii were processed immediately according to the modified enzymatic digestion protocol. Briefly, 100 individuals of *A. salina* were dried in oven at 60°C for 3 h. After 3 h, a homogenizing solution was added to the samples and incubated at 50°C for 15 minutes. Proteinase K (41 U/mg) was added and the samples were incubated at 50 °C for 2 hours. Then, we homogenized samples and we incubated again at 60° C for 20 minutes, after that samples were sonicated three times. After digestion the microplastic-containing suspensions were placed in Utermöhl chambers and the microplastics were examined at the inverted microscope (Axiovert 200, 20X magnification), counted, measured and categorized by colors and shape when possible in order to evaluate the abundances and sizes of micropastics ingested by *A. salina* during the experiment.

Prokaryotic abundance in surrounding seawater

We collected three replicates of 10 ml of seawater from the tanks. Total prokaryotic counts in water were performed using the SYBR Green I direct count procedure (Danovaro, 2009). Water samples were filtered onto 0.2 µm pore size filters (Anodisc filters (black-stained polycarbonate)) into the funnel with vacuum pressure no greater than 20 kPa (or 150 mmHg) to avoid destruction of cells. When the sample had passed through, filters were stained with 20 µl of SYBR Green I ((10000× in anhydrous dimethyl sulfoxide, Molecular Probes-Invitrogen) diluted 1:20 in pre-filtered TE buffer (pH 7.5) and incubated in the dark for 20 minutes. Then, to remove the excess of stain, filters were washed 3 times using 3 ml of Milli-Q water and finally filters were mounted onto microscope slides placing a drop (20 µl) of antifade solution (50% phosphate buffer (6.7 mmol L-1; pH 7.8) and 50% glycerol containing 0.5% ascorbic acid (Noble and Fuhrman, 1998))onto glass slide and mount the filter on the drop. Spot 20 µL antifade solution onto a clean coverslip, flip it over to cover the sample filter. Prokaryote direct counts were carried out under epifluorescence microscopy (magnification, 1000X; Zeiss filter set #09, 488009-9901-000, excitation BP 450–490 nm, beam splitter FT 515, emission LP 520). At least 20 microscope fields and 400 cells were counted randomly using epifluorescence microscopy with blue excitation.

Microbiome composition in C. rubrum exposed to microplastics

Corallium rubrum samples were collected to evaluate changes of microbiome composition from the beginning to the end of the experiment. After collection, coral samples were immediately frozen at – 20°C. Tissue corals were scraped from the skeleton and DNA extraction was performed using QIAGEN DNeasy Blood & Tissue Kit. In brief, samples were digested with proteinase K at 56 °C overnight or until the tissue was completely lysed, then samples were processed following the manufacturer's protocol. Finally, samples were held at − 20 °C before PCR amplification and sequencing. The molecular size and the purity of the DNA extracts were analyzed by agarose gel electrophoresis (1%) and the DNA was quantified by Nanodrop spectrophotometer (ND-1000). Primers used for PCR amplification of the 16S V3 region were 805R and 341F (Klindworth et al. 2013), with Illumina-specific primers and barcodes. Sequencing was performed on an Illumina MiSeq platform by LGC Genomics GmbH (Berlin, Germany).

Corals'microbiome was analysed for determining if microplastics at different concentrations could cause a shift in bacterial assemblages associated with coral tissues. Corals microbiome

was analysed at the start of experiment (T0) and at the end of experiment both for control nubbins (Ctrl Tf) and treatments (100 items L^{-1} Tf, 500 items L^{-1} Tf and 1000 items L^{-1} Tf). For bioinformatic analysis paired reads were joined, quality-filtered and assigned to taxa using the QIIME2 pipeline, as previously described (Bolyen et al., 2018). Amplicon sequence variants (ASVs) were identified through the DADA2 strategy (Callahan et al. 2016). Sequences were randomly resampled in the ASV table to enable comparison between samples, by normalizing the number of sequences between samples to the sample with the fewest sequences (n=15,000). Taxonomic affiliation of ASVs was performed within the QIIME2 pipeline using the VSEARCH-based consensus taxonomy plugin (Bokulich et al. 2018) on the SILVA database v132 (Quast et al. 2012) after the extraction of the portion of the reference 16S sequences amplified by the primers used for the analysis.

Statistical analyses

Differences in feeding rates and prokaryotic abundances were analysed through PERMANOVA (Anderson et al. 2008) according to a two-way experimental design. P- value was provided using unrestricted permutation of raw data. When low unique values in the permutation distribution were available, asymptotical Monte Carlo P-value were used instead of permutational P-values. Microbiome composition in corals was investigated by classification-clustering based on the Bray-Curtis similarity of transformed quantity data. All the analyses were performed using PRIMER 6 and PERMANOVA+ (Anderson et al. 2008). Significant differences in mRNA expression were determined using One-way ANOVA, with Tukey's Multiple Comparison Test to identify concentrations that elicited a significant effect relative to the control. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software).

Results

Mucus production, microplastics incorporated in mucus and prokaryotic abundances in mucus

A first signal of the impact of the microplastics was observed during the second day of exposure in corals exposed to 500 items L^{-1}. The quantity of mucus produced by the corals was larger at increasing concentrations of MPs (Fig. 1). We found that mucus entrapped ca. 43 MPs per ml of mucus with a size ranging from 300 to 1000 µm. We estimated that in our experimental systems coral mucus could entrap 2.23 % of the microplastics added to the systems. At the end of the experiment, prokaryotic abundance increased significantly at higher microplastics concentration than at lower concentrations ($4.04\pm 0.65x10^6$ vs. $2.36\pm 0.54x10^6$) (Fig.2).

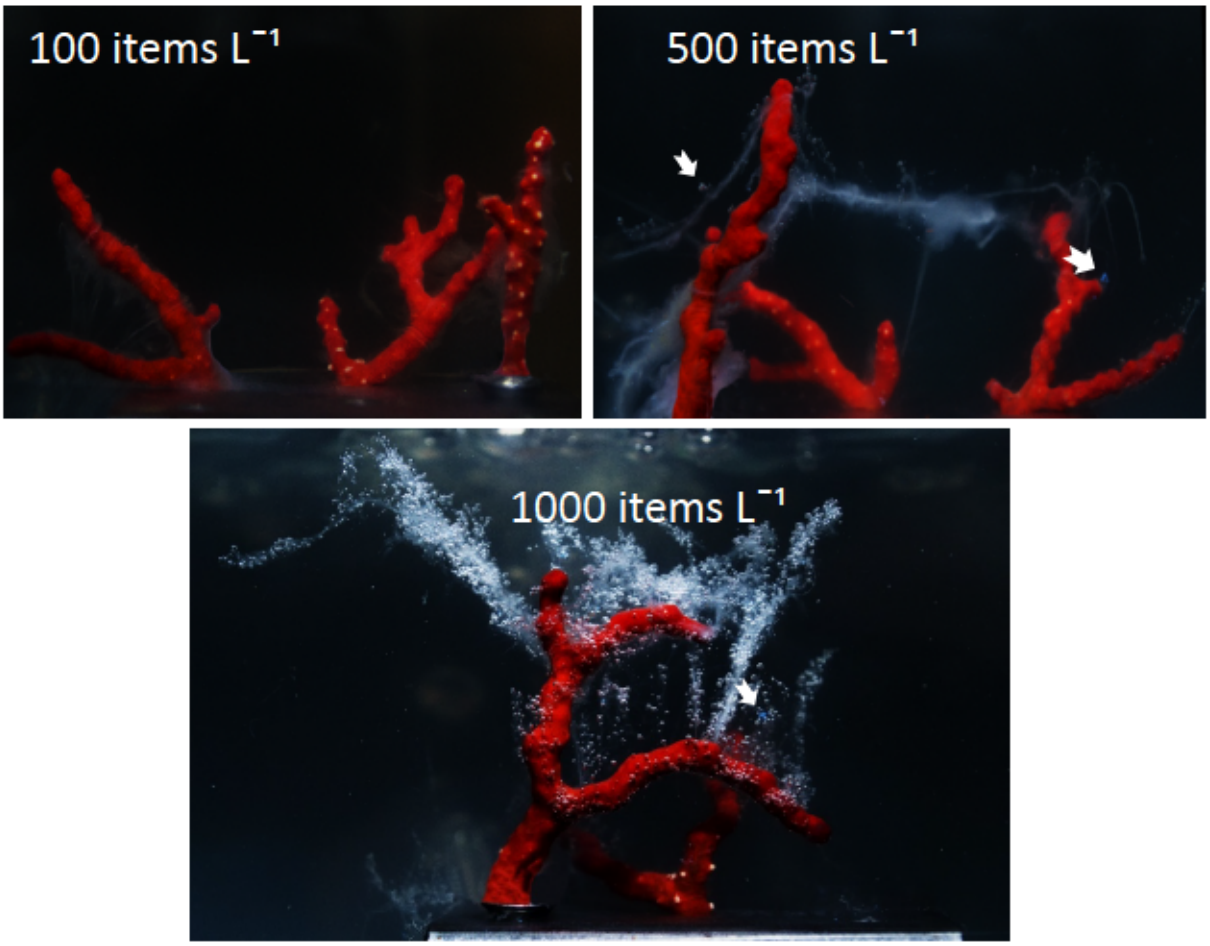


Figure 1. Photographs of mucus produced by corals exposed to microplastics. White arrows indicate microplastics items trapped by mucus

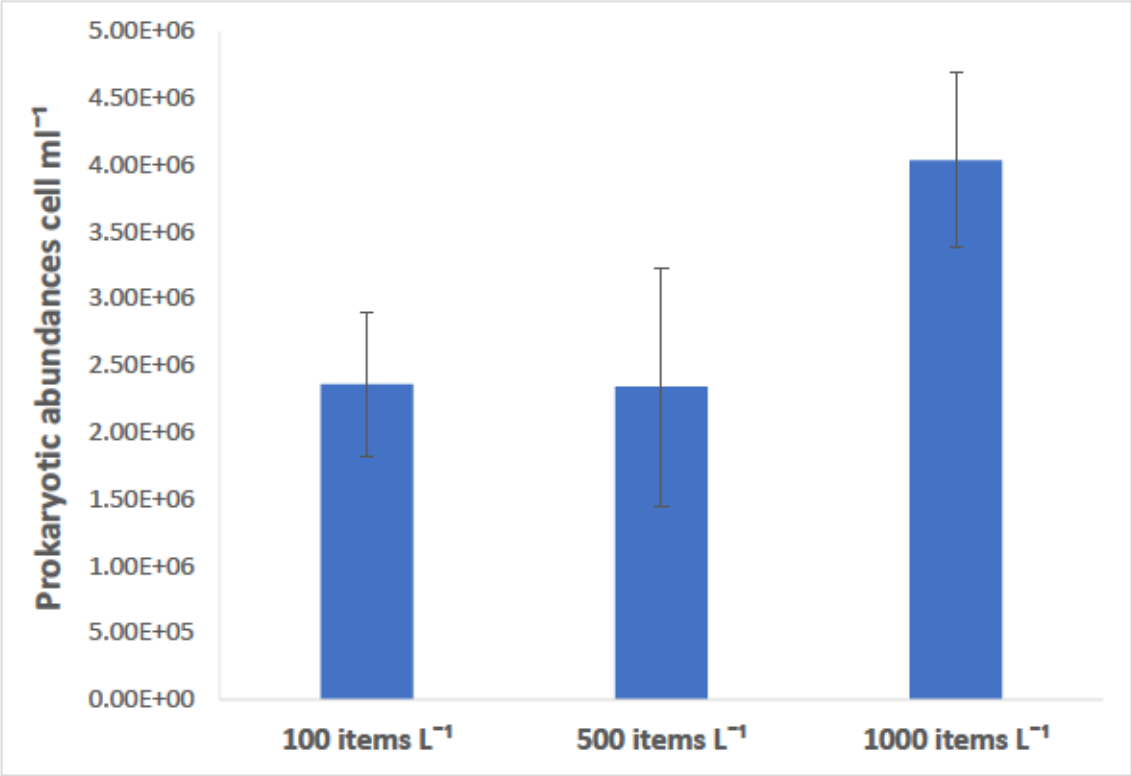


Figure 2. Total prokaryotic mucus abundances in corals exposed to different microplastics concentrations (100-500-1000 items L⁻¹).

Gene stress response

The expression levels of six genes (see the section Material and methods for more details) were followed by real-time qPCR to identify potential gene targets to better understand the effects of microplastics (100, 500 and 1000 items L^{-1}) at the molecular level, *C. rubrum*. These genes are involved in a broad range of functional responses, such as stress, detoxification process, and DNA repair. The effect on genes induced by microplastics is reported in Figure 3. At low microplastic concentrations (100 items L^{-1}), only the relative expression levels of the *MnSOD* gene resulted significantly higher respect to the control. The other genes analyzed did not show any differences in the expression levels compared to control. The relative expression levels of *cytb*, *mtMutS*, *hsp70, EF1* and *MnSOD* genes were significantly higher in corals exposed to 500 items L^{-1} respect to those not exposed to microplastics. Moreover, in *C. rubrum* branches exposed to the highest doses tested (1000 items L^{-1}), an up-regulation of *cytb, mtMutS, hsp60, EF1, MnSOD* genes was recorded.

