Microplastics in the Marine Environment and the Characterization of Their Attached Microbial Communities

Thesis

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Microplastics in the marine environment and the characterization of their attached microbial communities

by

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School of Life, Health and Chemical Sciences

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Abstract

Microplastics (MPs) pollution is increasingly recognized as a potential threat to the marine environment (Thompson et al., 2009) and for this reason MPs are included as indicators of the Marine Strategy Framework in order to reach a Good Environmental Status (GES) (Jahnke et al., 2013). In this thesis, MP distribution, occurrence at surface and its attached microbial community have been assessed and characterized using microscopy (light and electron) and High Throughput 16S rRNA gene sequencing (Illumina). Distribution and occurrence have been analysed as a function of water circulation, spatio-temporal variabilities and, most of all, distance from urban settlements and activities. Different time scales have been investigated, from the basin (Atlantic and Mediterranean sea) to sub-basin (Adriatic Sea) to coastal areas (Campania region, South Italy). In coastal areas seasonality has also been investigated. The so-called microbial plastisphere has been investigated in terms of community composition and presence of individual taxa, to be used as indicators of maturity and potential toxicity to humans. Since rivers are recognized as major sources of MPs to the sea, a comparison of freshwater to marine plastisphere members has highlighted differences but also similarities. In vitro production of biofilm attached to commercial microbeads has also been assessed during an experiment aimed at assessing the effect of plastics on the sea urchin *P. lividus*. In general, results point to the existence of “core” plastisphere prokaryotes, which are always present, also suggesting adaptive advantages of the attachment to MPs. Apart from these, communities appear to be influenced by local environmental and biogeographical factors at all scales investigated, also confirming previous observations. Variability in time and space is a factor to be considered when assessing MP pollution, especially in coastal area, and most of all, when considering the potential harmful effects of the attached microbes to ecosystems or humans.
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**List of Abbreviations**

- A.U. Arbitrary Unit
- ALK Resin Alkyd
- AS Alboran Sea
- BMAW Balearic Modified Atlantic Water
- BoP Bay of Pozzuoli
- CEL Cellophane
- Chl a Chlorophyll a
- ENACW Eastern North Atlantic Central Water
- EPDM ethylene propylene diene monomer rubber
- EPS exopolysaccharides
- EVA Ethylene vinyl acetate
- FO Frequency of Observation
- FT-IR Fourier-Transform Infrared Spectroscopy
- FTIR-ATR Fourier-Transform Infrared Spectroscopy in Attenuated Total Reflectance Mode
- GES Good Environmental Status
- GoN Gulf of Napoli
- HDPE High density Polyethylene
- HTS High Throughput Sequencing

19
IND Indurent Material
IR Polyisoprene
KEGG Kyoto Encyclopedia of Genes and Genomes
LDA Linear discriminant analysis
LDPE Light Density Polyethylene
LMWPE low-molecular-weight polyethylene
MAW Modified Atlantic Water
MB Microbead
MCE mixed cellulose ester
MP Microplastics
MPA Marine Protected Area
Mw Molecular weight
NAS North Adriatic Sea
NGO Non-profit organization
NH₄ Ammonia
NM Nautical Miles
NMDS Non-metric multidimensional scaling
NO₂ Nitrites
NO₃ Nitrates
NPCG North Pacific Central Gyre
NPSG North Pacific subtropical gyre
NRI Not-influenced by rivers
OTU Operational Taxonomic Unit
PA 6,6 Nylon 6,6
PA Particle-Attached
PA Polyamide
PBS Phosphate Buffered Saline
PC Polycarbonate
PCA Principal Component Analysis
PEA Poly-ethyl-acrylate
PET Polyethylene terephthalate
PFA Paraformaldehyde
Phaeo Phaeo-pigments

20
PO₄ Phosphates
PP Polypropylene
PS Polystyrene
PTFE Polytetrafluoroethylene
PU Polyurethane
PVA Polyvinyl acetate
PVA polyvinyl alcohol
PVC Polyvinylchloride
RDA Redundancy Analysis
RES Resin
RI River Influenced
SD Sarno Downstream
SD Standard Deviation
SEM Scanning Electron Microscopy
SiO₂ Silicates
SS Sarno Sea
SU Sarno Upstream
TIN Total Inorganic Nitrogen
TMAW Tyrrenhian Modified Atlantic Water
Chapter 1. Introduction
1.1 Plastics in numbers

In order to replace conventional materials, plastics have been chosen for their physico-chemical properties (e.g. light-weight, resistant, bioinert, etc) and its low cost of production, especially for packaging (Andrady et al., 2011). The world economy has raised plastic production to astonishing numbers. In 2015, the NGO “The Ocean Conservancy” reported 275 million metric tons of plastic produced all over the world. Hundred million of these metric tons were produced just from the 192 countries bordering the major oceans and seas of the world. Indeed, in 2017 world plastic production, even without considering PET, PA and polyacyl fibers, has reached 350 million tons (PlasticEurope Report, 2018). Of these, eight to twelve million tons of plastic litter is estimated to reach the ocean every year (Jambeck et al., 2015). From a global perspective, Europe is one of the most important markets for plastics (together with China and North America), with a constant production of synthetic polymers of 64.4 million tons per year and a plastic demand of 51.2 million tons per year (Plastics the facts, 2018). Within Europe, the leading countries in terms of demand are Germany, Italy, France, the United Kingdom, and Spain. The most relevant factors influencing the concentration of plastic in water are human population density in the area and proximity to the urban centre. This is due to increasing demographics favoring immigration to coastal regions, together with extensive fishing, recreational and maritime uses of the ocean (Ribic et al., 2010). For the marine environment it has been estimated that 80% of litter is delivered into aquatic systems by land-based sources: public littering, improper waste disposal, waste dump run-offs, tourism, industrial activity, and combined sewer systems contribute dramatically to the pollution of the aquatic environment with plastic (Andrady et al., 2011). Plastic in lakes and rivers may have different origins: tributaries, on-water activities, tourism, and improper dumping of disused or abandoned plastic wastes of terrestrial origin. Furthermore, stormwater events, rainwater drainage, flooding, and wind can collect and transport plastic that has been dispersed or generated on land to aquatic ecosystems (Faure et al., 2012; Bellasi et al., 2020). That is the reason why several entities have raised concerns regarding such threats, closely-linked to entanglements, ingestion of plastic by marine organisms, such as fish, seabirds, sea turtles, invertebrates, and marine mammals (Fossi et al., 2012; Lusher et al., 2015; Li et al., 2018). In addition, plastic is harmful because it can transfer many chemicals, like additives or other pollutants, that present health risks for humans and other species and limit reuse and recycling potential (Barnes et al., 2009; Worm et al., 2017).
1.2 Plastic dispersion

Rivers play an important role in the transport of plastic into lakes, seas, and the ocean. It is broadly accepted that the dominant input of plastic into oceans is from land-based sources, whereas only a minority is produced directly at sea from vessels, platforms, fisheries, and aquaculture (UNEP report, 2016). Lechner et al., (2014) reported that the Europe’s second largest river, the Danube, can release an average amount of 316.8 ± 4664.6 items per 1000 m³ into the Black Sea, which results in a mass load of 4.8 ± 24.2 g of plastic per 1000 m³. They estimated an average input of about 7.5 g per 1000 m³, resulting in a total entry of 4.2 tons per day at the average flow rate (1533 tons per year). A larger overview is given by the results of a European Commission DG Environment-funded project (SFRA0025, Van der Wal et al., 2015), indicating that the river Danube transports 20–30 tons of plastic litter per year to the North Sea and that the Italian Po River is estimated to transport about 120 tons of plastic litter per year to the Mediterranean Sea. Nevertheless, although high levels of plastic pollution are found in European rivers, the major inputs of plastic debris at the global level come from Asia. A recently published global model computed, considering geospatial information on waste management, population density, and hydrology, estimates that between 1.15 and 2.41 million tons of plastic are currently flowing into the ocean through riverine systems every year (Lebreton et al., 2017). The rivers which pollute the most, as predicted by the model and using information derived from observational studies, are located in Asia: the Yangtze, Xi, and Huangpu rivers (China) and the Ganges River (India and Bangladesh) occupy some of the top positions. Asian rivers represent 86% of total global input, whereas European rivers account for only the 0.28%, with a range of 2310–9320 tons of plastics discharged per year. Ninety-five percent of the plastics reaching the ocean is found in the top 5 meters of the water column (Eggers et al., 2020), also because most of plastics found in the fresh and sea waters are less dense than water and tend to float.

Despite the large oceanic accumulations such as the Pacific Garbage Patches attracting much media attention, the large part of plastic in the ocean is in the form of fragmented small pieces less than 5 mm in size, the MicroPlastics (MP). These are estimated to be around between 25 trillion macro- and 51 trillion MPs littering the ocean (Lebreton et al., 2018), and even if plastic macrolitter would no longer enter the seas, plastic pollution would continue to grow due to fragmentation of already existing plastic litter on the land and at sea,
via photo and oxidative degradation and physical fragmentation. Rivers and lakes are important sources of secondary MP via the fragmentation of the litter abandoned along their paths by weathering and mechanical disruption, similarly to what happens at sea (Kataoka et al., 2019).

1.3 Microplastics classification and composition

Microplastics are a highly heterogeneous mixture of different types of solid polymers with different densities, sizes and shapes. Primary MPs are manufactured as small particles, mainly by the cosmetic industry as scrubs or by the chemical industry as a precursor for other plastic products. Secondary MPs are made by the fragmentation of larger pieces due to physical (mechanical) and chemical processes, induced by light, heat, oxygen and also affected by biodegradation from the marine organisms. Fragments are the MPs most frequently found in aquatic ecosystems from litter and textiles, suggesting that secondary MPs are dominant (Virsek et al., 2016).