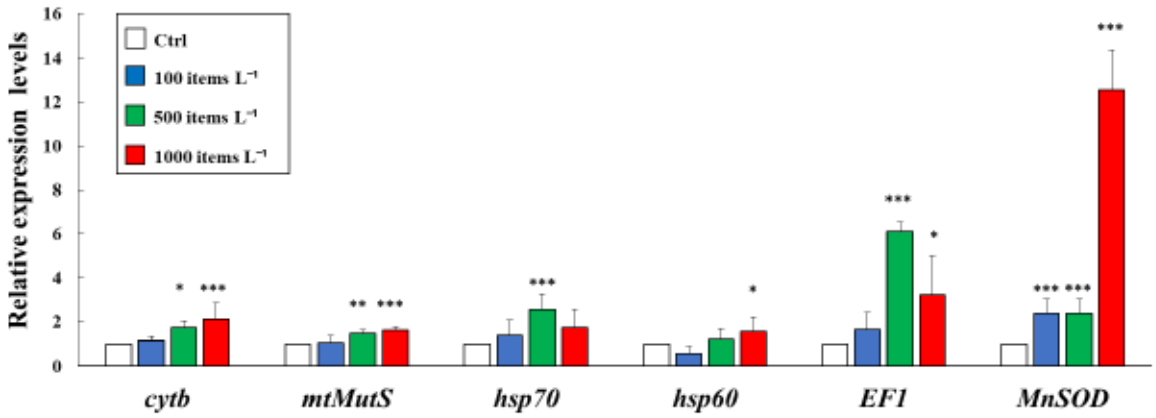


*Figure 3. The histograms show relative expression levels of six genes (cytb, mtMutS, hsp70, hsp60, EF1, MnSOD) followed by real-time qPCR in C. rubrum exposed to different microplastic concentration (100, 500 and 1000 items L^{-1}) and not exposed to microplastic (Ctrl). Data are reported as a fold difference of expression levels (mean ± SD), compared to the control colonies without microplastics. Means ± SD from three biological replicates. Significant differences compared to the control: *p<0.05, **p<0.01,***p< 0.001. One-way ANOVA (p< 0.05), with Tukey's Multiple Comparison Test*

Scanning Electron Microscopy (SEM)

During the entire experiment, corals in all treatments were subjected to tissue abrasion. At the beginning of the experiment (0d), no coral reported damage to the tissue, while after 7 days (7d) corals exposed to all three concentrations of microplastics showed a similar tissue damage equal to about 20 %, which was double than in the control. At the end of experiment (14d) corals exposed to 100 and 500 microplastic items L^{-1} showed, 23-27% of tissue damage, similar to the values reported after 7 days of exposure. Conversely, when exposed at 1000 microplastic items L^{-1} coral tissue resulted more damaged at the end (56%) than at the start of the experiment.

The percentage of tissue damage after 14 days of exposure to 1000 microplastic items L^{-1} was 5 times higher than in the control (Fig.4). Scanning electron microscopy images are reported in Fig. 5 A and B.

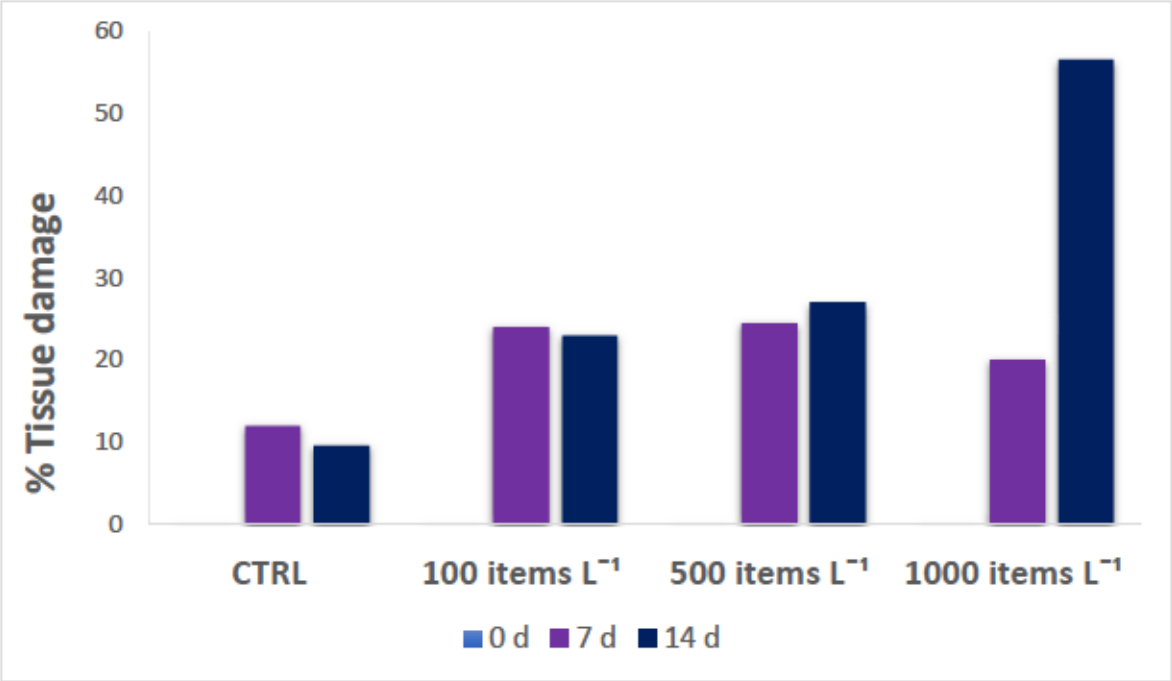
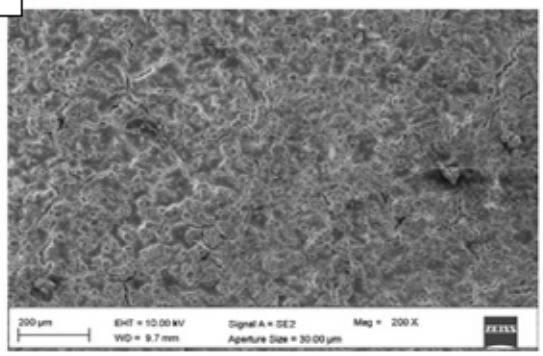


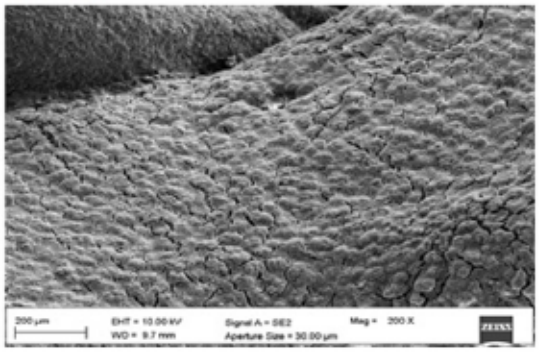
Figure 4. Percentage of tissue damage in corals exposed to different MPs concentrations (100-500-1000 items L^{-1}) and corals not exposed to MPs (CTRL).

A

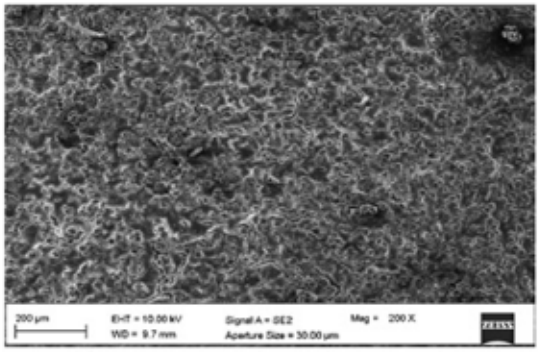
T0 CTRL



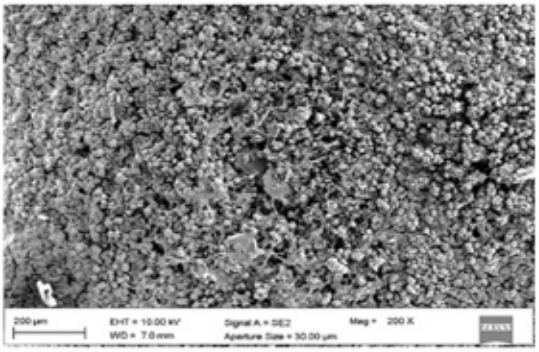
CTRL Tf



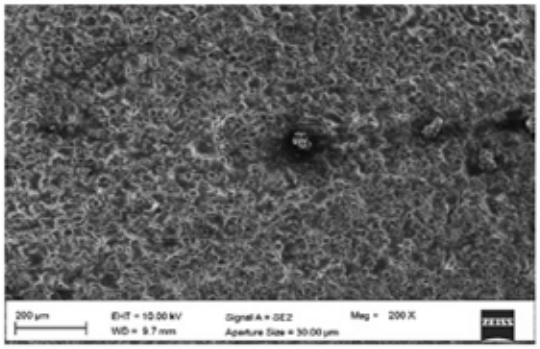
T0 100 items



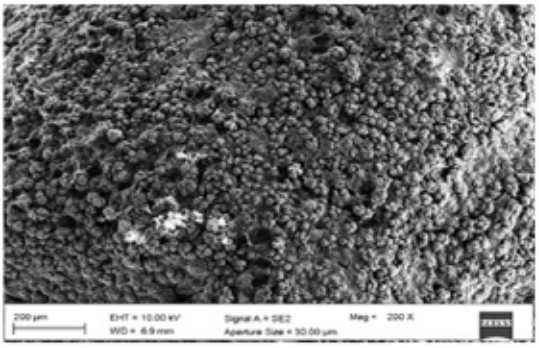
100 items Tf



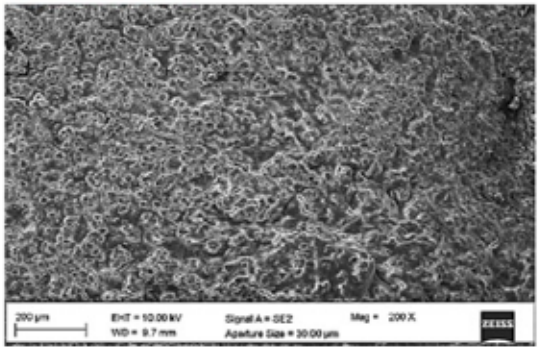
T0 500 items



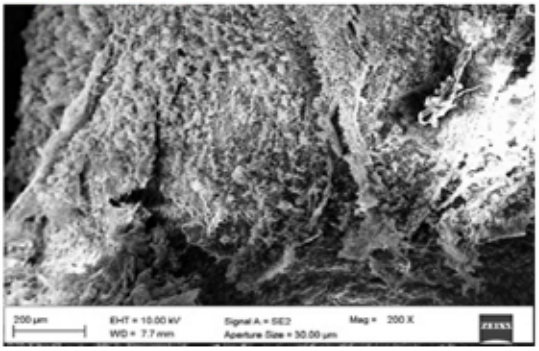
500 items Tf



T0 1000 items



1000 items Tf



B

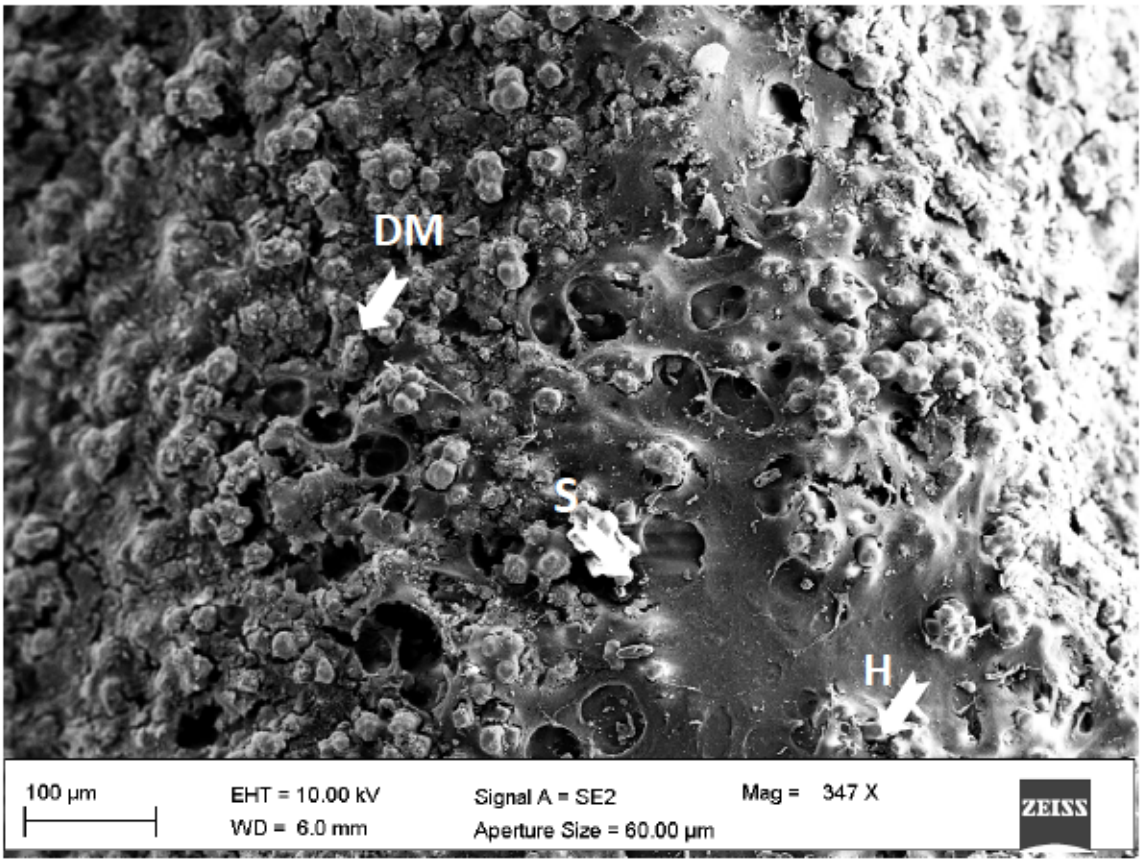


Figure 5. A) Scanning electron microscopy images of coral tissue exposed to different MPs concentrations (100-500-1000 items L^{-1}) and corals not exposed to MPs (CTRL), comparing the damage at the beginning (T0) and the end of experiment (Tf). B) Detail of the coral fragment exposed to 1000 itemsL-1. The red arrows indicate damaged (DM) and healthy (H) tissue and one of the sclerite (S)

Feeding rates of C. rubrum

A general decrease in the concentration of *Artemia salina* added to the systems containing corals and controls (without microplastics' addition) occurred both in the first (after 2 days) and second week (after 10 days; Fig 6 and 7).

In the first week of coral exposure to microplastics, the number of nauplii ingested changed significantly in all treatments and in the control (P value ≤ 0.01). In particular, a higher number of nauplii was removed when the corals were exposed to 100 and 500 microplastic items L^{-1} compared to the control and the treatment with 1000 microplastic items L^{-1} (Fig. 6).

In the second week of experiment we observed a decrease in the number of ingested nauplii in all treatments and control, but in corals not exposed to microplastics (Ctrl) this decrease was significant ($P < 0.007$) during 0h, 2h and 4h, while for corals exposed to 100 items L^{-1} the

number of naupli decreased significantly only after 4 hours of incubation (P = 0.014). In the corals exposed to 500 and 1000 items L^{-1} this decrease wasn't significant, suggesting that after ten days exposed to higher concentrations of microplastics (500-1000 items L^{-1}) strongly reduced their feeding while those exposed to lower concentration only slow down it (Fig. 7).

Figure 6. Mean feeding rate of Artemia salina nauplii during the second day of exposure at different sampling intervals (0 hours, after 2 and 4 hours). Clearance was evaluated for corals exposed to different MPs concentrations (100-500-1000 items L^{-1}) and corals not exposed to MPs (Ctrl).

Figure 7. Mean feeding rate of Artemia salina nauplii during the tenth day of MPs exposure at different time intervals (0 hours, after 2 and 4 hours). Clearance was evaluated for corals exposed to different MPs concentrations (100-500-1000 items L^{-1}) and corals not exposed to MPs (Ctrl).

Analyzing the ingestion rate of Artemia from corals we observed that in the controls it was maintained constant from the first to the second week of experiment (mean of 108.75 ± 5.30 naupli L^{-1} h^{-1}) whereas in all the other treatments, it significantly decreased. In particular, the clearance in the tank containing 1000 microplastic items L^{-1} was very low (37.50 ± 8.84 naupli L^{-1} h^{-1}). The corals exposed to 100 and 500 items L^{-1} showed a higher ingestion rate during the 2° day of exposure (279.17 ± 50.09 naupli L^{-1} h^{-1} and 235.00 ± 37.57 naupli L^{-1} h^{-1} respectively) but it strongly decreased during the 10° day of exposure (156.25 ± 15.47 naupli L^{-1} h^{-1} and 58.33 ± 11.79 naupli L^{-1} h^{-1} respectively). Instead, the corals exposed to higher microplastics' concentrations (1000 items L^{-1}) in the 2°day of exposure showed an ingestion rate quite similar to the Control (96.88 ± 2.21 naupli L^{-1} h^{-1}), and at the end of experiment it significantly decreased (37.50 ± 8.84 naupli L^{-1} h^{-1}; Fig. 8).

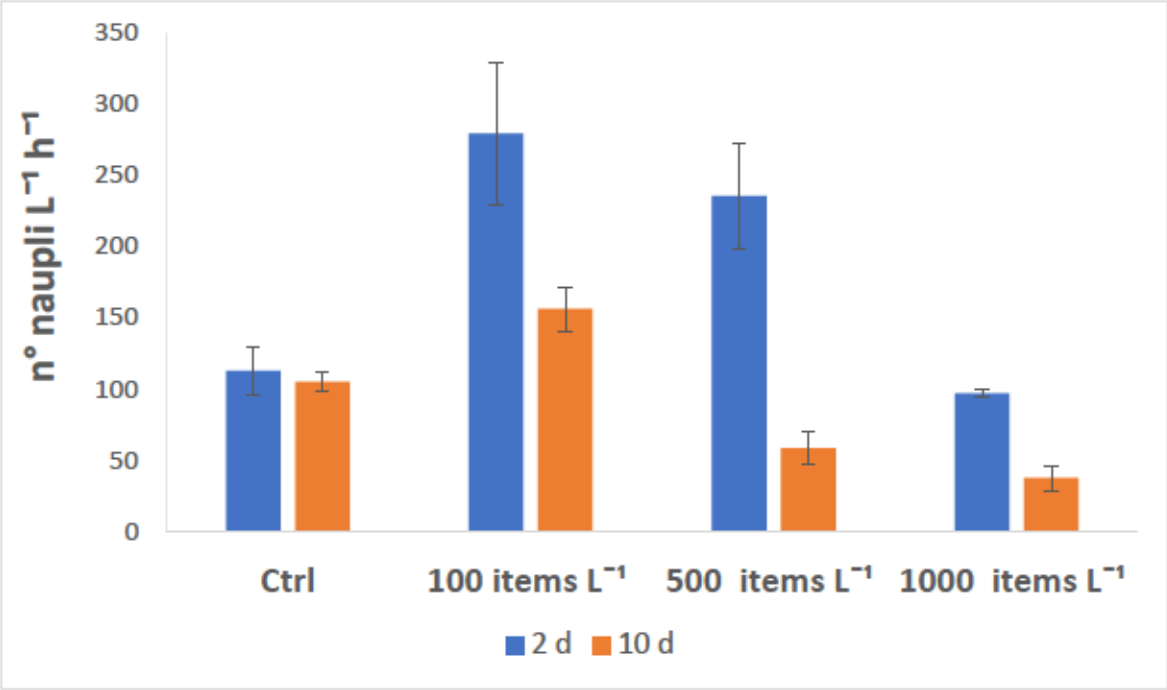


Figure 8. Ingestion rates in corals during the second and tenth day of different microplastics exposure (100-500-1000 items L^{-1})

Microplastics' ingestion from Corallium rubrum

At the end of the experiment we found 143.75 ± 8.83 (Fig. 9A) of ingested microplastic items with a mean range size of 418.13 µm ± 55.58 in the coral nubbins exposed to the highest concentration of microplastics (1000 items L^{-1}). The most ingested polymer by corals was polypropylene (PP), followed by polystyrene (PS) and polyethylene (PE) with the maximum size found of 1 mm (PET) and the minimum one of 100 µm (PVC), but for all polymers the smaller fraction (2-200 µm) was the most ingested one (Fig. 10). We estimated that the amount of microplastic items found within corals accounted for 5 % to the total amount added at the start of the experiment. Some images of microplastics ingested by *Corallium rubrum* were reported in Figure 9B.

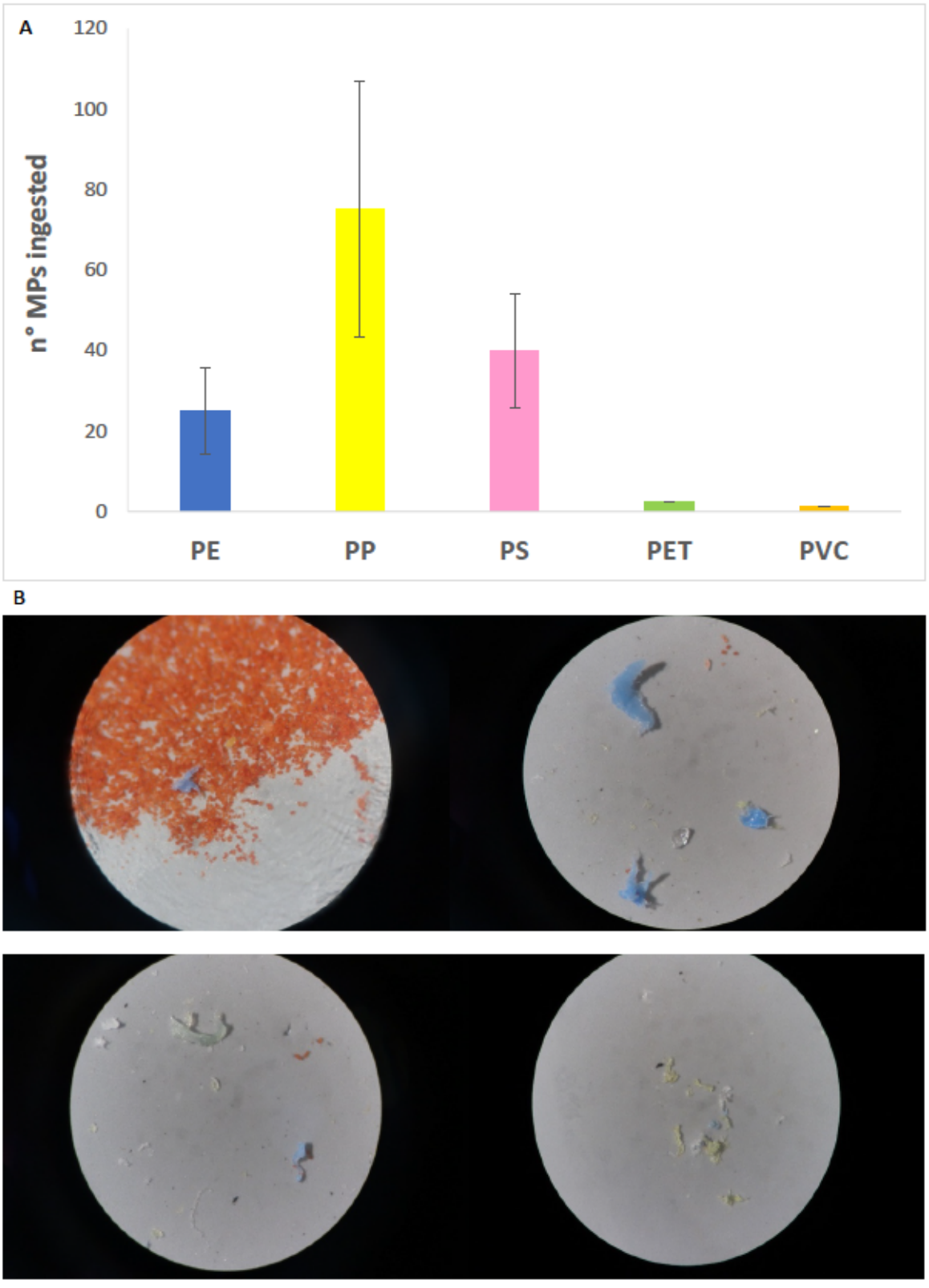


Figure 9. Number of different microplastics polymers ingested by Corallium rubrum fragment (A). Photographs of microplastic ingested by corals (B) (Zeiss Stemi 2000, 50X).

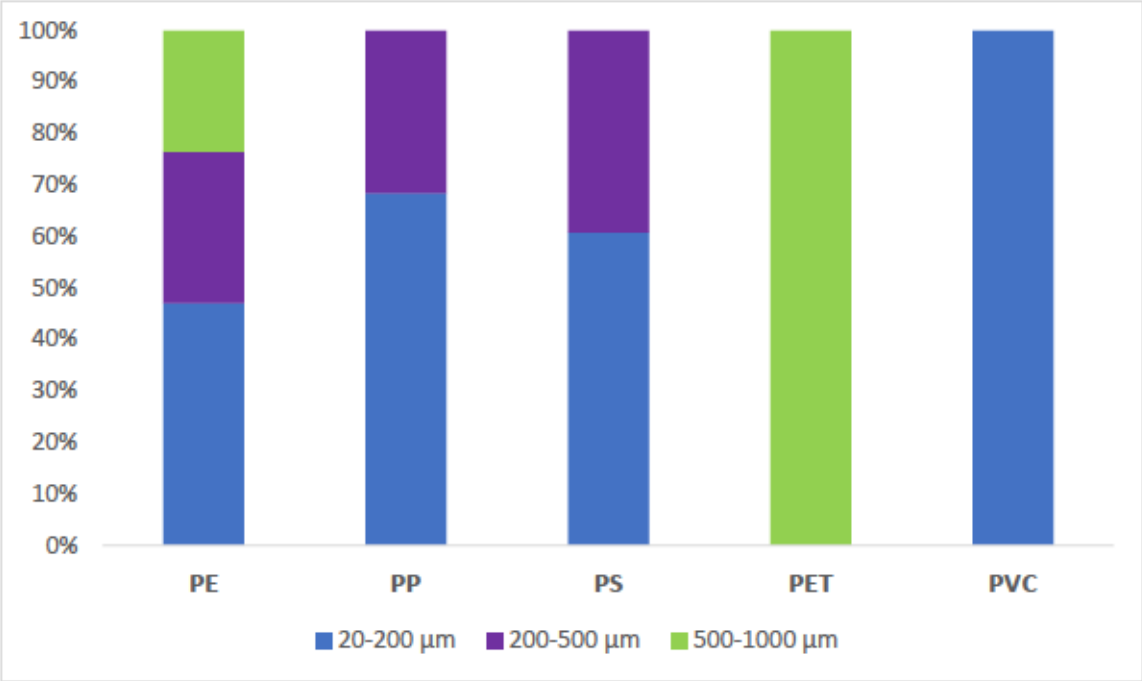
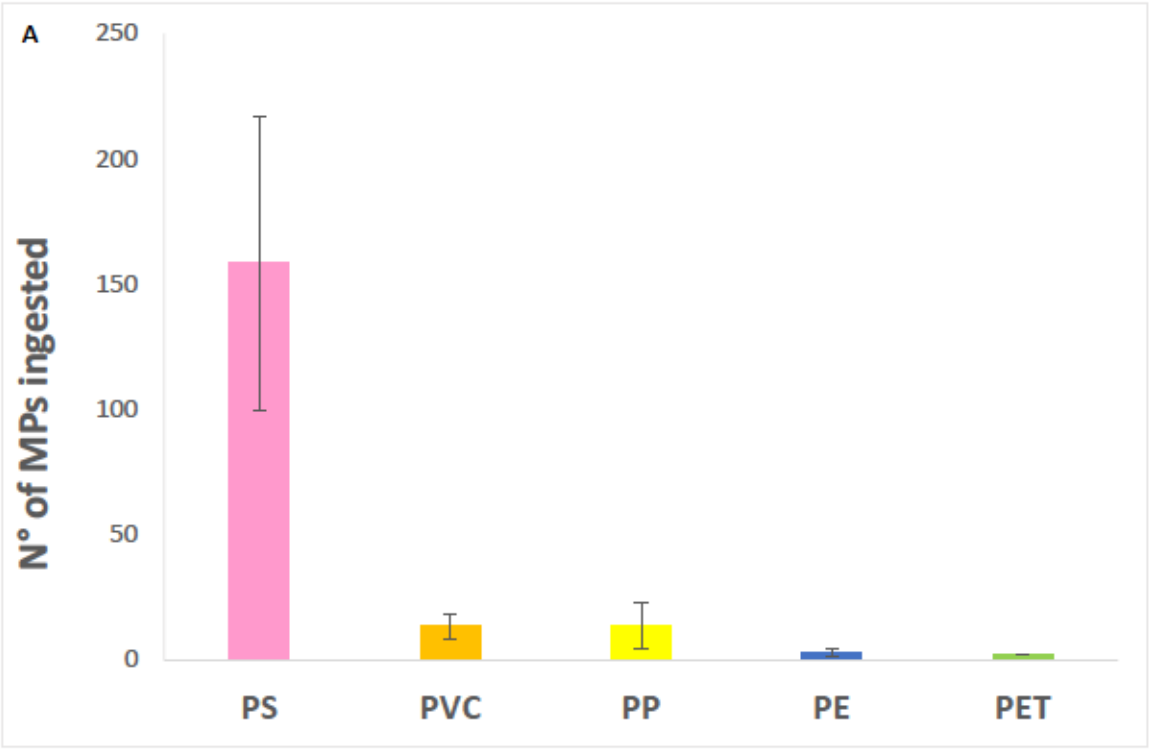


Figure 10. Contribution of different size of microplastics ingested by a fragment of Corallium rubrum.