Polyolefins (PE and PP) are the most abundant polymers floating in the sea (Suaria et al., 2016, 2018) and primarily come from land-based sources (Andrady et al., 2011), but many other plastic polymers are found, including polystyrene (PS), polyvinylchloride (PVC), polyamide (PA), polyethylene terephthalate (PET) and polyvinyl alcohol (PVA) (Table 1.1). Their environmental fate in the oceans depends mostly on their density. Polymers denser than seawater (e. g. PVC) sink, while those with lower density (e. g. PE and PP) will float at the surface (Avio et al., 2016). Some of these polymers contain fillers and plasticizers to change the original structure to market needs, that can also modify initial polymer density (Worm et al., 2017).
Table 1.1. Plastic polymers with their density range (g cm\(^{-3}\)) and positive/negative buoyancy as relative to freshwater. Data from Frias et al., 2019 and https://omnexus.specialchem.com/polymer-properties/properties/density#values

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Polymer</th>
<th>Density (g cm(^{-3}))</th>
<th>Buoyancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>Polystyrene</td>
<td>0.01 - 1.06</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>/</td>
<td>Resin</td>
<td>0.561</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
<td>0.85 - 0.92</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>C(<em>{25})H(</em>{52})</td>
<td>Wax</td>
<td>0.88 - 0.94</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>LDPE</td>
<td>Low density Polyethylene</td>
<td>0.89 - 0.93</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>IR</td>
<td>Polyisoprene</td>
<td>0.90 - 0.91</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>EVA</td>
<td>Ethylene vinyl acetate</td>
<td>0.93 - 0.95</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>HDPE</td>
<td>High density Polyethylene</td>
<td>0.94 - 0.98</td>
<td>Positive (↑)</td>
</tr>
<tr>
<td>PA</td>
<td>Polyamide</td>
<td>1.12 - 1.15</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PA 6,6</td>
<td>Nylon 6,6</td>
<td>1.13 - 1.15</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly methyl methacrilate</td>
<td>1.16 - 1.20</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinil acetate</td>
<td>1.19</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
<td>1.20 - 1.22</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
<td>1.20 - 1.26</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>/</td>
<td>Resin Alkyd</td>
<td>1.20 - 1.36</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene Terephthalate</td>
<td>1.38 - 1.41</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinil chloride</td>
<td>1.38 - 1.41</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>/</td>
<td>Cellophane</td>
<td>1.42</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>EPDM</td>
<td>ethylene propylene diene monomer rubber</td>
<td>1.5 - 2.00</td>
<td>Negative (↓)</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
<td>2.10 - 2.30</td>
<td>Negative (↓)</td>
</tr>
</tbody>
</table>
1.4 Microplastics in the aquatic environment

Over the past few years, a significant effort has been made to quantify MPs in the oceans, mainly the floating ones (Table 1.2, modified from Avio et al., 2016). Ocean gyres and other convergent zones are important areas of debris and micro debris accumulation, and wind mixing affects their vertical distribution (Kukulka et al., 2012). In the last four decades, MP concentrations have increased by two orders of magnitude in the North Pacific Central Gyre (NPCG) (Goldstein et al., 2012). However, in the North Pacific subtropical gyre (NPSG) MP distribution is more spatially variable, with two orders of magnitude less (Goldstein et al., 2013). In this last case, MPs appear to concentrate in the centre of the gyre (5.38 particles m$^{-3}$ Eriksen et al., 2013). Oceanographic features strongly affect the distribution of MPs and upwelling regions represent convergence zones for marine debris including MPs, even in coastal areas (Doyle et al., 2011), where they are modulated by local weather conditions (Moore et al., 2002; Lattin et al., 2004). In general, MP concentrations increase towards inshore, as a result of the terrestrial inputs and particle resuspension from sediments (Lattin et al., 2004). MPs can also be transported by ocean currents towards offshore (Reisser et al., 2013). Regular sampling schemes found MPs in the northeast Atlantic Ocean to be widespread and abundant both at the sea surface (2.46 particles m$^{-3}$, Lusher et al., 2014) and in biota (3.3 particles g$^{-1}$, Courtene-Jones et al. 2017) in the coastal pelagic zones, while were found surprisingly less abundant in the proximity of urban areas (Frias et al., 2014). As for river runoff, high variability has been found among Portuguese and British estuaries, as at the former sites were found less MPs than the latter (0.04 particles m$^{-3}$, Frias et al., 2014; 1.5 particles m$^{-3}$, Maes et al., 2017).
Table 1.2. Average measurements of floating MP items reported from different geographical areas (modified from Avio et al., 2016).

<table>
<thead>
<tr>
<th>Location</th>
<th>Average measurement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Pacific (central gyre)</td>
<td>334.3 items m⁻²</td>
<td>Moore et al., 2001</td>
</tr>
<tr>
<td>Northeast Pacific (South California)</td>
<td>8 items m⁻³</td>
<td>Moore et al., 2002</td>
</tr>
<tr>
<td>Northeast Pacific</td>
<td>0.004–0.19 items m⁻³</td>
<td>Doyle et al., 2011</td>
</tr>
<tr>
<td>North Pacific subtropical gyre</td>
<td>0.021–0.448 items m⁻²</td>
<td>Goldstein and Goodwin, 2013</td>
</tr>
<tr>
<td>North Pacific subtropical gyre</td>
<td>0.16 items m⁻²</td>
<td>Law et al., 2014</td>
</tr>
<tr>
<td>South Pacific subtropical gyre</td>
<td>0.027 items m⁻²</td>
<td>Eriksen et al., 2013</td>
</tr>
<tr>
<td>Australian coast</td>
<td>0.00085 items m⁻³</td>
<td>Reisser et al., 2013</td>
</tr>
<tr>
<td>East China Sea</td>
<td>0.167 ± 0.138 items m⁻³</td>
<td>Moore et al., 2002</td>
</tr>
<tr>
<td>Yangtze estuary</td>
<td>4137.3 ± 2461.5 items m⁻³</td>
<td>Zhao et al., 2014</td>
</tr>
<tr>
<td>South Korea coast</td>
<td>13 +–11 items m⁻²</td>
<td>Song et al., 2015</td>
</tr>
<tr>
<td>Northwest Atlantic</td>
<td>0 – 1.331 particles m⁻²</td>
<td>Wilcox et al., 2020</td>
</tr>
<tr>
<td>Northeast Atlantic (Celtic Sea)</td>
<td>2.46 items m⁻³</td>
<td>Lusher et al., 2014</td>
</tr>
</tbody>
</table>

In terms of river microplastics pollution, the mentioned Yangtze River in the Wuhan region, the largest city in central China, showed a MP concentration of 2516.7 ± 911.7 particles per m³ (Zhao et al., 2014), an incredibly high number compared to the 0.3168 particles per m³ found in the Danube (Austria) and 0.028 particles per m³ found in the Tamar Estuary (England) (Bellasi et al., 2020). As mentioned before, North America has similar contribution to European MPs pollution and there is a confirmed link between population density in the river basin, land use, and MP concentration in the estuary of Chesapeake Bay, United States (5534 – 297,927 pieces km⁻², Yonkos et al., 2014). Other examples of MP concentrations in rivers are shown in Table 1.3.
Table 1.3. MP concentrations in rivers around the world.

<table>
<thead>
<tr>
<th>Location</th>
<th>Items m$^{-3}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jiaojiang (China), Yangtze River</td>
<td>100 - 4100</td>
<td>Zhao et al., 2015</td>
</tr>
<tr>
<td>Hanjiang (China)</td>
<td>1660 - 8925</td>
<td>Wang et al., 2017c</td>
</tr>
<tr>
<td>Pearl River (China)</td>
<td>379 - 7924</td>
<td>Lin et al., 2018</td>
</tr>
<tr>
<td>Danube (Austria)</td>
<td>0.3168</td>
<td>Lechner et al., 2014</td>
</tr>
<tr>
<td>Tamar Estuary</td>
<td>0.028</td>
<td>Sadri et al., 2014</td>
</tr>
<tr>
<td>Great Lakes Tributaries (US)</td>
<td>1.9 - 3.2</td>
<td>Baldwin et al., 2016</td>
</tr>
<tr>
<td>North Shore Channel (US)</td>
<td>1.67 - 10.36</td>
<td>Hoellein et al., 2017</td>
</tr>
<tr>
<td>Higgen's Creek (US)</td>
<td>1.94 - 17.93</td>
<td>McCormick et al., 2014</td>
</tr>
</tbody>
</table>

1.5 Microplastics in the Mediterranean Sea

The Mediterranean is a semi-enclosed sea, with inputs of Atlantic water originating from the Strait of Gibraltar and numerous large rivers (i.e. Po, Ebro, Nile), and characterized by high variability of surface currents and diverse instabilities (Cincinelli et al., 2019). By estimating both terrestrial and marine inputs, Lebreton and coworkers (2012) modeled the transport and distribution of floating debris in the global ocean, identifying the Mediterranean Sea as a potentially important accumulation area, with 23,150 tons of floating plastics (Eriksen et al., 2014). Plastic accumulation likely results from significant discharges combined with a limited export to the Atlantic Ocean, resulting in the Mediterranean Sea as a convective basin and a sink for Atlantic floating plastic (Cozàr et al., 2015).