Microplastics ingested by Artemia salina

The experiment with nauplii of *A. salina* and microplastics revealed that at the end of experiment, these can ingest on average 1.89± 0.75 microplastic fragments per each individual, without considering competition with corals (Fig. 11A). Polystyrene was the most ingested polymer by *A. salina;* also others polymers were ingested but to a lesser extent.

The mean size of microplastics polymers found after enzymatic digestion in *A. salina* was 29.62 µm ±16.99. The size of microplastics ingested did not change among the different polymers. Some images of microplastics ingested by *A. salina* were reported in Fig. 11B.



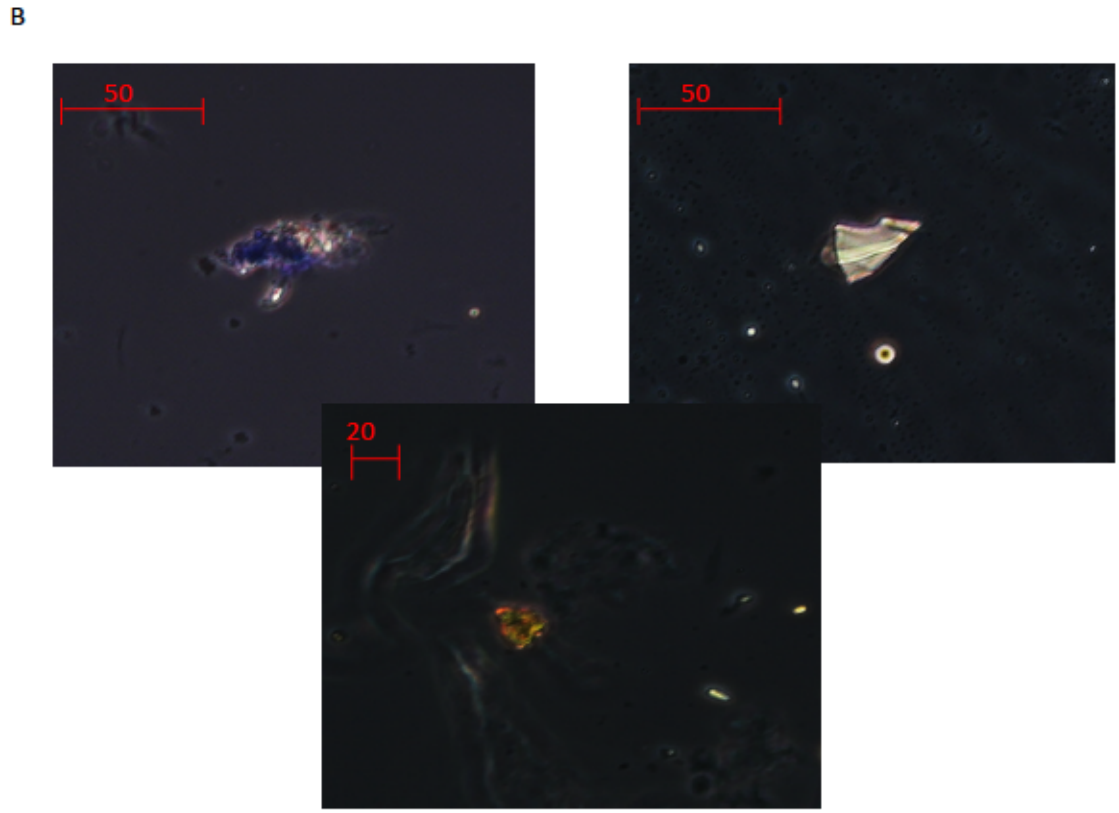


Figure 11. A) Number of different microplastics polymers ingested by Artemia salina. B) Photographs of microplastic found after enzymatic digestion of artemia (Zeiss Stemi 2000, 50X)

Prokaryotic abundance in surrounding seawater

While in corals unexposed to MPs (Ctrl) prokaryotic abundance didn't change significantly at the end of the experiment (14d) when compared with the beginning (0d), the corals exposed to different treatments of microplastics showed significant differences ($P< 0.01$) (Fig. 12). At the beginning of experiment prokaryotic abundances were quite similar in all samples (on average, $2.04 \pm 0.26 \times 10^6$ cells ml^{-1}) but at the end of experiment, the corals treated with 500 and 1000 items L^{-1} showed significant differences ($3.75 \pm 0.58 \times 10^6$ cells ml^{-1} and $2.90 \pm 0.54 \times 10^6$ cells ml^{-1}). Also significant differences in prokaryotic abundances were observed at the end of experiment between control and all the treatments ($P< 0.05$).

A further proof of the effect of the microplastic on prokaryotic abundances in seawater has been obtained with the tanks that contained only microplastics at the higher concentration without corals (CTRL MPs 1000 items L^{-1}). We found indeed that only microplastics supply produces an increase in prokaryotic abundances at higher microplastics concentration especially (1000 items L^{-1}) were added ($P< 0.005$).

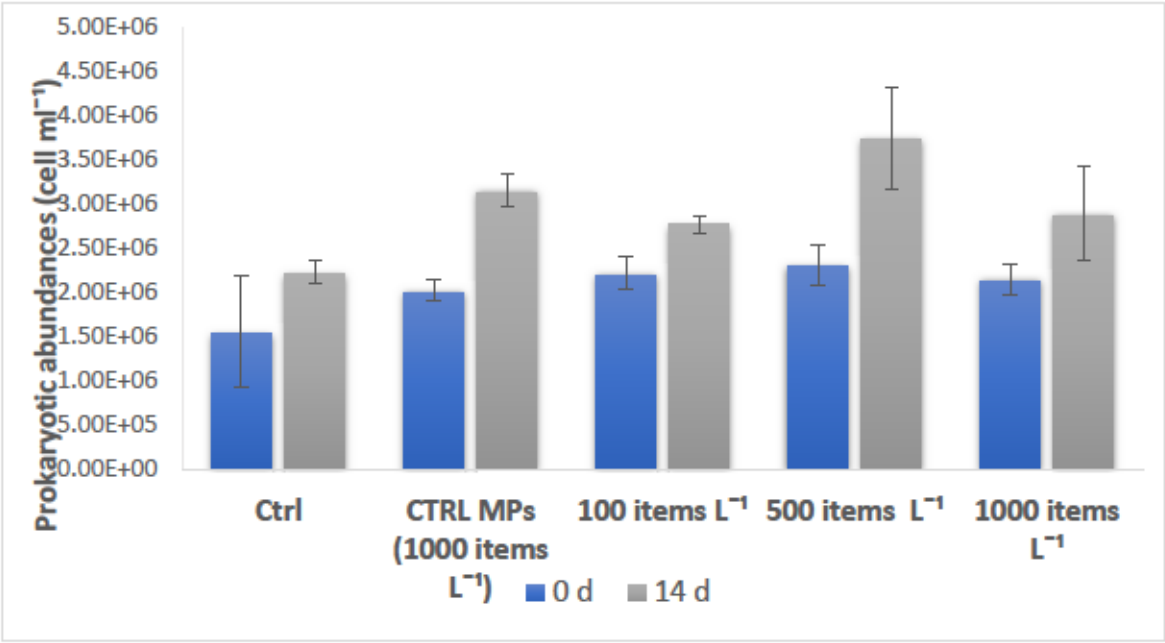


Figure 12. Total prokaryotic abundances in surrounding seawater at the beginning (0 d) and end of experiment (14 d) in corals not exposed to microplastics (Ctrl) and corals exposed to different microplastics concentrations (100-500-1000 items L^{-1}). The label CTRL MPs (1000 items L^{-1}) indicate an additional control that contains only microplastics without corals.

Microbiome composition in C. rubrum exposed to microplastics

Microbiome analysis revealed that at the start of experiment (T0) all corals showed a very low OTU's (Operational Taxonomic Unit) richness with 47.50 (±7.78) OTUs observed. At the end of experiment OTUs richness increase slightly in coral not exposed to microplastics (77 ±7.78 OTU obsverved) (Ctrl Tf), but considerably in corals exposed to 100, 500 and 1000 items L^{-1} showed the highest OTU's richness with 146 (± 39.60), 105 (± 4.95) and 140.25 (±24.40) OTU observed respectively (Fig.13). A similar pattern was demonstrated by Shannon index that increase at the end of experiment in corals treated with microplastics, suggesting that MPs influence the bacterial diversity increasing it (Fig. 14)

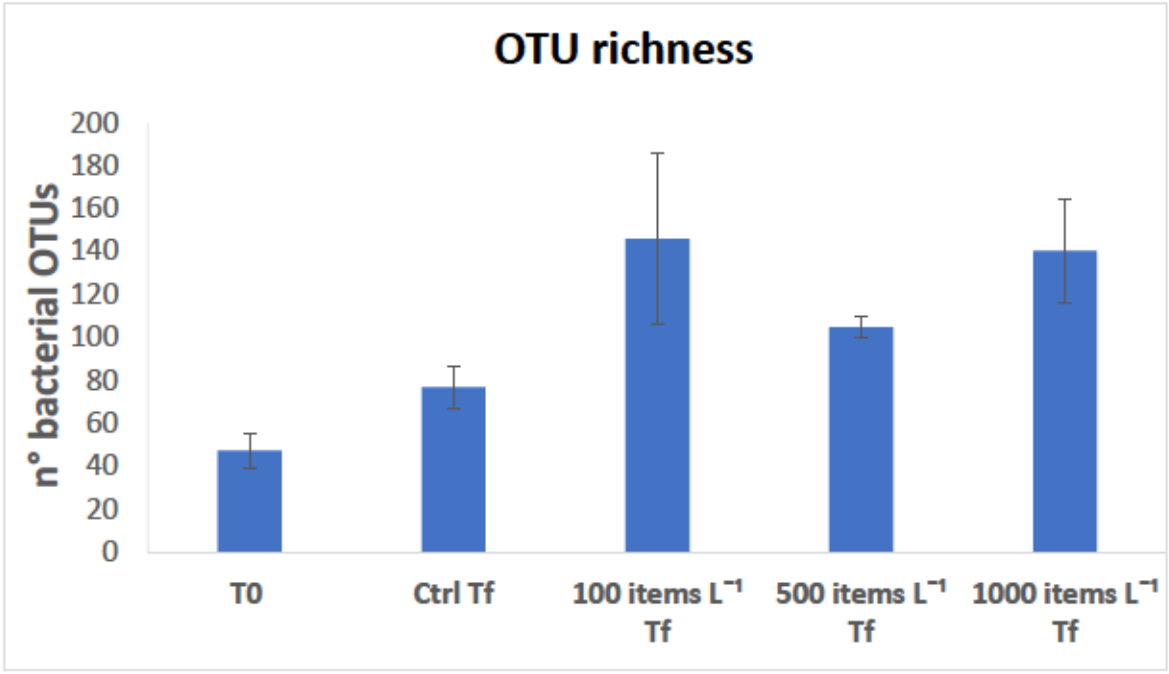


Figure 13. Number of OTUs observed at the beginning (T0) and in the end of experiment in corals not exposed to microplastics (Ctrl Tf) and corals exposed to different microplastics concentration (100-500-1000 items L^{-1} Tf).

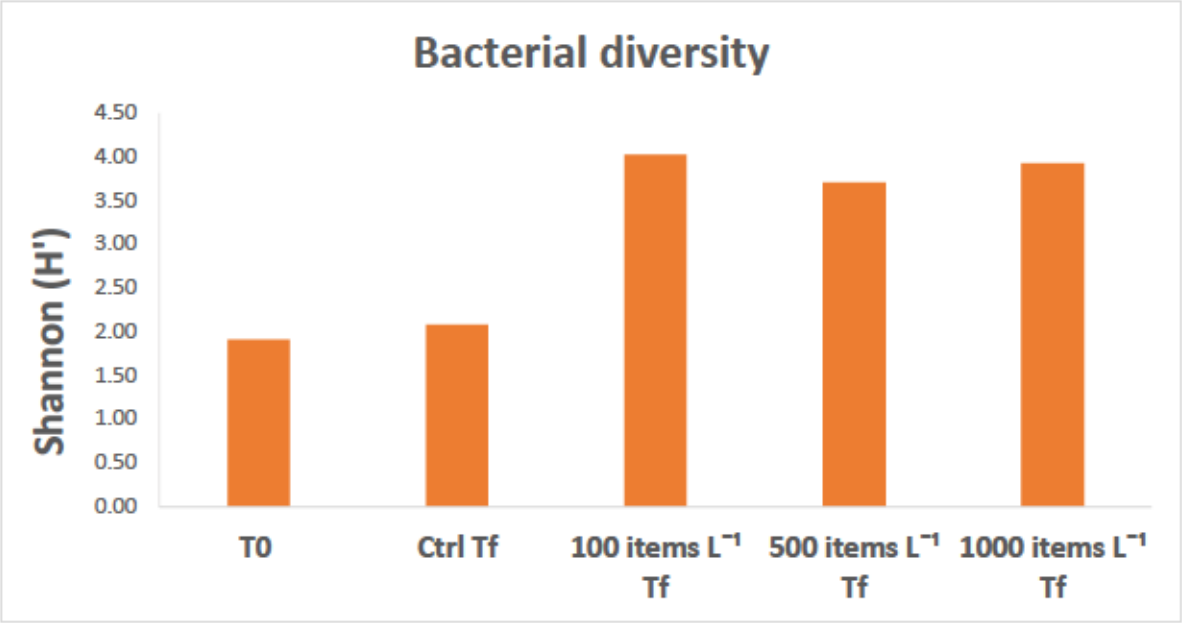


Figure 14. Bacterial diversity (Shannon index) at the beginning (T0) and in the end of experiment in corals not exposed to microplastics (Ctrl Tf) and corals exposed to different microplastics concentrations (100-500-1000 items L⁻¹ Tf).