From the end of 1970’s, the interest of MPs in the Mediterranean seas increased. The very first publications were focused on beaches (Shiber, 1979) and seawater (Morris, 1980). In the last ten years (period 2010-2020) there was a strong positive correlation between the number of articles published, which supports the general impression of a growing interest in MPs research in the field. In the Alboran Sea, influenced by the inflow of Atlantic waters with its dense commercial shipping lanes, fishing activity and terrestrial sources, which may influence MP inputs the concentration reported by de Haan et al. (2022), 0.10 items m$^{-2}$ were found. Most recently, Camins et al. (2020) reported an average of 1.12 MPs m$^{-2}$ in the
Balearic Sea, which was lower than the concentrations reported for the same region in 2018 (0.9 MPs m\(^{-2}\), Ruiz-Orejon et al., 2019). As for the Gulf of Lion, Schmidt et al. (2018) sampled areas close to wastewater treatment plants and river mouths several times for a period of 2 years and reported mean concentrations of 0.112 MPs m\(^{-2}\). Those concentrations were similar to the results obtained from the earlier trawling study in the same region (0.13 MPs m\(^{-2}\), Fauve et al., 2015). Along the Italian coast, MP concentrations at surface are similar to those reported for the North Pacific Central Gyre (0.27 particles m\(^{-3}\); Collignon et al., 2012), also in areas distant from the coast (0.15 particles m\(^{-3}\); de Lucia et al., 2014). Instead, low concentrations were found at surface near Corse Island (0.012 particles m\(^{-3}\); Collignon et al., 2014), possibly due to strong winds and currents actively distributing particles at surface (Collignon et al., 2012). Few information on MPs in the Gulf of Napoli is available. A technical report from CNR-ISMAR from a sampling cruise in summer 2017 indicates 0.26 MPs m\(^{-3}\) at Punta Campanella and 3.56 MPs m\(^{-3}\) close to the city of Portici (CNR-ISMAR, 2017). High concentrations were found near Ischia (0.49 MPs m\(^{-3}\)), probably depending on the fact that the currents that arrive in this area come from the Gulf of Napoli, densely populated and full of activities together with a high human presence on the island itself in the summer period (Pini et al., 2018). Few data are also available from the Sarno river, one of the most polluted river in Europe (Baldantoni et al., 2018), very much contaminated by organic substances, with a huge difficulty to detect the presence of MPs (De Falco et al., 2020). The latter sites (Bay of Pozzuoli, Cilento area and Sarno river) will be investigated in this PhD thesis. The Adriatic Sea is often considered as one of the most polluted regions of the Mediterranean Sea (Suaria et al., 2016), in part because of the extensive marine traffic and touristic centers in the region, but also because of the large riverine inputs from the river Po (Vianello et al., 2018), which flows through various industrial regions and opens to a wide delta in the northern part of the Adriatic. There has been a wide range of MPs concentrations reported in the Adriatic Sea, from low numbers in the central Adriatic (up to 0.0004 MPs m\(^{-2}\), Capriotti et al., 2021) to very high concentrations (up to 41.3 MPs m\(^{-3}\) Gajšt et al., 2016, Virsek et al., 2017). The city and port of Trieste and the river Po are among the top ten sources of marine litter (Liubartseva et al., 2016). Further East, Gündoğdu et al. (2018) reported that high concentration of MPs in the Levantine Sea which increased after rain (average 0.535 MPs m\(^{-2}\)).
1.6 The missing ocean plastic sink

A discrepancy has been revealed between the calculated amount of plastic predicted to have entered the oceans since the 1960’s and the amount measured by monitoring surveys. This has been attributed to errors in calculations (e. g. Weiss et al., 2021), who demonstrated that river fluxes calculated in past works are overestimated and the residence times of plastics of any size at the sea surface are higher than expected (from days to years). Other works demonstrated, instead, that there is an enormous lack of information related to plastic sinking processes, and have renamed this phenomenon as the “missing 99%” (Cozàr et al., 2015; Van Sebille et al., 2015).

One of the theories on why such a discrepancy exists, is by the effect of biofouling, playing a role in increasing density and promoting the vertical transport of plastics towards the seafloor. Large plastics sink when biofouled by hydroids, barnacles, bryozoans, or brown, green, and red algae (Holmström, 1975, Ye and Andrady 1991, Amaral-Zettler et al., 2021). MPs, instead, are mostly colonized by prokaryotic and eukaryotic microorganisms, which may increase the density of buoyant plastics and have MPs to sink as well. Indeed, Amaral-Zettler et al., (2021) showed how the plastisphere community can change density of MPs, even for low density polymers, and make them sink.

In turn, as soon as MPs exit the euphotic zone, photosynthesis cannot take place anymore and the biofilm will probably reduce its density and, hence, float again (Figure 1.1). This putative path has been numerically modelled by Kooi et al., 2017 and proposed as a possible solution to the “missing 99%” theory. Nevertheless, the absence of accurate experimental measurements—i.e., realistic Plastisphere thickness and density values—complicates current sinking models, which could help in predicting the fate of plastic pollution in the environment. However, a consensus is missing within the scientific community.
In the past few years, more attention has been devoted to the microbial communities colonizing MP particles, also in the hope to identify potential degraders. It has been reported that plastic substrates are easily colonized by microorganisms and that these communities are different from the ones thriving in the surrounding seawater (Zettler et al., 2013; Reisser et al., 2014; Amaral-Zettler et al., 2015; De Tender et al., 2015). While free floating planktonic organisms rely on distant and transient microbe–microbe exchanges, the build-up and cycling of nutrients between phototrophic and heterotrophic microorganisms within biofilms is more effective, increasing interactions and use of hotspots of nutrients and organic matter. It is therefore not surprising that organisms belonging to the Plastisphere are more productive than their planktonic counterparts (Bryant et al., 2016, Wright et al., 2020). However, different discriminants have been found to shape the plastisphere prokaryotic communities. The most important seem to be biogeography (Amaral-Zettler et al., 2015), seasonality (Oberbeckmann et al., 2014, others), and, to a lesser extent, the polymer type (Zettler et al., 2013, Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015; Eich et al., 2015; Debroas et al., 2017). The first two have been found more often as the relevant discriminants as they include the environmental conditions which seem to have a direct link to the colonization of the community (Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015). The first colonizers, instead, may be influenced by the chemical structure of plastics, and so by polymer type, just in the very first phase of colonization (Datta et al., 2016; Mincer
et al., 2016), but the communities on different substrates converge over time as their biofilms mature (Amaral-Zettler et al., 2020). SEM microphotographs show both prokaryotes and eukaryotes as first colonizers of MPs surfaces, possibly due to their fast adaptation to emerging ecological niches and novel habitats (Zettler et al., 2013; Reisser et al., 2014; Oberbeckmann et al., 2014; Eich et al., 2015). Few articles have reported quantitative abundances of these two types of microorganisms, which could be relevant especially in terms of their contribution to enhanced primary and secondary productivity of different marine regions (Reisser et al., 2014). Abundances can be also relevant in order to verify the hypothesized role of plastics-associated microorganisms as vector of HABs (Masò et al., 2003) and/or pathogens like *Vibrio* and *Aeromonas* genera, Campylobacteraceae family and *Aeromonas salmonicida* species (Zettler et al., 2013; Oberbeckmann et al., 2019; Amaral-Zettler et al., 2020).

Specific members of the microbial Plastisphere have already been identified, also with the help of amplicon 16S sequencing, in different marine environments bacteria belonging to Proteobacteria and Bacteroidetes families like Flavobacteriaceae, Saprospiraceae and Rhodobacteriaceae (Oberbeckmann et al., 2016; De Tender 2017) and the genus *Vibrio* (Zettler et al., 2013, others). It has been hypothesized also the presence of some bacterial members may be used as proxy of biofilm formation phase on plastics: alpha- and gammaproteobacteria appear to be members of primary biofilm colonizers, while Bacteroidetes as secondary biofilm colonizers in the marine environment (De Tender et al., 2017). As for diatoms, several genera are commonly reported to thrive attached to MPs, including *Mastogloia*, *Nitzschia* and *Amphora* but also *Navicula* and *Cocconeis* (Zettler et al., 2013; Reisser et al., 2013; Dudek et al., 2020). Other works have found also other phototrophs like Cyanobacteria, Chlorarachniophytes and *Ulva* sp. (Zettler et al., 2013; Bryant et al., 2016). Fungal diversity in the plastisphere remains relatively underexplored, but molecular surveys have been of fundamental help to better describe them. It has been reported that they can live attached, in general, to plastics but also wood (Kettner et al., 2019). When they are found as members of epiplastic communities, this is usually because they co-occur with diatoms (Lacerda et al., 2019), using the latter as food but also as organisms inducing biofilm formation (De Tender et al., 2017). Among other functions, it has been reported also that fungi can degrade polymers (Paço et al., 2017; Russell et al., 2011), while other trophic roles include decomposition, parasitism, predation, symbiosis and pathogenesis (Amaral-Zettler et al., 2020). Other evidence of (micro)organisms found to colonize and survive, even at long time exposure and long distances, on MPs, are ciliates,
small flagellates, radiolarian and choanoflagellates. The latter are also evidences of ecological interactions on MPs as they feed on bacteria and other microbes (Amaral-Zettler et al., 2020).