In all coral nubbins we found a "core" microbiome that contributed for 88.40% to the microbial assemblages mostly represented by Spirochaetaceae (34.13%), Rhodobacteraceae (21.03%), an unkowm family of Gammaproteobacteria (5.49%), Alteromonadaceae (5.40%), Hahallaceae (3.79%), Vibrionaceae (3.68%) and Flavobacteriaceae (1.47%) and by other families with lower contribution (< 1%) (Fig. 15). The microbial assemblages associated with corals unexposed to MPs at the start (Ctrl T0) and at the end of the experiment (Ctrl Tf) were dominated by Spirochaetaceae family (62% and 71.42%, respectively), while in the treated corals this family had subjected to a general decreasing compared to Ctrl Tf in corals the exposed to 100 items L^{-1} (39.18%), 500 items L^{-1} (5.35%) and 1000 items L^{-1} (17.52%). However, in all treatments we found an increase of Rhodobacteraceae (from 11% to 41%, Fig. 16). At the end of experiment, all corals exposed to microplastics showed 8 shared families: Nannocystaceae, uncultured bacterium (Phylum TM6 Dependentiae), Porticoccaceae, Halomonadaceae, Cytophagaceae, Bacteriovoracaceae and two Unknow families, which belong to the order Cytophagales and the others to the class Alphaproteobacteria Incertae Sedis. Considering the single treatments, exclusive families were present for each treatment with a similar number of exclusive families for each treatment (13 for treatment with 100 items L^{-1}, 11 for treatment with 500 items L^{-1} and 14 for treatment with 1000 items L^{-1}). However, different unknown families were present in 100 and 1000 items L^{-1} treatments, and in particular

the treatment with the highest microplastics concentration also presented the highest number of unknown families (6 unknown families).

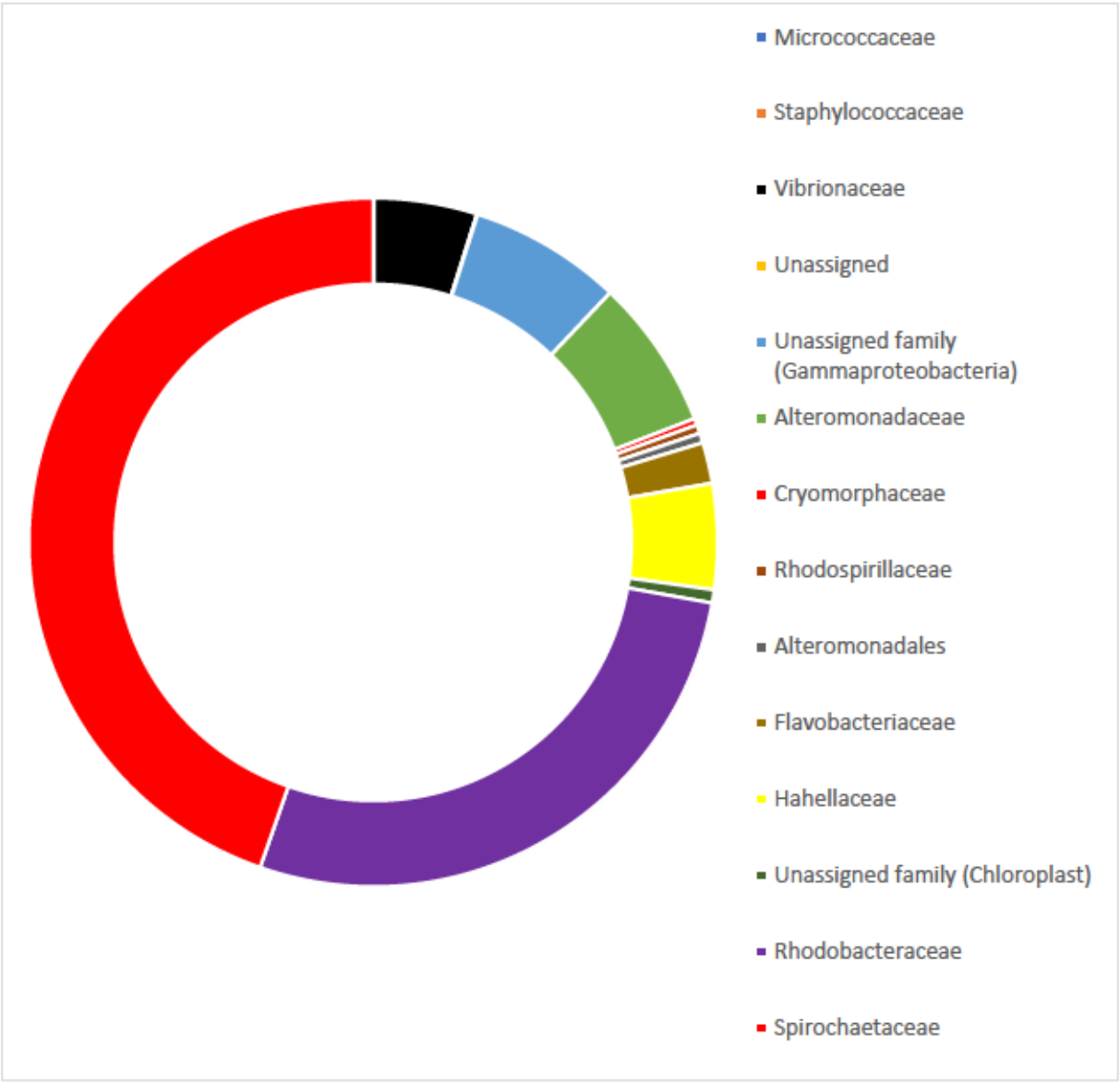


Figure 15. Core microbiome present in corals not exposed to microplastics and corals exposed to different microplastics concentration (100-500-1000 items L^{-1})

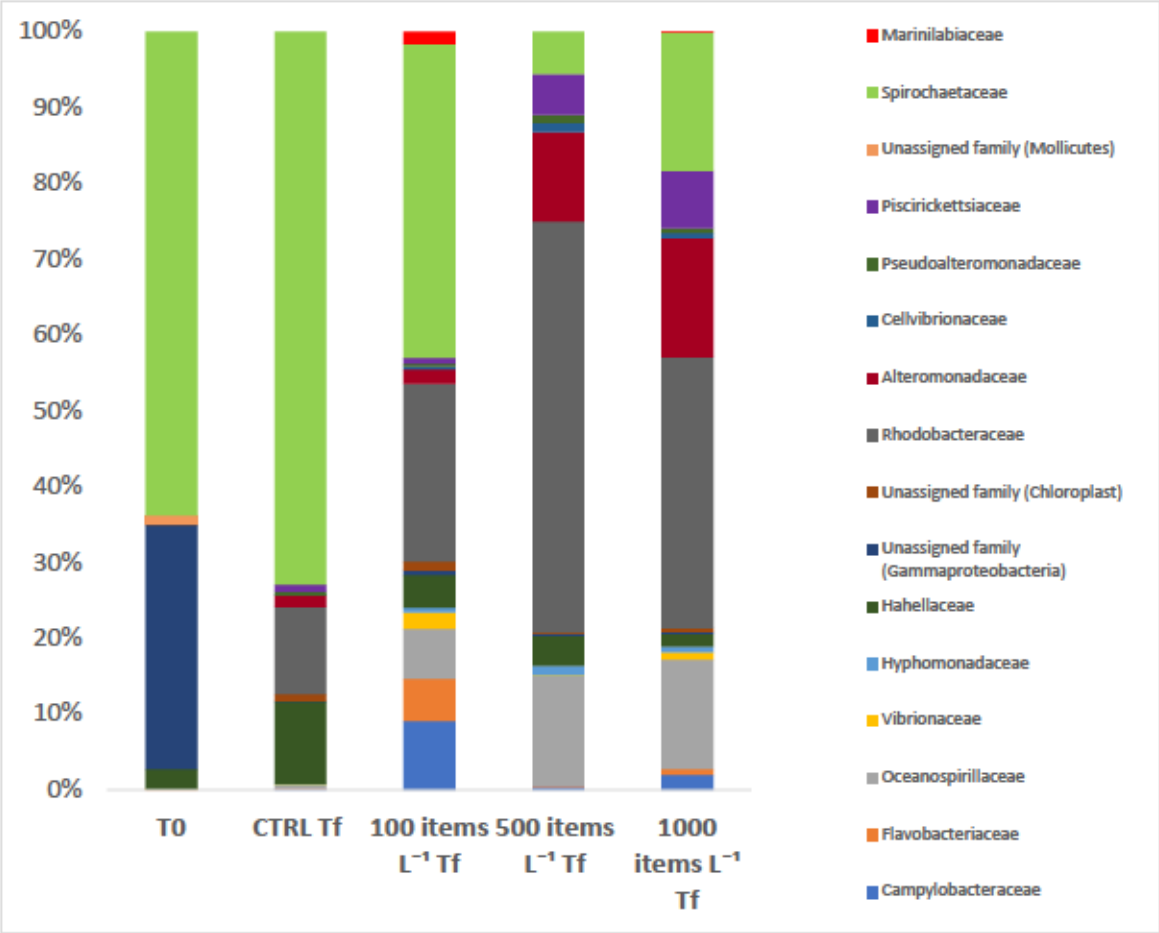


Figure 16. Taxonomic composition at the beginning (T0) and in the end of experiment in corals not exposed to microplastics (Ctrl Tf) and corals exposed to different microplastics concentration (100-500-1000 items L⁻¹ Tf).

This shift in corals microbiome has been observed by using a Bray-Curtis analysis. Cluster analysis showed that at the end of experiment microbial assemblages associated with *Corallium rubrum* exposed to microplastics differed from those present at the start of experiment (T0) and in the Control at the end of experiment (Ctrl Tf). Indeed, Ctrl T0 and Ctrl Tf clustered together with a similarity higher than 65%, instead corals treated with different microplastics concentrations clustered apart (similarity percentage of 45%), especially those added with 500 and 1000 microplastics items L^{-1} (Fig. 17).

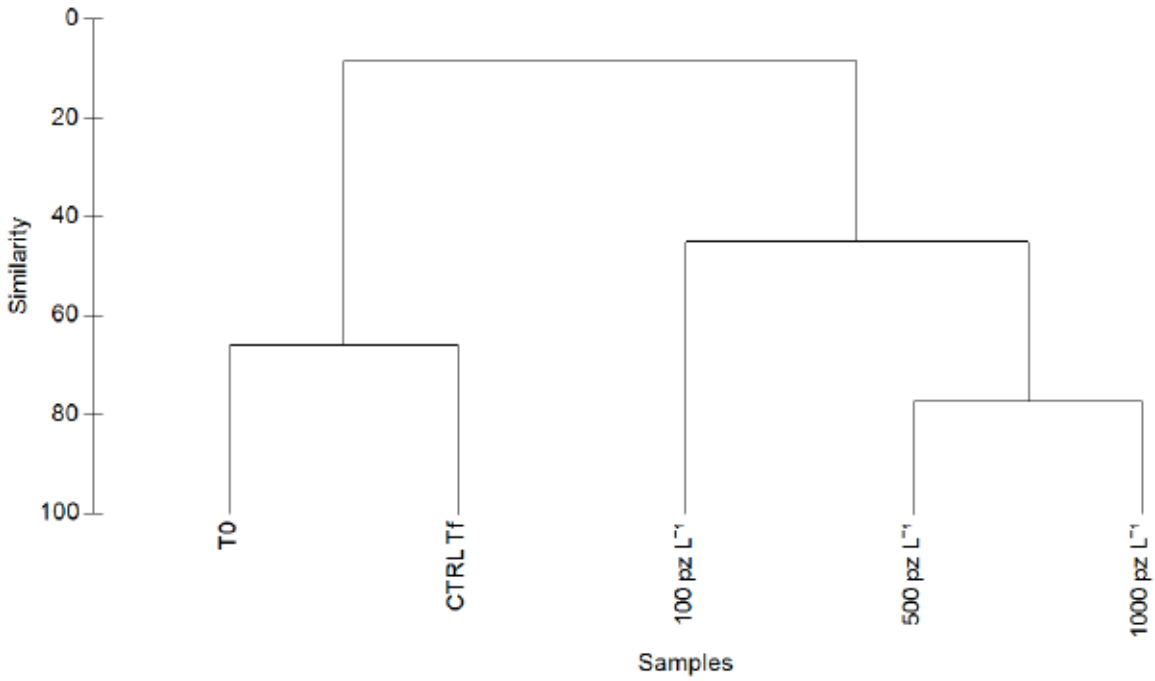


Figure 17. Cluster analysis of microbiomes at the beginning (T0) and in the end of experiment in corals not exposed to microplastics (Ctrl Tf) and corals exposed to different microplastics concentrations (100-500-1000 items L⁻¹ Tf).

Discussion

In the last years microplastics have been considered as contaminants of emerging concern also for tropical corals (Lamb et al. 2018; Hankins et al. 2018; Reichert et al. 2018; Allen et al. 2017; Hall et al. 2015) despite they are not new contaminants in marine environment.

Information about the impact of microplastics on shallow water organisms and tropical coral reefs is growing, conversely available studies on deep-water species, including corals, are still limited. *Corallium rubrum* is an important habitat-forming species that provides structural complexity to benthic communities in the mesophotic zone of the Mediterranean Sea and can extend down to 800 m depth (Cerrano et al. 2013). However, commercial over-exploitation of *C. rubrum*, ocean acidification and elevated seawater temperature are posing serious risks to this species (Bramanti et al. 2013; Cerrano et al. 2000).

Due to the emerging concern caused by marine microplastic pollution, in the present study we investigated the responses of *Corallium rubrum* colonies exposed to different microplastics' concentrations in order to better understand the potential risk for this deep-water habitat forming species.

Corallium rubrum colonies were exposed for two weeks to a combination of plastic polymers (PE, PP, PS, PVC and PET) and concentrations (100 items L^{-1}, 500 items L^{-1} and 1000 items L^{-1}).

Corals exposed to microplastics were visually stressed by microplastic contamination compared to the control, especially at higher concentrations. Indeed, we observed large production of mucus especially in the treatment with 1000 items L^{-1}. Previous studies reported that mucus represents one of the first signals of coral stress and an immediate response to the changed environmental conditions (Jatkar et al. 2010). In addition, mucus owns protective properties since acts as a barrier against potential pathogens (Ritchie et al. 2006; Bourne et al. 2007; Glasl et al. 2016). In the present study indeed, we found that mucus contained not only prokaryotic cells but also microplastics, suggesting that *C. rubrum* especially when exposed to higher levels of microplastics produced mucus to protect itself.

We also conducted analyses of expression of genes involved in folding new polypeptide chain, antioxidant activity, DNA repair, protein synthesis and electron transport systems, which are important components of the cellular defense system against toxic compounds (Goldstone et al., 2006). We observed that microplastics, especially at higher concentrations (500-1000 items L-1), altered the expression levels of most of the genes considered, confirming the cellular stress of *C. rubrum*.

Microplastics damaged the integrity of the coral tissues, generating further stress to the animals. In this regard, SEM analysis demonstrated that microplastics abraded the corals' tissue generating its tearing in some points where the skeleton and sclerites were visible (Figure 4B). In particular, the fraction of damaged tissue increased when the corals were exposed to 1000 items L^{-1} with values exceeding 50%. Previous studies reported that regeneration mechanisms can rapidly repair small lesions (e.g. 0.1 to 1 cm denuded axis) but when lesions are greater, healing time increases proportionately to lesion size (van Woesik 1998, Bavestrello et al. 1999). Therefore, we can suggest that high microplastic contaminations can not only damage coral tissue but hinder their recover potential.

During the experiment, microplastics negatively altered coral feeding rates. In fact, corals unexposed to microplastics continued to feed during the experiment without undergoing variations in the rate of ingestion, while corals treated with MPs showed a slowdown in the ingestion at the end of the experiment with a more marked effect on corals treated with the

highest concentration of microplastics. The reason of the feeding rate decrease can be due to the direct and/or indirect (i.e., mediated by the ingesting of prey containing microplastics) ingestion by corals.

Chemical digestion analyses of the corals revealed that each fragment contained 144 ± 9 microplastics with a preferential size range of 20-200 μm (modal value: 100 μm) at the end of the experiment. The fragments ingested by corals were preferentially represented by polypropylene, polystyrene and polyethylene. The size of fragments ingested by corals is compatible with the size of their prey. On the basis of these results, we can argue that *C. rubrum* exchanges plastics for food ingested them directly. Previous investigations suggested that despite corals rely on chemoreception to capture prey and their feeding responses are stimulated by prey (Lenhoff and Heagy, 1977), however other available information reveals that anthozoans can mistake microplastics for prey because these polymers can contain phagostimulants involved in chemosensory control during prey capture and feeding (Allen et al. 2017).

In the present study, we also observed that the coral' prey, *A. salina,* ingested microplastics.

Considering the feeding rates and the number of microplastics ingested by *A. salina* we estimated that corals were able to potentially remove all the plastics present in the systems (1000 fragments L^{-1}). Coral's prey ingested plastic polymers regardless of their typology but in a narrow range size (10-58 μm; modal value 25 um). This suggests a size selection of plastic polymers by *A. salina* and a potential transfer to corals of fragments < ca. 60 μm, smaller than those directly ingested directly by *C. rubrum.*

A documented effect in stressed corals is the change in the distribution and composition of the associated microbial assemblages (Ziegler et al. 2016).

Our findings also indicate an increase in prokaryotic abundance in seawater surrounding corals and an increase of bacterial diversity associated with them when exposed to different concentrations of microplastics compared to the control at the end of experiment.

The term microbiome is now commonly used to refer to the consortium of microorganisms associated with coral (Sweet and Bulling, 2017). To distinguish between the healthy state of the coral-associated microbial community, the term 'pathobiome' has been recently suggested for coral diseases (Sweet and Bulling, 2017). This term has been introduced as a growing number of studies to refer to shifts in the consortia of microbial communities linked with gross

signs of compromised host health, rather than pointing at a single causative agent (reviewed in Vayssier-Taussat et al., 2014). Generally, certain bacterial groups become more dominant in the pathobiome due to the complex interactions between environmental stress and the coral host (Pollock et al., 2017; Vega Thurber et al., 2009).

Recent information on hermatypic corals revealed that plastic debris could also directly introduce resident and foreign pathogens or may indirectly alter beneficial microbial symbionts (Lamb et al. 2018) for example cross-ocean bacterial colonization of polyvinylchloride (PVC) is dominated by Rhodobacterales (Dang et al. 2008), a group of potentially opportunistic pathogens associated with outbreaks of several coral diseases (Soffer et al. 2015).

Spirochaetes and Rhodobacteraceae typically belong to microbial assemblages associated with corals in natural environment (Morrow et al. 2012, Rodriguez-Lanetty et al. 2013, van de Water et al. 2016). Indeed, they were present in all red corals (here defined as core microbiome) despite Spirochaetes decreased in the treatments whereas Rhodobacteraceae decreased compared to the controls (T0, Ctrl Tf), suggesting that microplastics could modify natural microbiomes. High levels of Rhodobacteraceae have been associated with several coral diseases (Mouchka et al. 2010) and it has been reported that Rhodobacteraceae bacteria rapidly colonize compromised tissues (Pollock et al. 2017). In addition, members of the Rhodobacteraceae family are known to readily form biofilms in marine environments indicating their role as opportunists rather than primary pathogens (Witt et al. 2011, Elifantz et al. 2013).

Microplastics impact can also be highlighted by the appearance (in corals exposed to all microplastics concentrations) of bacterial families involved in injured or dead corals, such as TM6 Dependentiae (Yeoh et al. 2015), which were exclusively found in the red corals exposed to microplastics but not in the controls. Other bacterial families appeared only in corals exposed to microplastics such as Porticocacceae (one of the microbial families containing hydrocarbon degrader taxa; Bælum et al., 2012; Hazen et al., 2010; Brakstad & Lødeng, 2005; Dubinsky et al., 2013; Gutierrez et al., 2015) Halomonadaceae, Cytophagaceae, Bacteriovoracaceae families (previously associated with anthropogenic impacts in corals microbiome; Ziegler et al. 2016).

The Desulfovibrionaceae family was exclusively found in corals exposed to the higher microplastics concentration (1000 itemL^{-1}). Members of Desulfovibrionaceae have been reported to be involved in death of corals and associated with lesions characterized by anoxic conditions (Carlton and Richardson, 1995; Glas et al., 2012; Richardson et al., 1997; Sato et al., 2016).

These findings reveal that microplastics can alter the natural microbiomes of the *C. rubrum* and can favour the increase of taxa associated with coral diseases or anthropogenic stressors, which can represent an additional factor altering the corals' health already impaired by physical and functional damage.

Conclusions

Overall, our findings highlight that microplastics can strongly affect deep-water communities, such as *Corallium rubrum,* hampering a wide range of important functions for the maintenance of its health. Indeed, microplastics negatively induced functional stress as revealed by the mucus production and alteration in the expression level of stress response proteins. Moreover, microplastics caused tissue damage and reduced the feeding rate (especially at higher concentrations 1000 items L^{-1}) due to direct and indirect ingestion of microplastics. In this regard, we found that *Artemia salina* could represent a vector of smaller microplastics for *C. rubrum*, revealing that these can be transferred along the trophic webs. The health of the red corals can be additionally hampered by the appearance/increase of bacterial taxa reported to be present during coral diseases, especially at the highest microplastic concentrations.

Finally, we can conclude that microplastics may represent a further anthropogenic factor impacting deep-water communities including long-living and habitat-forming species such as *C. rubrum* with negative consequences for habitat integrity, biodiversity and ecosystem functioning.

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6. DIVERSITY OF MICROBIAL ASSEMBLAGES COLONIZING PLASTICS IN DEEP-SEA HABITATS

Introduction

Plastics fragments are considered to be very stable and highly durable compounds (Sivan 2011) and may persist for a long time (hundreds to thousands of years) in the aquatic environment (Barnes et al., 2009). Plastics are biodegraded aerobically in nature, anaerobically in sediments and landfills and partly aerobically and partly anaerobically in composts and soil. Carbon dioxide and water are produced during aerobic biodegradation and carbon dioxide, water and methane are produced during anaerobic biodegradation (Gu et al., 2000). Dominant groups of microorganisms and the degradative pathways associated with polymer degradation are often determined by the environmental conditions. When O_2 is available, aerobic microorganisms are mostly responsible for destruction of complex materials. In contrast, under anoxic conditions, anaerobic consortia of microorganisms are responsible for polymer deterioration (Shan et al. 2008).

Due to the chemical stability of synthetic polymers, buoyant microplastics may persist in the water column over a timescale of weeks to months, eventually sinking and accumulating in sediments (Woodall et al., 2014). The sinking rate of a particle is affected by its buoyancy, which, in turn, depends on the size and composition of the biofilm on its surface (Artham et al., 2009; Lobelle and Cunliffe, 2011). In the environment, microplastics provide a surface for biological colonization (Harrison et al., 2011; Ghosh et al., 2013), including unique microbial consortia immersed in an extracellular matrix of polymers (polysaccharides, glycoproteins and proteins; Costerton et al., 1995). Biofilms are primary sites for carbon and nutrient transformations and form the base of food webs, contributing to ecosystem functioning (Battin et al. 2016). Once in the marine environment, microbes in few minutes colonize plastic debris, which can include pathogenic, toxic, invasive or plastic degrading-species (Oberbeckmann et al., 2016). As synthetic polymers are not water soluble, biofilm-forming bacteria degrade such materials more efficiently than planktonic strains (Webb et al., 2009). In the photic zone, degradation of plastics is greatly facilitated by photolysis (Andrady et al., 1998); therefore, intensive degradation would occur at the water surface (Cole et al., 2011). However, most of the microplastics are dispersed in the water column (Gorokhova, 2015), where UV-driven degradation is limited, whereas bacteria-mediated degradation could be more important. In bottom sediments, biodegradation by bacteria and other microorganisms would predominate

due to the low temperature and lack of sunlight (Rummel et al., 2017). The extent of bacteria-mediated degradation in situ is yet to be proven (Bryant, 2017).

Plastics colonization has been reported in a number of studies starting in the 1970 (Carpenter et al. 1972; Colton et al. 1974) where authors mentioned diatoms and others microbes on the debris, but Zettler et al. 2013 was the first study that focused to the so called "Plastisphere" (the diverse assemblage of taxa that inhabit the thin layer of life on the outer surface of Plastic debris) with a comprehensive characterization of microbial communities living on plastics in the open ocean with an emphasis on bacteria. It reported that bacteria growing on polyethylene and polypropylene debris from the Sargasso Sea mainly belonged to the classes Alpha- and Gammaproteobacteria similar to reports from other marine areas (Oberbeckmann et al., 2014, 2016; De Tender et al., 2015; 2017). Recently, De Tender et al. (2017) analyzed 98 datasets from various locations across the North Sea, Atlantic and Pacific oceans describing the microbial communities associated with polyethylene recovered. Despite the variability introduced by different sequencing platforms, seasons and environmental conditions, a clear pattern was observed, where the composition of bacterial taxa displayed a high level of consistency within a given marine region, suggesting that particular bacterial taxa are able to successfully colonize plastic materials. The most dominant classes found on plastics recovered from the Atlantic and the Pacific areas were Alpha- and Gammaproteobacteria, whereas Flavobacteria and Gammaproteobacteria dominated plastic litter communities collected in the North Sea. At the family level, Rhodobacteraceae dominated all regions except for the North Sea, where Flavobacteriaceae were the most abundant.

Despite the large amount of available information on microbial assemblages colonizing plastic debris floating in water column, studies on microbial biofilm of plastics collected in benthic ecosystems, including deep-sea sediments, are practically absent.

In this study we analysed microbial assemblages present on different macroplastics collected in the S. Gregorio and Tremestieri submarine canyons (Ionian Sea) and surrounding sediments by using High throughput sequencing (Amplicon Sequencing). This research aimed at identifying taxonomic composition of microbial assemblages on different plastic items in the deep-sea floor to understand their origin with respect to surrounding habitat and explore the presence of taxa potentially degrading plastics.

Materials and Methods

Samples Collection

Plastics were collected during an oceanographic cruise on February 2016 in S. Gregorio Canyon (206 m depth) and Tremestieri Canyon (347 m depth; Ionian Sea) by using a box-corer. Two fragments of macroplastic were sorted with sterile forceps and immediately frozen at -20 °C. Sediments samples were collected at the same sampling stations of plastics during the same deployment and they were frozen immediately at -20 °C.

FT-IR Spectroscopy analyses

The actual polymer composition of the fragments was determined by FT-IR analysis. Under laminar flow hood, each fragment was cut using a sterile lancet, rinsed with ultrapure sterile Milli- Q water to eliminate sediment residues and then putted on CaF_2 windows support to allow transmittance analyses. FT-IR measurements were carried out using a Perkin Elmer FTIR Spectrometer Spectrum GX1 interfaced with a Perkin–Elmer Autoimage microscope and equipped with a photoconductive 0.25 mm Hg-Cd-Te (MCT) array detector, operating at liquid nitrogen temperature and covering the entire IR spectral range from 4000 to 700 cm^{-1}. The Spectrum Autoimage 5.1.0 software package (Perkin Elmer, Waltham, MA, USA) was used. On each sample, deposed on CaF_2 windows, both the microphotograph, obtained by means of a microscope television camera, and the corresponding average spectrum were collected. Spectra were acquired in transmission mode, at room temperature, with a spectral resolution of 4 cm^{-1} and a spatial resolution from 30 x 30 to 50 x 50 μm^2. Each spectrum was the result of 64 scans. Background spectra were acquired on clean portions of CaF_2 windows and ratioed against the sample spectrum. For data handling was used software packages Spectrum 5.3.1 (Perkin Elmer). Each sample was compared with reference scans from plastics of known composition present in Spectrum 5.3.1 libraries.

Microbiome composition in plastic fragments and sediments

DNA extraction from plastic samples were performed using QIAGEN DNeasy Blood & Tissue Kit with some modification. In brief, plastic samples (≈25 mg) were cut in small fragments and digested in proteinase K at 56 °C overnight. Then, samples were subjected to ultrasound treatment (three 1-min treatments using a Branson Sonifier 2200; 60W), after that they were processed as per the manufacturer's protocol. Following DNA extraction, samples were held at − 20 °C prior to sequencing analyses. Instead for sediment samples microbiome extraction were

performed using QIAGEN DNeasy Power Soil with some modification. Before starting with official protocol, we introduced three steps with Washing Solution to purify sediment matrix and allows a better DNA extraction. Then, sediment samples (1 g each one) were processed as per the manufacturer's protocol. Following DNA extraction, samples were held at − 20 °C prior to sequencing analyses.