Several microorganisms can be active plastic degraders, via inducible extracellular enzymes acting in depolymerization of synthetic polymers (Wei et al., 2017). Enzymes from plants can also hydrolyse the ester bonds in PET and PUR (Chen et al., 2013; Wei et al., 2014c; Schmidt et al., 2017) and some enzymes involved in the metabolism of plant lignin are also able to degrade PE (Sivan, 2011; Restrepo-Flórez et al., 2014). The problem here is that environmental conditions in the ocean may not allow efficient or complete biodegradation. In order to be taken up by microorganisms and be oxidized intracellularly, the polymers must have a low molecular weight (Mw). To this end, plastics that are hydrolysable (that is, with backbones consisting of components other than just C–C or C–H; for example, PET, polyurethane (PU) and polycarbonate) are more likely to be more suitable substrates for microbial degradation in the environment than are the non-hydrolysable polymers most commonly encountered in the pelagic marine environment (PE, PP and expanded PS) (Amaral-Zettler et al., 2020). The discovery of PETase, an enzyme that hydrolyses plastic polymers such as PET, in the bacterium Ideonella sakaiensis, and the subsequent recovery of related enzymes from marine and terrestrial metagenomes in public databases, indicates that a PET-degrading capacity may be ubiquitous in those environments (Yoshida et al., 2016). Abiotic factors like UV irradiation, oxygen, temperature, together with chemical oxidants, play a crucial role in the degradation of PE and PP. A mesophilic marine beach soil-derived Pseudomonas strain incubated with low-molecular-weight polyethylene (LMWPE) as a sole carbon source is often cited as the best example of the potential for biodegradation of polyethylene (Danso et al., 2019). Although this and other studies suggest possibilities for microbial solutions to plastic pollution, LMWPE does not occur commonly in the marine environment, and conditions in the ocean result in very slow rates of degradation. While numerous authors describe degradation of chemical additives by microorganisms, no enzymes degrading polystyrene, polyamide, polyvinylchloride, polypropylene, ether-based polyurethane, and polyethylene are known, and these represent more than 80% of annual plastic production. It is therefore evident that further research is needed in this field (Danso et al., 2019).
1.8 Aims of the work

In this context, the aim of this PhD work was to investigate the different factors, in particular the spatial and temporal variabilities, contributing to the MPs distribution in coastal areas, in regards to MP characteristics, together with their associated microbial community. As for the latter point, the aim was to assess discriminants shaping the microbial community, if they do change or there is the presence of a strict group of microbes, a “core”, collecting MPs directly from the environment. From the spatial point of view, different areas were investigated from coastal areas (Campania coast, South Italy, and North Adriatic) to North Atlantic. As for the temporal point of view, annual and interannual (seasonal) features have been investigated. As incubation experiments have had a relevance in the plastisphere research, in collaboration with Dr. Anna Palumbo and Carola Murano and in the framework of the SZN Flagship Project MicroMare, it was carried out an experiment of exposure of sea urchins to polystyrene microbeads, with the aim to investigate the effects of plastisphere on the latters after incubation in natural seawater. More in particular with the hypothesis that the biofilm modulates and enhances the toxic effects of polystyrene on the sea urchins feeding on them.
Chapter 2.  
Materials and Methods
In this Chapter the general procedures commonly used to obtain the data presented in the thesis are described. Deviations from these are reported in each Chapter or subchapter, when pertinent.

### 2.1 Sampling and ancillary parameters

At each sampling site, a CTD cast was performed using a SeaBird SBE19 or 901 probe (SeaBird Electronics, USA). Discrete samples for chlorophyll and dissolved inorganic nutrients were taken from Niskin bottles on a Rosette sampler. The determination of photosynthetic pigments has been performed by HPLC and nutrients (Total Inorganic Nitrogen as the sum of NO$_3$, NO$_2$ and NH$_4$, PO$_4$ and SiO$_2$) all analysed by Dr. Maria Saggiomo and Francesca Margiotta of the Marine Analytical Facility of the Stazione Zoologica Anton Dohrn, RIMAR Department. Samples for dissolved inorganic nutrient analyses (ammonia, nitrites, nitrates, silicates and phosphates) were immediately stored in 20 ml high-density polyethylene vials at −20 °C until the analyses, which were carried out with a five-channel continuous flow autoanalyzer (Flow-Sys Systea), according to Hansen and Grasshoff (1983). For chlorophyll a (Chl a), 200–540 ml of seawater was filtered onto GF/F filters and immediately stored in liquid nitrogen until the analysis. Chl a was analysed according to Holm-Hansen et al. (1965) with a Shimadzu RF-5301 PC spectrofluorometer, daily calibrated with a Chl a standard solution (from Anacystis nidulans; Sigma).

### 2.2 Microplastics collection and isolation

Plastics were collected using a 0.6 × 0.16 m rectangular hyponeuston net (also named “manta” net, Figure 2.1A) with a 333-μm mesh size (Oceomic, Spain). The net presented two steel “wings” providing stability and buoyancy, allowing sampling of the top 20 cm layer of the water column. In the center of the opening of the manta net, a flowmeter was placed (Model 23.090, KC Denmark A/S, Denmark) to estimate the volume of water sampled (Figure 2.1B).
The net was towed usually for 30 minutes at an average speed of 2 knots or less. Sometimes, when the meteorological conditions were not optimal, the manta net was towed for 20 minutes. At the end of the tow the manta net was retrieved, rinsed with running seawater and the material recovered from the cod end (Figure 2.1C). Once removed, the cod-end content was sieved through two sieves of 5000 and 300 μm in sequence in order to isolate the material in the desired size range. In order to characterize the plastisphere, the largest plastic pieces (usually ranging in number from 6 to 11 pieces larger than 1 mm) within 300 and 5000 μm of plastic were removed with sterile forceps, rinsed with 0.22 μm filter-sterilized seawater and cut into three sub-pieces for DNA extraction, Scanning Electron Microscope (SEM) and chemical composition analysis by Fourier-Transform Infrared Spectroscopy (FT-IR), respectively. The remaining pieces in the smaller sieve, together with all organic and inorganic material, including zooplankton and other organisms retained, were then poured into a glass container, fixed with 70% ethanol and stored at 4°C for subsequent analysis.

While the Manta net was in the water, 1 to 2 L of surface seawater was filtered through 0.22 μm, 47 mm diameter, mixed cellulose ester (MCE) filters (GSWP04700, Millipore, USA) in triplicate, to collect microorganisms suspended in the ambient surface water (free-living microbes, as opposed to plastic-attached ones). All the equipment was cleaned, and all items were stored in clean covered (during work) or sealed (for storage) Petri dishes.

Plastic and seawater filters for downstream DNA analysis were immediately placed in 1.5 ml Eppendorf tubes filled with Puregene lysis buffer (Qiagen, Valencia, CA) and frozen at 38°C for subsequent analysis.
–20 °C. Plastic samples for SEM were fixed in 4% Paraformaldehyde (PFA) for 2–23 h, then transferred to 50% ethanol in Phosphate Buffered Saline (PBS) and kept at –20 °C. Finally, plastic samples for FT-IR were placed in individual 1.5 ml Eppendorf tubes.

The sampled area and the water volume filtered during each trawl were calculated using the frame dimensions and the tow distance (derived from the flowmeter readings). MP concentrations were expressed as particles m$^{-3}$. The volume sampled was calculated by multiplying the towing distance (referring to flowmeter readings times a conversion factor, specific for each flowmeter, as indicated from the manufacturer, Model 23.090, KC Denmark A/S, Denmark, in our case).
2.3 Microplastics counting and classification

Once in the lab, samples were poured into a glass Petri dish and analyzed with the use of a stereomicroscope (Leica CLS 150 XE, using a 20 - 80x zoom) for the count and classification of MP particles. Cotton clothes and laboratory coats were worn to minimize contamination by synthetic fibers. The laboratory was aired before the analysis and then closed to minimize airflow that could increase airborne contamination. All the equipment and the working counters were cleaned prior to analysis and particles were stored in clean covered (during work) or sealed (for storage) Petri dishes.

Microplastic particles were distributed into six categories according to their visual features, four of them belonging to secondary MPs and two to primary ones (Virsek et al., 2016). The most abundant were usually secondary MPs divided into fragments, films, foams and filaments. Among these, the most numerous were usually fragments (Figure 2.2A): they were tough and thick, with sharp edges and an irregular shape and they came in different colors. The films (Figure 2.2B) were also irregular but they appeared thin and flexible and usually transparent. Foams were soft, irregular and from white to yellow (Figure 2.2C). Filaments (Figure 2.2D) were also very abundant and composite in shape, dimension, thickness and color. As for primary MPs there were two types: pellets and granules. Pellets (Figure 2.2E and G) were usually irregular and around 5 mm in diameter. They were usually flat on one side and they could be of different colors. Granules (Figure 2.2F) had a regular round shape, usually 1 mm ca in diameter and they came in white, beige, or brown (Virsek et al., 2016).
Unfortunately, a standardized methodology for MP extraction, quantification, characterization, and toxicity has not been properly set up yet. As a consequence, the data related to microplastics around the world are generated using a diverse array of sampling and extraction equipment and methodology of sampling and identifying microplastics of differing size ranges and report data in a range of units (e.g., pieces or mass per km² and/or m³ Cozár et al., 2015; Avio et al., 2016). Some methods may be designed to be more technical, and others may be created to facilitate citizen science initiatives, i.e. collection by non-professionals. In order to choose the best protocol, it was important to consider temporal and spatial scales, because MP distribution is highly variable across space and time (Paradinas et al., 2021). Second, it was important to choose a method that could sample the exact size-range of interest, effectively extracting MPs from the media without dissolving or melting the material of interest and in order to identify and confirm the many different types of MPs. An overriding priority was to describe the data collected to prevent ambiguity. The most important aspect in this case was using consistent units so that we could synthesize data across studies to ask questions about broader contamination. It is well-known that plastics are associated with unique cocktails of chemicals and which increase their load of chemicals when they entered aquatic habitats via sorption. In addition, they gain a fouling community that hitchhiked on their surface. Methods related to the fate and occurrence of
these chemicals and communities were useful to understand issues related to fate and effects. As plastics travel around the oceans, they represent a vector of many different chemicals and/or biological communities. In general, questions related to effects became increasingly important due to widespread contamination by MPs. As such, the need for accepted methods for measuring effects and using them in assessments of risk is increasingly acknowledged (Rochman et al., 2017).

In this study, MPs were collected from the hyponeuston using the manta net (with a 333-micron mesh size) and counted by the stereomicroscope, following the ISPRA Protocol (ISPRA, Programmi di Monitoraggio per la Strategia Marina Art. 11, D.lgs. 190/2010) for assessing microplastics pollution (units used were items m⁻³). Once retrieved from the manta net, MPs were analysed with Fourier-Transform Infra-Red Spectroscopy for chemical identification. This has not been possible for all samples, due to the impact of Covid-19 outbreak on access to analytical facilities.

2.4 Scanning Electron Microscope (SEM) analysis and coverage estimates

Preserved plastic samples were dehydrated on ice through resuspension in a series of increasing concentrations of ethanol: 10 min each in 70%, 85%, 95%, followed by 3 × 15 min in 100% ethanol. Samples were then critical-point-dried using a Leica EM CPD300 (Leica Microsystems, Inc. USA). Then the pieces on metal stubs were sputter-coated with 15 nm of platinum using a Polaron SC7640 (Thermo VG Scientific, USA) and visualized on a JEOL 6700F microscope (Jeol Inc., USA) at the Functional Analyses and Bioimaging Facility of the SZN. The coverage of plastic piece surfaces by biofilm was qualitatively estimated at 500x magnification using percentage of colonization from 0% of coverage, Figure 2.3 A to 100% coverage, Figure 2.3 B (Amaral-Zettler et al., 2021).
For each randomly selected field, two levels of magnification were used: 500x for diatoms and other relatively larger organisms and 1500x for prokaryotes. Cell counts were processed from acquired digital images using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD http://imagej.nih.gov/ij/, 1997-2012). All counts from the different fields were averaged (technical replicates) and expressed as cells mm$^{-2}$, with each area of a single field of view being 0.062 mm$^2$. In order to randomly sample the surface of each plastic piece and to avoid biases due to patchiness, the longer dimension of each plastic piece was divided by 250, which was the length, in microns, of the represented side of the square view of the SEM at 500x, in order to calculate the number of fields encompassed (N) along the whole piece. An electronic random number generator freely available on the Internet (www.random.org) was used to generate random numbers corresponding to the N fields to be analyzed. Diatoms were assigned to the finest taxonomical level possible, using Tomas et al. (1997) and Avancini et al (2006) and also with the support of Drs. Diana Sarno, Lucia Porzio, Maria Cristina Buia and Cecilia Totti as diatom taxonomy experts. Since it was not possible to distinguish Bacteria from Archaea based on morphology only, in this thesis the term “prokaryotes” has to be considered inclusive of both Bacteria and Archaea.

Figure 2.3. Coverage of surface of microplastic pieces. A) Coverage 0, no colonization of plastics; B) Coverage 4, 100% of colonization of the plastic surface.
2.5 DNA Extraction, Sequencing and Bioinformatic analyses

DNA was extracted from either single plastic pieces or filters using a modified bead-beating approach (Zettler et al., 2013) in combination with the Puregene Tissue DNA extraction kit (Qiagen, Valencia, CA). The sizes of DNA extraction products were visually checked on 0.8% agarose gel electrophoresis and quantified with Nanodrop spectrophotometer. The extracted DNA was sent to the CGBM Sequencing Facility of the Dalhousie University, Canada, for 2×300 paired-end (PE) Illumina MiSeq sequencing using the primers 515F-Y (5’-GTGYCAGCMGCCGCGGTAA) and 926R (5’-CCGYCAATTYMTTTRAGTTT) of the 16S rDNA gene (Parada et al., 2015). The raw data in fastq format were preprocessed by the Bioinforma service at SZN using the pipeline implemented in QIIME 2 (Bolyen et al., 2019). In particular, for the de-noising step the DADA2 (Callahan et al., 2016) tool was used, setting as minimum read length 280 nt (forward reads) and 260 nt (reverse reads). For the taxonomic classification, the SILVA database (v. 132) was used (Quast et al., 2012). OTUs assigned to mitochondria, eukaryotes and unknown were removed. To enable comparison between samples, the cleaned dataset was randomly subsampled to the smallest number of sequences in one sample, using the function ‘rarefy’ in vegan R package (Oksanen et al., 2019). Bray–Curtis Dissimilarity matrix was computed (vegdist in vegan) and used for subsequent ordination analyses. Non-metric multidimensional scaling (NMDS) was performed using the metaNMDS function in vegan and the dendrograms presenting hierarchically organised samples were built with hclust and average method (Ramette 2007; Buttigieg and Ramette 2014). Alpha diversity indexes (OTUs observed, Chao and Shannon) were calculated with R package phyloseq (McMurdie et al., 2013). Wilcoxon test was used to test statistical differences in the abundances of taxa in different conditions. In particular, the presence of signals for differences at temporal, spatial scale and also for different polymers were tested (PE, PP, etc.). Venn diagrams were obtained using the Venny open-source online website (Oliveros, J.C. (2007-2015) - An interactive tool for comparing lists with Venn diagrams). Linear discriminant analysis (LDA) effect size (LefSe, Segata et al., 2011) was performed with a standard LDA threshold score of 3.5. This tool determines the features (in this case, e.g. the taxa) most likely to explain differences between classes (in this case between free and attached communities, or communities belonging to different polymers). First, the Kruskal-Wallis test rank sum test on classes was used, then, if
subclasses of different classes were present, the pairwise Wilcoxon test was applied. Finally, the LDA was built with the class to determine the relevant features. Chloroplast sequences were extracted and reassigned to eukaryotic groups using PhytoREF Database. PhytoREF is a resource to discover, assess and monitor the diversity of photosynthetic eukaryotes from high-throughput sequencing (Decelle et al., 2015). Finally, Mothur software (https://www.mothur.org) was used to plot the different resulting taxa at the taxonomic profiles (Chappidi et al., 2019).

2.6 Polymer identification

The analysis of the microplastic chemical composition was performed both at CNR-IAMC Palermo (Capo Granitola) by Dr. Fabio D’Agostino and at ENEA Centro di Ricerche CASACCIA SSPT-PROTER-BES by Dr. Maria Sighicelli. The analyses were carried out without treatment, using a Nicolet iS5 FTIR Spectrometer (purchased by Thermo Scientific) in ATR mode (Diamond). The spectra were acquired in absorbance mode from 4000 to 600 cm$^{-1}$ with a resolution of 4.0 cm$^{-1}$ and collecting 32 scans. In order to identify the polymers, the obtained spectra were compared with Hummel Polymer Sample Library and the results were shown reporting compound name and correlation percentage. Some examples of the MPs found in environmental samples are in Figure 2.4.

Figure 2.4. Examples of spectra analysed by ATR-FTIR of MPs found in environmental samples. Blue lines describe the environmental sample while the red ones the standard spectrum from the comparison library. A) Polyethylene PE; B) Polypropylene atactic PP; C) Polystyrene atactic PS.
2.7 Growth on Polystyrene microbeads

In order to allow growth of microbial biofilm on plastic beads to be exposed to sea urchins in ecotoxicological experiments, fluorescent polystyrene microbeads (MBs, 441 excitation/485 emission, micro-PS) of 45 µm in diameter were purchased from Polysciences (Warrington, USA). Four x 10⁴ MBs L⁻¹ were incubated in a glass Erlenmeyer flask for one week in 1 L of unfiltered natural seawater collected from a coastal site in the Gulf of Napoli (40° 49’13.8” N, 14° 18’09.7” E) in a temperature-controlled culture cabinet chamber (Angelantoni, Italy; temperature: 18±1°C; light 100 µmol; LD 12:12 cycle) on an orbital shaker. After one week, 500 ml were centrifuged with an Allegra 5r centrifuge (Beckman Coulter, CA, USA) at 3500 rpm speed for 10 minutes. The pellet was then collected on a 47 mm filter of 10-micron pore size to retrieve MBs. The supernatant was filtered onto 0.22 µm, 47 mm diameter, mixed cellulose ester (MCE) filters (Millipore, USA) and processed as indicated above for prokaryotic DNA sequencing (sections DNA Extraction, Sequencing and Bioinformatic analyses, mentioned above). Growth of biofilm (or lack of) was verified by Scanning Electron Microscopy (SEM). The same procedure was followed in order to retrieve the beads and the free-living prokaryotes from the tanks of the sea urchin incubations (see Chapter 6).
CHAPTER 3.

Introduction to the sample sites
Microplastics were collected in different times and at different places around the Mediterranean Sea and in the North Atlantic. The three major areas interested were the Several sites were also repeatedly sampled along the coasts of Campania region, namely: Bay of Pozzuoli, Gulf of Napoli, Sarno river plume and path, Cilento area. The North Adriatic Sea was also sampled during an oceanographic cruise, while several stations were also sampled along a transect from the Azores (Portugal, North Atlantic) to Catania (Italy).