The molecular size and the purity of the DNA extracts were analyzed by agarose gel electrophoresis (1%) and the DNA was quantified by Nanodrop spectrophotometer (ND-1000). Primers for PCR amplification of the 16S V3 region were 515F-Y and 926R, particularly well-suited for marine samples according to Parada et al. (2016), with Illumina-specific primers and barcodes. Sequencing was performed on an Illumina MiSeq platform by LGC Genomics GmbH (Berlin, Germany).

Bioinformatic analyses

Paired reads were joined, quality-filtered and assigned to taxa using the QIIME2 pipeline, as previously described (Bolyen et al., 2018). Amplicon sequence variants (ASVs) were identified through the Deblur strategy (Amir et al., 2017). Sequences were randomly resampled in the ASV table to enable comparison between samples, by normalizing the number of sequences between samples to the sample with the fewest sequences (n=30,000). Taxonomic affiliation of ASVs was performed within the QIIME2 pipeline using the VSEARCH-based consensus taxonomy plugin (Bokulich et al. 2018) on the SILVA database v132 (Quast et al. 2012) after the extraction of the portion of the reference 16S sequences amplified by the primers used for the analysis. Microbiome composition in sediments and plastics were investigated by classification-clustering based on the Bray-Curtis similarity of transformed quantity data. All the analyses were performed using PRIMER 6 (Anderson et al. 2008).

Results

FT-IR Spectroscopy analyses and microbiome composition in plastic fragments and sediments

The two unknown fragments collected during sediment sampling were identified, by infrared spectroscopy (FT-IR), as plastics based on spectra compared to known standards (Fig. 1). They corresponded to polyvinyl plasticized (PVC-P) and polyethylene (PE).

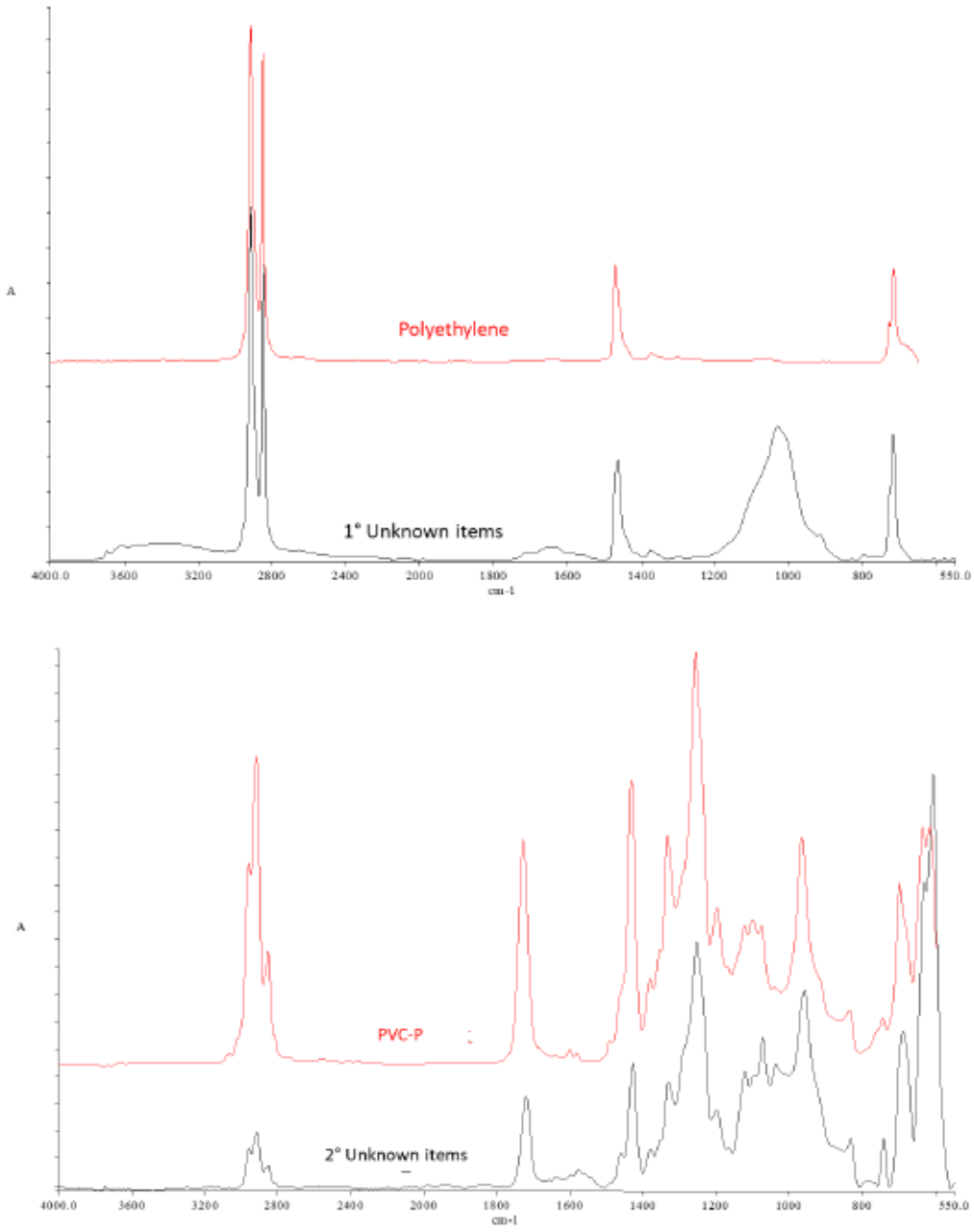

Figure 1. Identification of plastics using FT-IR spectroscopy. The first spectrum is related to the plastic found in S. Gregorio Canyon (206 m) and the second one to Tremestieri Canyon (347 m).

We found 880 and 1030 bacterial OTUs from PE and PVC-P, respectively. In the sediments surrounding the two polymers we found 2884 and 3502 OTUs, respectively (Fig. 2). Shannon index was higher in the PE than in PVC-P (8.65 vs. 9.45). Such values were lower than in the sediments (9.98 and 10.30, respectively; Fig. 3). Evenness was higher in PVC-P (0.94) than in the other samples, where values were similar (on average, 0.88) (Fig. 3).

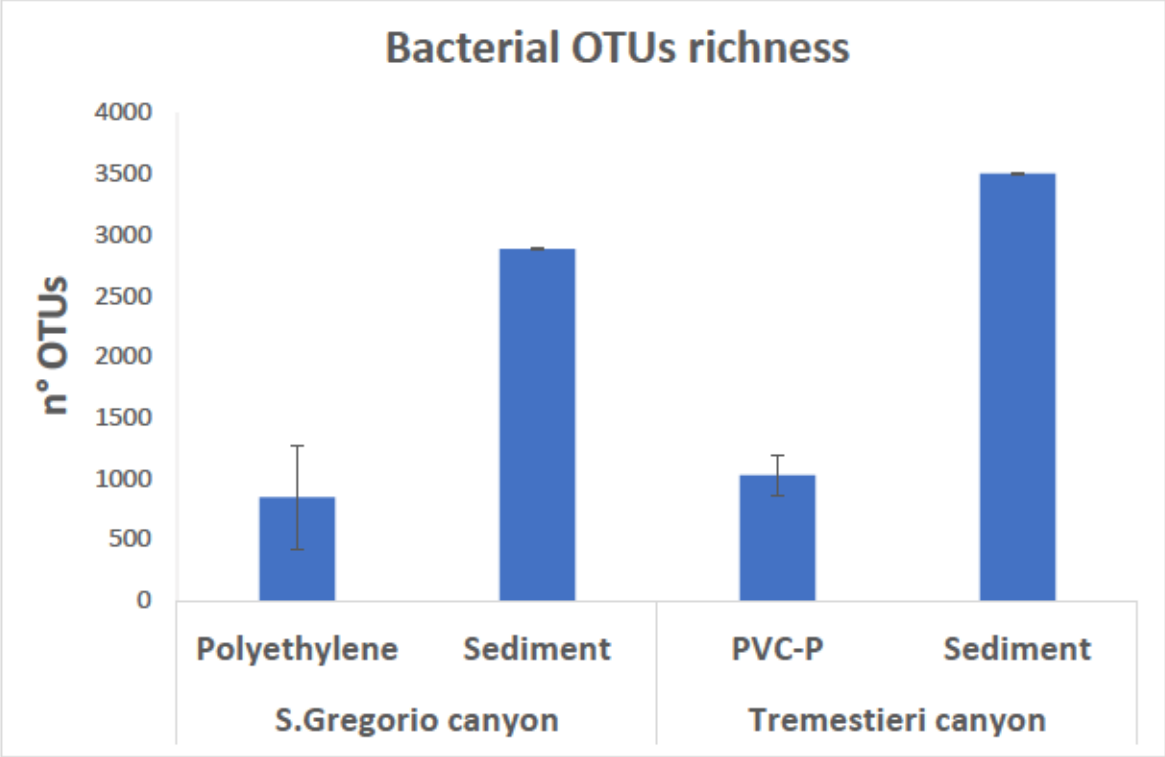

Figure 2 OTUs richness in polyethylene (PE), polyvinyl plasticized (PVC-P) and surrounding deep-sea sediments

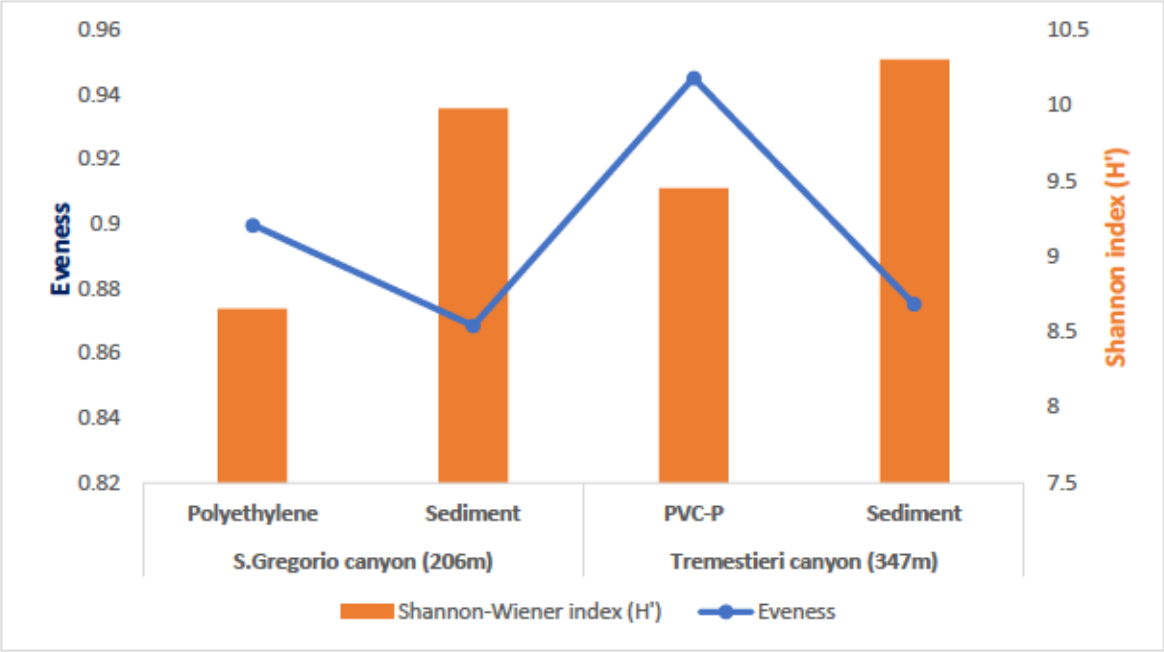

Figure 3 Shannon index and Eveness in polyethylene (PE), polyvinyl plasticized (PVC-P) and surrounding deep-sea sediments

Six families (Methylobacteriaceae, Streptococcaceae, env.OPS 17, Propionibacteriaceae, Burkholdriaceae, and an unknown family of Class Candidatus Falkowbacteria) were shared among the two plastics (Fig. 4) affiliated with different phyla such as Protobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Parcubacteria. Burkholdriaceae represented the most abundant families (88.76%), followed by Streptococcaceae (3.61%), env.OPS 17 (2.98%), Methylobacteriaceae (2.91%), Propionibacteriaceae (1.73%) and by an unknown family (1.11%).

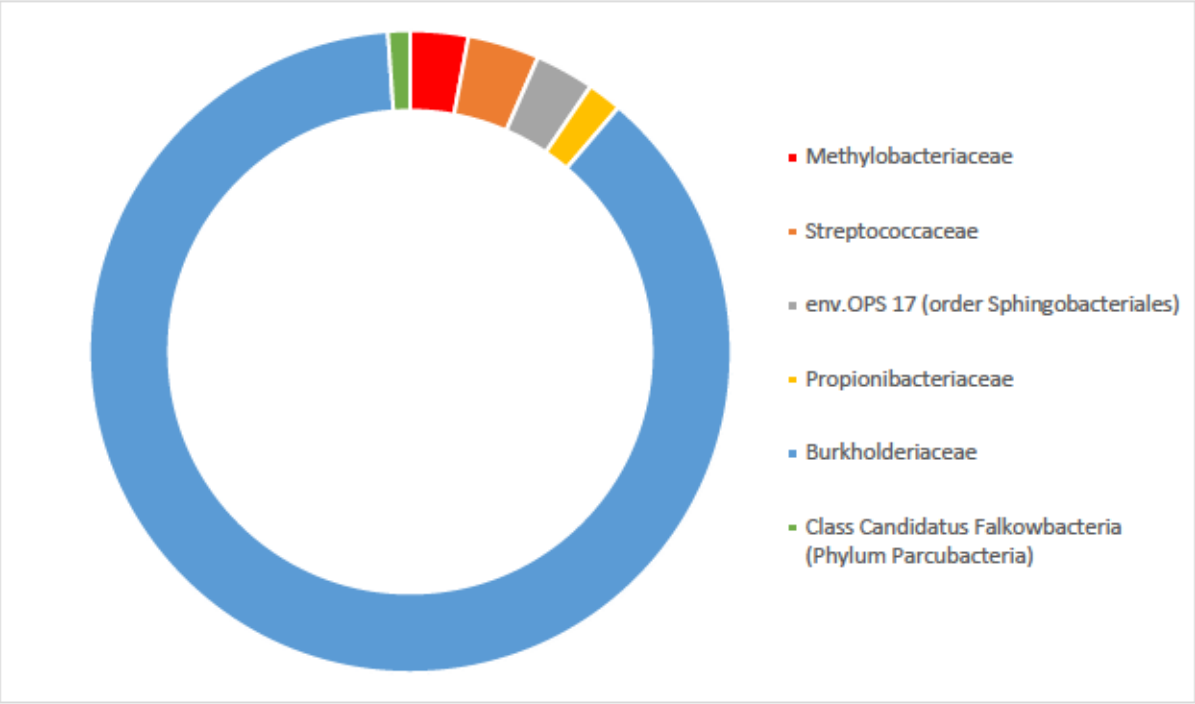

Figure 4 Shared families in Polyethylene (PE) and polyvinyl plasticized (PVC-P)

Both sediment samples and plastics were characterized by exclusive families that contributed in different way.