3.1 The Gulf of Napoli

The Gulf of Napoli is a coastal embayment with an average depth of 170 m and an area of approximately 870 km² (5.8 surface/volume ratio). The littoral area of the Gulf of Napoli is heavily influenced by land runoff from a very densely populated region (Cianelli et al., 2012). However, due to the complex physiography and bottom topography, the inner shelf is strongly coupled with the offshore waters of the Tyrrhenian Sea. This results in the tight intertwining of two subsystems within the Gulf: a eutrophic coastal zone and an oligotrophic area similar to the offshore Tyrhenian waters (Carrada et al., 1980). The location and width of the boundary between the two subsystems vary over the seasons and determines a highly dynamic system with exchanges between the two subsystems (Casotti et al., 2000). This reflects into high biological variability, where intense spring and autumn phytoplankton blooms, mainly contributed by diatoms, represent the main feature (D’Alcalà et al., 2004; Scotto di Carlo et al., 1995; Zingone et al., 1990; Zingone et al., 1995). Cyanobacteria show a repetitive bloom in the summer, possibly due to release of grazing pressure (Modigh et al., 1996). Despite this general pattern, a high interannual variability is evident in the timing and extent of peaks and minima (Ribera d’Alcalà et al., 2004, Cianelli et al., 2017; Zingone et al., 2019).

No information on MPs in the Gulf of Napoli is available, but a technical report from CNR-ISMAR from a sampling cruise in summer 2017, indicates 0.26 MPs m⁻³ at Punta Campanella and 3.56 close to the city of Portici, our st.1 (Mezzelani et al., 2017). Floating MP concentrations, distribution, and characteristics of the associated microbial plastisphere were investigated with the aim of assessing their space and time distribution, identifying 48
possible accumulation areas, assessing putative differences between polymers in terms of attached prokaryotes, trying to identify common members of the microbial plastisphere, to be interpreted as obligate plastic-associated organisms.

MPs were collected in January and July 2018 (MP18A and MP18B, respectively), August and September 2019 (MP19A and MP19B, respectively) and January 2020 (MP20A) using an hyponeuston “manta” net at three stations in the Gulf of Napoli. The three sampling sites were, respectively, in front of the small town of Portici (st. 1, 0.5 nm from the coast), in the middle of the bay at the Long Term Ecological Research station “MareChiara” (st. 2, 2 NM away from the coast) and at the Marine Protected Area of Gaiola (st.3, 0.5 nm from the coast, Figure 3.1).

Figure 3.1. Sampling stations in the Gulf of Napoli; st.1-Portici (urbanized area); st.2-MC, long term ecological research station; st.3- Marine Protected Area Gaiola.
3.2 Bay of Pozzuoli

The Bay of Pozzuoli (BoP) is a large bay (surface area ca 33 km² and volume ca 2 km³) located in the northern part of the Gulf of Napoli. The BoP has a narrow continental shelf and an average depth of 60 m, with a maximum depth of 110 m (Somma et al., 2016). Its circulation is strongly influenced by the general tyrrhenian patterns, mostly flowing northwards, but it is also strongly affected by local wind stress (Menna et al., 2007). The BoP is not subject to regular and/or intense terrestrial runoff, but freshwater inputs are at times detected in the bay from both the Volturno River mouth, located outside of the Bay to the North, and the Napoli city area, with numerous urban outlets to the South. Local human activities from the densely settled areas of Pozzuoli and Baia and the industrial district of Bagnoli dramatically affected the environment in the recent past. In particular, during the 20th century, the site of Bagnoli was long used for industrial activities including an important steel plant (in operation from 1910 to the early 1990s), an asbestos materials manufacturing plant, also producing cement and fertilizers. In addition, the BoP also hosts urban sites with residential houses, fisheries and agricultural sites, as well as mussel farms, along with recreational and touristic sites, such as beaches, thermal baths, and important archaeological ancient roman sites (Romano et al., 2018). The industrial area of Bagnoli has been widely investigated since the late 1990s, when it was classified as SIN (“Sito di Interesse Nazionale” – Site of National Interest) for environmental reclamation of disused and heavily polluted coastal sites. The initial studies focused on sediments, highlighting high levels of metals, PAHs and PCBs in the area close to the industrial settlements (Trifuoggi et al., 2017). Metals are also due to hydrothermal sources while PAHs and PCBs are due to the past industrial activities. Recent studies have revealed a substantial PAHs contamination over the entire BoP where also the Baia-Pozzuoli industrial and port areas are significant contributors to heavy metal pollution (Margiotta et al., 2020). Until now, no info on MPs pollution have been reported from the BoP.

MPs were collected during 4 surveys in July 2018, and February, July and October 2019 on board of the R/V Vettoria in the framework of the ABBaCo Project (MIUR - Fondo Integrativo Speciale per la Ricerca - determina CIPE - GU n.56 8.3.2017). In each survey, manta trawls were carried out to address the MPs horizontal distribution and the characterization of the plastisphere community at established stations. Stations AB2–5 were aligned along a transect just outside the town of Bagnoli (AB2) to outside of the bay (AB5).
near the westernmost part of the bay, Capo Miseno. Station AB1, whereas, was positioned in the westernmost part of the GoN, close to a mussel cultivation area. All the stations were positioned around 0.5 nm from the coast, Figure 3.2.

Figure 3.2. Sampling stations in the Bay of Pozzuoli; st. ab1; st. ab2; st. ab5.
3.3 North-Adriatic Sea

The North Adriatic Sea (NAS) borders to the west by the Italian peninsula and to the east by the Balkans, represents the northernmost part of the Mediterranean Sea (excluding the Black Sea), and is characterized by shallow waters (with an average depth of 35 m), regularly and gradually sloping towards South-East up to the 100 m isobath. The circulation of the NAS is predominantly cyclonic and consists of a current incoming direct NW, which flows off the eastern edge (Eastern Adriatic Current) balanced by an outgoing current (Western Adriatic Current) which flows close to the Italian coast (Russo and Artegaioni, 1996). The first introduces relatively warm and highly saline waters into the basin, while the second transports more dilute waters laden with fine sediments to the southernmost regions of the basin. Despite its small volume, the NAS alone receives about 20% of the fresh water of the whole Mediterranean, with a contribution coming mostly from the Po river, Italy’s largest river with a length of 673 km and a drainage basin of 71,000 km² (Boldrin et al., 2005). The Po river flows through one of the most productive agricultural and industrial areas in Italy, entering the northern Adriatic Sea through a large delta with five tributaries (Maistra, Pila, Tolle, Gnocca, and Goro), differing in terms of their water discharge and solid loads. Another contribution of freshwater is received by the NAS from the lagoon of Venice which covers a surface area of around 550 km², at an average depth of 1 m. The total exchange between the sea and the Venice lagoon is 385 million of cubic meters of water per day, receiving about 33 m³ s⁻¹ of water from nine main rivers. The drainage basin covers 1,870 km² of densely populated areas, with about 1,500,000 inhabitants, where intensive agriculture and important industrial activities are located (Canu et al., 2002).

Distribution models predict MPs to accumulate in correspondence with river discharges and shipping lanes (Liubartseva et al., 2016), and the NAS has both such features, with MP concentrations as high as 3 MP m⁻² (Vianello et al., 2018). The most common polymers found as floating MPs are low density polymers (especially PE, PP, EVA and PS) classified as transparent irregular fragments organized in patchy distribution linked to hydrodynamic and meteorological drivers, as the northerly bora wind, dominates in winter (Vianello et al., 2018). Other abundant MPs come as a result of fragmentation of mussel nets (Basili et al., 2020). Basili et al., (2020) identified bacteria and diatoms as main components of the
microbial plastisphere. The most common bacteria belonged to Gammaproteobacteria, Bacteroidetes, in particular to the genera *Pseudoalteromonas*, *Alteromonas* and *Dokdonia*. While as for the minor contributors, the phyla Actinobacteria and Cyanobacteria. The microbial communities observed in the area show a biogeographical distribution and do not show a polymer specificity (Basili et al., 2020). During February 2019, eight stations were sampled in order to assess the MP horizontal distribution and the plastisphere community, using a manta hyponeuston net. A total of 10 manta trawls were conducted with two stations sampled twice (N3 and S1). A storm, with strong winds interrupted the cruise from February 23rd to 24th (Figure 3.3). Stations N1, N5, C10, VE02 and PTAA were sampled only before the storm, station PAL only after the storm, while stt. N3 and S1 were sampled both before and after the storm. N1 (16 NM from the coast), N3 (10 NM from the coast), N5 (6NM from the coast) and S1 (4 NM from the coast) were part of transect related to the Po river delta. VE02 (1.5 NM from the coast), PTAA (7 NM from the coast) and C10 (16NM from the coast) a transect related to the Venice lagoon. Finally, Paloma station (PAL, 5 NM from the coast) was sampled as an oligotrophic station, close to the city of Trieste.
Figure 3.3. Map of the area with the sampling sites. The double dots at stations N3 and S1 indicate the double sampling, before and after the storm.
3.4 The Cilento and Vallo di Diano Marine Protected Area

The Gulf of Salerno shows a continental shelf, about 9–20 km wide, with a sharp shelf break, 120 to 180 m deep. This area is about 100 m deep and is characterized by small bays with soft sediments alternating with rocky shores and cliffs. Specific habitats are associated with each geomorphological type and are highly diversified based on the different substrate. The marine coastal area is highly colonized by seagrasses, especially *Posidonia oceanica*. The marine protected area of “Santa Maria di Castellabate” was established in 2009 and classified as of “very good” environmental quality. The whole area extends over an area of about 138 km² down to a depth of 100 m (Figure 1) and is characterized by a deep well-mixed surface layer in winter and a shallow seasonal thermocline in summer. Strong winter winds, mainly from N-NNE, produce a cyclonic circulation, while weaker summer winds, mainly from SSW-S, induce an anticyclonic circulation (D’Angelo et al., 2020).

During May 2018, six stations were sampled within the MPA, in correspondence of the town of Santa Maria di Castellabate. The stations were selected according to art. 11 “Programmi di Monitoraggio” del d.lgs. n. 190/2010, considered in the Directive 2008/56/CE (Marine Strategy Framework Directive, MSFD) for assessing MP pollution and were located 0.5, 1.5, 6 nautical miles off the coast along two transects (Figure 3.4), from Punta Licosa (stt. 1,2,3) and from Punta Tresino (stt. 4,5,6). At each of the stations, a sample was collected using a manta trawl.
Figure 3.4. Map of the Cilento area studied.
### 3.5 Sarno river

The Sarno River is located in the South-West of Italy (Campania region), and its catchment basin hosts an area strongly urbanized and interested by industries and cultivations. Surface and groundwater reservoirs make the Sarno flatland ideal for numerous industrial activities, and the favourable climate, together with the high agronomic quality of its soil makes the area one of the most fertile in Italy (Baldantoni et al., 2018). In 1988, the Sarno River basin has been declared “area at high risk of environmental crisis“ (SIN) by the Ministry of the Environment, as it is a part of the region where high human impact has compromised environmental quality and the sustainable use of its rich natural resources (Ministry Council's Decree, 1994; De Pippo et al., 2003). The Sarno river is considered the most polluted river in Europe (Montuori et al., 2015; Pepi et al., 2016) and one of the ten most polluted rivers in the world (Cicchella et al., 2014). Public health conditions are precarious as the basin's inhabitants consume low-quality water directly and are also exposed to a cocktail of other pollutants due to the use of water in the food chain, via field irrigation and animal feed. Thus, the risk of pollution-related diseases is increased compared to other regions (Bonomo et al., 1999).

MPs are of great public concerns for its ubiquitous presence and persistence in the aquatic environment. The global presence of marine MPs has been confirmed in recent years, but there is less data concerning freshwater systems, even though rivers can act as vectors for the transport of litter into the ocean (Li et al., 2018). In general, the main input of plastic into the sea is from terrestrial sources, as public littering, improper waste disposal, waste dump run-offs, tourism, industrial activity together with inadequate sewer systems (Andrady et al., 2011, UNEP report, 2016). Storms, rainwater drainage, flooding and winds can collect and spread plastics over a wide area.

On January 24th, 2020, three manta net tows were carried out at 2 sites along the Sarno river (Sarno Upstream, SU and Sarno Downstream, SD) and on January 15th, 2020, in the marine coastal area just outside the river’s mouth (Sarno Sea, SS; Figure 3.5). Sarno Upstream was positioned at one of the sources of the river Sorgente Palazzo, 30 km from the sea, while Sarno Downstream station was positioned at the last physical barrier between river and sea, around 1 km away from the river’s mouth, Sarno Sea. The latter station was positioned 0.5 NM from the coast, away from river plum.
Figure 3.5. Map of the sample sites related to Sarno river (upstream and downstream) and in the marine coastal area.
3.6 Microplastic transit cruise from Azores to Sicily

In the Northeastern Atlantic Ocean, the surface main water mass, denominated Eastern North Atlantic Central Water (ENACW), also referred to as the “tropical” ENACW (ENACWt, Bashmachnikow et al., 2015), originates from a westward re-circulation of the Azores Current near the Iberian Peninsula (Bashmachnikow et al., 2015). The ENACW enters the Mediterranean Sea through the Strait of Gibraltar (width ~13 km; sill depth ~300 m), and determines a complex system of anticyclonic eddies in both the Alboran Sea and the Algerian basin, along the Algerian current. ENACW deepens as it progresses towards the eastern Mediterranean basin transforming into the Modified Atlantic Water (MAW), which then flows through the Sicily Strait or enters the Tyrrhenian Sea northward (Sioukou-Frangou et al., 2010). Less saline ENACW has higher nutrient concentrations than the Mediterranean Water (MW; Bashmachnikow et al., 2015), and this results in a net nutrient transport from the Mediterranean to the Atlantic Ocean with the first acquiring an oligotrophic character, increasing from West to East. The nutrient deficit is only partially compensated by river runoff, atmospheric depositions and nitrogen-fixation (Bethoux et al., 1998, 2002; Krom et al., 2004; D’Ortenzio and Ribera d’Alcala, 2009; Garcia-Martinez et al., 2019). Recently, it has also become evident that the Atlantic Water flowing into the Mediterranean Sea is not as impoverished as previously thought in terms of nutrient load (Garcia-Martinez et al., 2019). These general circulation patterns make the Mediterranean Sea a “miniature ocean” with features similar to larger oceans with a strong influence of structures at mesoscale, which contribute to complexity and peculiarity of the Mediterranean basin (Bethoux et al., 1999). These mesoscale structures, like eddies, wind-driven Ekman currents, geostrophic currents and wave-induced Stokes drift drive the surface ocean circulation, also play a role in marine debris accumulation (Onink et al., 2019). By estimating both terrestrial and marine inputs, Lebreton and coworkers (2012) modeled the transport and distribution of floating debris in the global ocean, identifying the Mediterranean Sea as a potentially important accumulation area, with 23,150 tons of floating plastics (Eriksen et al., 2014). Plastic accumulation likely results from significant discharges combined with a limited export to the Atlantic Ocean, resulting in the Mediterranean Sea as a convective basin and a sink for Atlantic floating plastic (Cozàr et al., 2015). In the Atlantic Ocean, MP concentrations are similar to those reported in other areas like the North Pacific Central Gyre.
Numerous studies report that there is a large disparity between the quantity of plastic predicted to have entered the oceans and the amount measured by monitoring studies, sometimes referred to as the “missing 99%” (Cozàr et al., 2015; Van Sebille et al., 2015). This observation calls for better monitoring campaigns and also studies on the physical and biological routes of plastics dispersal. Between 8000 and 20000 litter items km$^{-2}$ have been estimated to sit on the deep seafloor of the Mediterranean Sea (Pierdomenico et al., 2019). At specific sites, these figures are even larger, like in the Messina Strait (Canals et al., 2021), or in submarine canyons between the southern tip of the Italian Peninsula and Sicily, where ca 106 litter km$^{-2}$ are estimated to be present (Canals et al., 2021). These large quantities, mainly consisting of macroplastics (>25 mm) are due to dumping and waste mismanagement, although a considerable proportion is represented by microplastics, estimated at 13.5% (Koelmans et al., 2017). These latter are found at depth due to physical fragmentation combined with biofouling, which decreases their buoyancy and promotes their vertical transport towards the seafloor. This mechanism has been observed for large buoyant plastics (Ye and Andrady, 1991) and is expected to be so for smaller pieces as well and has also been numerically modelled (Amaral-Zettler et al., 2021). In the past few years, increasing attention has been paid to the “Microbial Plastisphere”, a term used to define as the microorganisms thriving on plastic (Zettler et al., 2013). Examples of these communities have been studied also in the Mediterranean Sea (Dussud et al., 2018; Delacuvellerie et al., 2019; Amaral-Zettler et al., 2021) highlighting the relevance of prokaryotes and eukaryotes as main components, especially prokaryotes and diatoms. This is not surprising, due to the closeness of microbes in biofilms, which is expected to foster interactions and effective nutrient cycling. Unfortunately, few articles report quantitative abundances of these two types of microorganisms (Reisser et al., 2014), and only a few estimate their contribution to primary and secondary productivity of different marine regions, which is reported to be particularly high (Bryant et al., 2016; Wright et al., 2020). Other works, instead, shed light on the importance of biogeography of the microbial plastisphere showing that its members form different communities between, for instance, the Pacific and the Atlantic Ocean (Amaral-Zettler et al., 2015) and between different areas of the Mediterranean Sea (Amaral-Zettler et al., 2021). This last work highlights also the ecological interactions among the community, and the relevance of protozoans and fungi, together with bacteria and diatoms. These interactions are expected to be dependent also on the selective role of different
substrates in terms of hydrophilicity (Oberbeckmann et al 2018) or physical complexity, offering shelter from turbulence and wave action (Reisser et al., 2014). These data were obtained during a cruise on board the RV Pelagia, led by Prof. Linda Amaral Zettler of the Royal Netherland Institute for Sea Research (NIOZ). The expedition started at Ponta Delgada in the Azores (Portugal) and ended in Catania (Italy). During the cruise 21 stations were sampled for microplastics and other parameters (Figure 4.1). The aim of this subchapter is to assess differences in microbial plastisphere on MP between the Atlantic Ocean and the Mediterranean Sea, based on prokaryotes and diatoms densities and morphological groups identified by Scanning Electron Microscopy (SEM). On board on the RV Pelagia of the Royal Netherland Institute for Sea Research (NIOZ), twenty-two manta-net trawls were carried out from the Azores to Sicily from July 27th to August 7th 2018 (Figure 3.6).

Figure 3.6. Map of the stations sampled during the cruise. The numbers describe the manta trawls carried out during the expedition (22).