Polyethylene presented 16 exclusive families on a total amount of 237 families, with the most abundant belonging to Chitinophagaceae, Christensenellaceae, Coriobacteriaceae, while polyvinyl plasticized presented 2 exclusive families (Parachlamydiaceae and Microbacteriaceae) on a total of 242 families. Sediments surrounding PVC-P were characterized by 25 exclusive families, instead sediments surrounding PE presented 17 exclusive families (Fig. 5).

At the OTU level, we found 1 unknown OTU in PVC-P that affiliated with the domain of Bacteria, and 7 unknown OTUs in polyethylene, 2 of which belonged to the Archaea domain and 5 to the Bacteria domain.

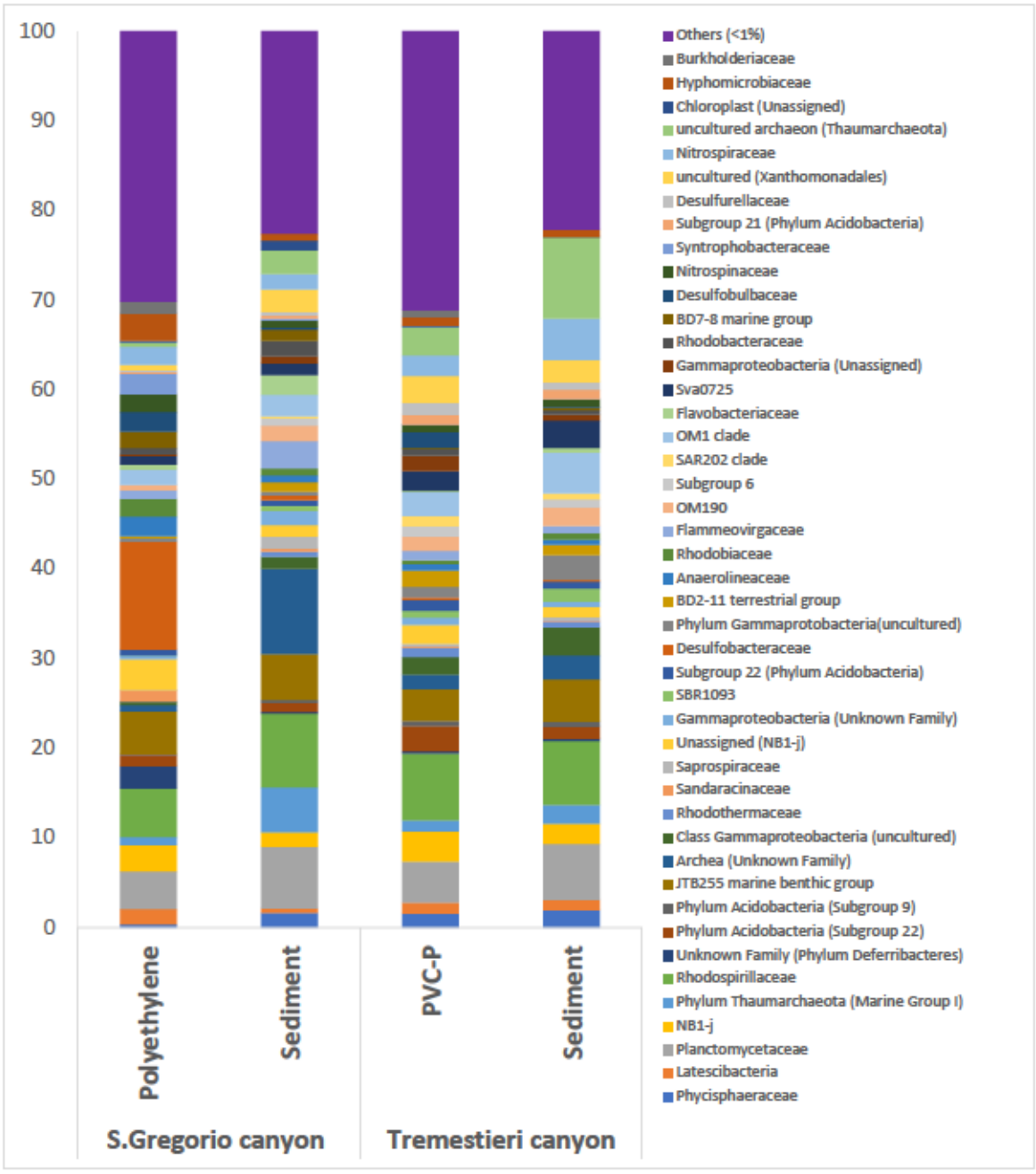

Figure 5 Taxonomy of microbiome of polyethylene (PE), polyvinyl plasticized (PVC-P) and surrounding deep-sea sediments

Bray-Curtis analysis showed that microbiome assemblages associated with polyethylene (PE) clustered apart with a similarity of ca. 65% from the other microbiome assemblages. Polyvinyl plasticized (PVC-P) and the deep-sea sediments were similar for ca. 80 % (Fig. 6).

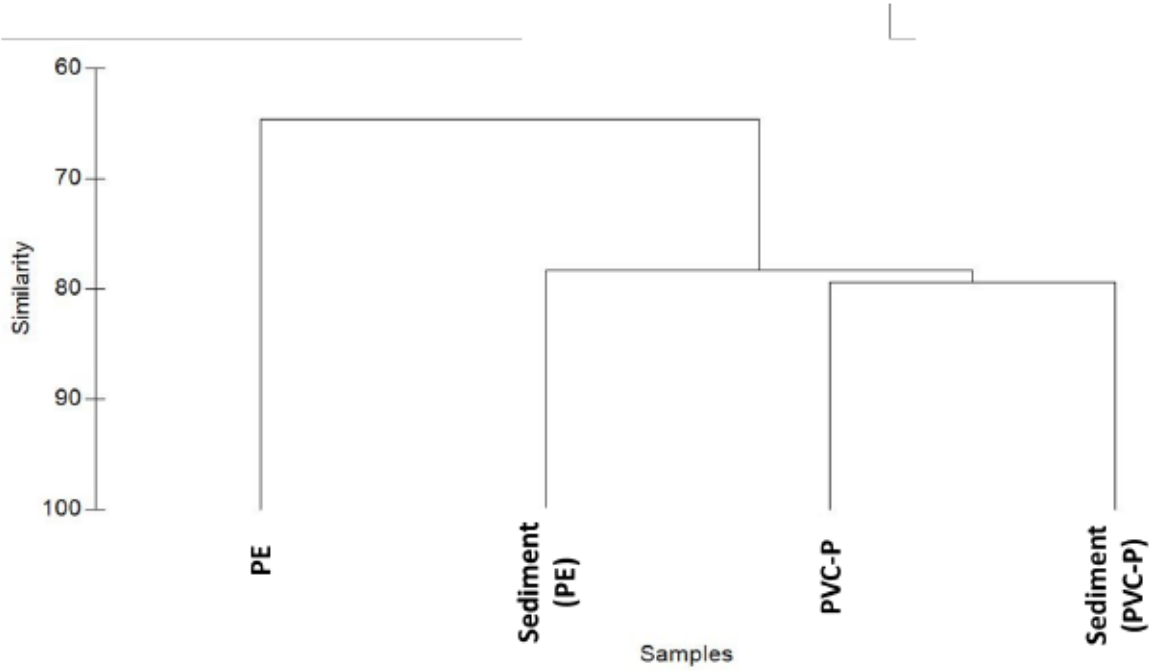

Figure 6 Cluster analysis comparing polyethylene (PE), polyvinyl plasticized (PVC-P) and surrounding deep-sea sediments

Discussion

Plastic items found in deep-sea sediments, polyethylene and PVC-P are commonly used in packaging and other single-use plastic applications. We hypothesized that man-made substrates, being physically and chemically different from deep-sea sediments, can have the potential to select for and support distinct microbial communities. This, indeed, was observed in previous studies comparing microbial assemblages in floating plastic fragments and in surrounding sea water (Zettler et al. 2013; Debroas et al. 2017).

Despite the importance of sediments as a sink for accumulation of plastic debris (Browne et al. 2011; Barnes et al. 2009; Thompson et al. 2004; Bergmann et al. 2017; Classens et al. 2011), only few laboratory studies have been carried out to investigate microbial biofilm on plastics incubated in seiments (Harrison et al. 2014, Nauendorf et al. 2016).

Studies conducted in pelagic ecosystems reported contrasting results on the bacterial richness on plastics floating in sea water. Indeed, investigations in NE Atlantic Ocean revealed a lower richness of bacterial taxa in the plastics rather than in surrounding seawater (Zettler et al. 2013),

whereas the opposite was observed in coastal and open water areas from Pacific, Atlantic and North Sea (Bryant et al. 2016, De Tender et al. 2015). However, in most of the studies conducted microbial composition of plastics and surrounding environment was different (Zettler et al. 2013, Debroas et al. (2017).

Our study showed that sediments were characterized by a higher number of OTUs than in the plastic fragments. Previous investigations explained the lower bacterial richness on plastics with a potentially more selective and specialized bacterial assemblage than in surrounding sea water (Zettler et al. 2013). In the present study, bacterial taxa belonging to Protobacteria, Alpha and Betaproteobacteria present both in PE and PVC-P have been already reported for other different man-made substrates (Dussud et al. 2018, Harrison et al., 2014, Bryant et al., 2016).

Polyethylene was characterized by a higher number of exclusive families than PVC-P, which were not found in the sediments. In PE we found exclusive members of Chitinophagaceae (Bacteroidetes), Christensenellaceae (Firmicutes) and Coriobacteracee (Actinobacteria). In particular, the Chitinophagaceae family was found in polyethylene plastic debris collected in different pelagic ecosystems (De Tender et al. 2017, Zettler et al. 2013) and have been detected also in areas characterized by sewage discharge (Ziegler et al. 2016). In PVC-P we found only exclusive members of Microbacteraceae (Actinobacteria), Parachlamydiaceae (Chlamydiae). These families have been previously associated with different plastic substrates (De Tender et al. 2017). The Parachlamydiaceae family, which naturally infect amoebae, can suggest the potential transport of pathogens through plastics items (Greub et al. 2002) rising concerns on the potential role of plastic debris as a disease vector (Zettler et al. 2013).

The reason of the higher number of exclusive families in PE than in PVC-P, could be ascribed to the simpler chemical structure a lower molecular weight of PE that results in a higher susceptibility to microbial degradation than in PVC-P (Yamada-Onodera et al., 2001). Polyvinyl chloride (PVC) is a strong plastic that resists abrasion and chemicals (Shah et al. 2008).

The difference in assemblage composition between plastics and sediments may be attributed to several factors, including differences in substrate features (plastics vs. sediment), polymer types, nutrient concentrations (Zettler et al. 2013) and provenance that can select specific assemblages.

In this study, we found a lower similarity between microbial assemblages in PVC-P and PE (ca. 65%) than between PVC-P and sediments (ca.80%). This suggests that the peculiar characteristics of PE can select specific families of bacteria which cannot be supported by PVC.

In PE we also found 7 unknow prokaryotic taxa (including also Archaea) whereas only one unknown taxon was detected in PVC-P. We hypothesize that these unknown taxa could represent new species able to use PE, which is one of the most commonly used polymer in packaging and single use plastics, thus providing a starting point for a better understanding of the potential role of prokaryotes in the biodegradation of plastic polymers.

Conclusions

This study provides new insights into microbial assemblages colonizing deep-sea plastic debris. Despite a lower α- bacterial diversity was found on the plastic fragments, these were characterized by exclusive bacterial families not found in the sediments leading to hypothesize that specific bacteria are able to grow on these artificial substrates.

The composition of microbial biofilm changed depending on the plastic polymer, potentially due to the different physicochemical properties of the plastics which also selected different new taxa.

A major future challenge will be to investigate the changes and ecological interactions occurring between microbial communities and plastic fragments and identify new prokaryotes degrading specific plastic polymers.

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7. CONCLUSIONS

The results of the present thesis indicate that the specific procedure developed for deep-sea sediments is highly reliable and allow us to identify the majority of the plastic polymers even those characterized by very small dimensions (2 μm).

This protocol applied to deep-sea sediment samples showed that microplastics are present and highly diversified in a wide range of habitats from different geographical areas (i.e., Atlantic, Southern and Arctic ocean and Mediterranean and Black Sea) and although the dataset needs to be expanded, sediments from Atlantic Ocean appeared to be the most contaminated ones. Nylon, polypropylene, polyethylene and polyester were the most abundant polymers in deep-sea habitats.

Mediterranean canyons can act as conduits of microplastics favouring their accumulation in deep-sea benthic ecosystems, but this is not a general rule for all canyon systems investigated. The mechanisms that can influence accumulation of microplastic are still unknown and topographic features and other environmental factors, including oceanographic processes, could influence microplastic accumulation and composition in these remote ecosystems.

Deep-sea benthic communities, such as nematodes, can ingest microplastics especially when the contamination levels are higher, with potential negative impacts on benthic food web functioning.

Microplastics can also strongly affect deep-water communities, such as *Corallium rubrum,* hampering a wide range of important functions for the maintenance of its health, thus threating biodiversity and ecosystem functioning.

Finally, metagenomic analyses revealed that different plastics could act as substrate for microorganisms, and in particular these can be colonized by novel and unique bacterial and archaea taxa which could represent their degraders, offering new cues for investigating their potential in biotechnological applications.