At each station, the manta net was towed for 30 min and MP items collected and processed as described in Chapter 2. Station coordinates and sampling dates are reported in Table 3.1. From Station 1 to Station 11, the distance towed ranged between 0.9 and 7.6 Nautical Miles (NM), while from station 13 to station 22, it ranged between 0.9 and 1.5 NM. The variation in tow distances was due to challenging meteorological marine conditions.
Table 3.1. Stations, date of sampling, location and coordinates of starting point of manta tows.

<table>
<thead>
<tr>
<th>Station</th>
<th>Date</th>
<th>Geographical area</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-001</td>
<td>2018-07-27</td>
<td>NW Atlantic</td>
<td>38.25667</td>
<td>-23.8217</td>
</tr>
<tr>
<td>MT-002</td>
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<td>NW Atlantic</td>
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<td>MT-006</td>
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<td>NW Atlantic</td>
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<td>MT-007</td>
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<td>NW Atlantic</td>
<td>36.73</td>
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<td>MT-008</td>
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<td>MT-009</td>
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<td>NW Atlantic</td>
<td>36.005</td>
<td>-8.681</td>
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<tr>
<td>MT-010</td>
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<td>MT-011</td>
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<td>3.549</td>
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<tr>
<td>MT-022</td>
<td>2018-08-07</td>
<td>Strait of Messina</td>
<td>38.085</td>
<td>15.53</td>
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</tbody>
</table>
CHAPTER 4.
Spatio-temporal variability of MP distribution and microbial plastisphere in coastal areas
Coastal marine ecosystems are continuously exposed to anthropogenic impacts from densely urbanised areas, tourism, recreational and commercial fishing activities, among others (Coll et al., 2012; Halpern et al., 2012; UNEP, 2016). This constant exposure of coastal areas to human activities increases the risk of mismanaged waste entering the marine environment, especially plastic (Compa et al., 2020). As MPs are now widely recognized as marine pollutants, it is fundamental to define common methodologies to quantify their presence in all the environmental matrices, seawater among these. It is also important to evaluate their geographical distribution, transport mechanisms and residence times, factors related both with the physical characteristics of particles (density, size shape buoyancy) and with the hydrodynamic forcings (winds, tides and currents) which also play a role in marine debris accumulation (Onink et al., 2019; Ruiz-Orejon et al., 2019; Compa et al., 2020). Despite the well-known subtropical ocean gyres acting as accumulation areas for floating plastic debris, the spatial distribution of these particles in the Mediterranean Sea is affected mainly by the variability of the surface circulation which hampers the formation of stable retention zones (Ruiz-Orejon et al., 2019). In addition, temporal variability has also been often recorded, even within the same seasons (Compa et al., 2019; 2020; Pereiro et al., 2019). In addition to the wind, tides and density currents induced by river discharges add variability to the shelf circulation. The freshwater discharges have also been observed to induce a significant circulation over the inner shelf (Mendoza et al., 2020, Lazure et al., 2006; Ferrer et al., 2009). However, the potential influence of the nearshore dynamics in the understanding of spatio-temporal distribution of floating plastic and its potential effects on the retention of plastics represents currently a gap in knowledge (Compa et al., 2020). Hence sampling campaign data and marine circulation data are fundamental for any MP study (Pini et al., 2018).

Similar trends of variability are linked to the community of microbes colonizing the surface of MPs, the so called “plastisphere” (Zettler, Mincer, & Amaral-Zettler, 2013). When a piece of plastic enters the ocean, microbes colonize it within hours (Harrison et al., 2014), and from there on different factors contribute on shaping the community. Geographic location and environmental parameters appear to be the primary influences shaping plastisphere communities, but studies using environmentally collected samples are rare (Amaral-Zettler et al., 2020). Studies show that ocean-basin scale (Amaral-Zettler et al., 2015), coastal areas (Amaral-Zettler et al., 2021) and riverine environments (Oberbeckmann et al., 2014) differences exist. A work related to the North Sea (Oberbeckmann et al., 2014)
showed differences in terms of spatial distribution and environmental conditions. The data of this work shows that microbial communities on plastic vary in structure and composition with regards to geographical location and season, especially, also influenced by temperature and oxygen concentrations. Moreover, also polymer type can be another parameter to take in account when discussing about discriminants shaping the microbial communities.

Plastisphere communities were found different among the North Pacific and North Atlantic gyres (Amaral-Zettler et al., 2015) and revealed that regardless of point of entry, microbes that inhabit the Plastisphere tended to reflect their local surroundings more than their potential origins, also exhibiting a latitudinal gradient in species richness in the North Atlantic. Moreover, a work related to all the Italian coast, demonstrated that the Mediterranean Plastisphere varies regionally (Amaral-Zettler et al., 2021). For example, differences were found among samples originally sampled in Adriatic and Ligurian Sea. Nevertheless, the overall pattern corresponded well with distinct Mediterranean hydrodynamic provinces and their role in structuring marine populations, as mentioned above related to MP distribution, is a relevant parameter and should be taken into account when discussing results. Differences were found also related to environmental conditions for example between rivers and ports where were found clear differences but also some overlap. Microbial communities from river water and river plastic were distinct from each other and from marine counterparts, with port water and plastic clustering between river and marine systems. Understanding connectivity between Plastisphere communities in freshwater and marine systems is an important step in understanding the contribution of rivers to attached microbial communities in marine systems (Amaral-Zettler et al., 2021).

It must be noted that these analyses were conducted with samples collected over different seasons, so some of the effects are undoubtedly due to seasonal and interannual factors (Amaral-Zettler et al., 2021). These researches demonstrate that there is a clear need to consider variability along spatial and temporal gradients, considering the movement of plastics from lakes to rivers onward to the sea, as well as the original nature of the polymer, to better understand dynamic variation in the structure, diversity and ecological roles of plastisphere microbial communities with marine plastic pollutants (Oberbeckmann et al., 2014; Amaral-Zettler et al., 2021).
As mentioned above, temporal variability both in MPs concentrations and plastisphere community composition is very high. In this subchapter different areas were sampled repeatedly with the aim of understanding seasonal variability.

### 4.1 Temporal variability on MPs distribution and characteristics

Regarding the Gulf of Napoli, MP concentrations ranged up to one order of magnitude, from 1.12 to 12.08 MP m\(^{-3}\). Highest concentrations found were during summer 2019 (both August and September, Figure 4.1.1). The lowest during the two winter period times (January 2018 and 2020, Table 4.1.1). Throughout the years monitored, MPs ranging between 1000-5000 µm (Figure 1), called large MPs (Thompson et al., 2009), accounted for 79.7% of total MPs. Small MPs (ranging among 300-1000 µm, Thompson et al., 2009) accounted for 16.2% of total MPs. The other MPs were all less than 333 µm, our minimum size limit of the mesh size of the manta-net used. This confirm a high efficiency of the device used, since less than 4% of the MPs were smaller than the manta net size. Three out of five times large MPs accounted for more than 80% contribution of MP most common size fraction (Figure 4.1.1), and the only times that this did not happen is when the highest MP concentrations were found (August and September 2019).

Table 4.1.1. MP concentration related to the different stations of the Gulf of Napoli (st.1,2 and 3) at the different samplings (with total average and standard deviation). Data are in MP m\(^{-3}\). No manta trawl carried out in July 2018 at station 3.

<table>
<thead>
<tr>
<th></th>
<th>St.1</th>
<th>St.2</th>
<th>St.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan-18</td>
<td>1.12</td>
<td>1.78</td>
<td>1.27</td>
</tr>
<tr>
<td>Jul-18</td>
<td>4.66</td>
<td>1.60</td>
<td>-</td>
</tr>
<tr>
<td>Aug-19</td>
<td>9.46</td>
<td>8.39</td>
<td>1.29</td>
</tr>
<tr>
<td>Sep-19</td>
<td>12.08</td>
<td>9.89</td>
<td>5.32</td>
</tr>
<tr>
<td>Jan-20</td>
<td>1.13</td>
<td>1.42</td>
<td>1.78</td>
</tr>
<tr>
<td>Average</td>
<td>5.69</td>
<td>4.61</td>
<td>2.41</td>
</tr>
<tr>
<td>dev.st</td>
<td>4.94</td>
<td>4.16</td>
<td>1.95</td>
</tr>
</tbody>
</table>
Figure 4.1.1. Percent contribution (y-axis) of Large (1000 to 5000 µm, in blue) and Small (333 to <1000 µm, in orange) MPs at different sampling dates (averages).

Fragments were the most common type of MPs found, averaging 63.4% of the total MP found, followed by films (25.2%), filaments (6.9%) and foams (4.4%) (Figure 4.1.2). The remaining 0.2% accounted for the so-called primary MPs, clearly not affecting the Gulf of Napoli, in granules or pellets. Some exceptions were found when in January 2020, very similar values were found comparing fragments and films. In the same season – but different year, 2018 – was found a high contribution of films. As for MP colour (Figure 4.1.3), during two samplings in 2018, coloured MPs dominated, both in January and July. A true shift of this trend was found from the year 2019. Indeed before, colored MPs were more abundant, but from that year on, transparent MPs dominated. On the contrary, white has always been the least found colour, even though, noteworthy is the peak found in st.3 in September 2019.
Temporal trends of MPs characteristics were found using a Principal Component Analysis plotting together physical microplastic characteristics and sampling times (Figure 4.1.4). In January 2018 the highest concentrations of large, colored MPs suggest recent entrance of these MPs into the sea, since these are considered less impacted both from hydrodynamism and photodegradation (Andrady et al., 2011). From July 2018 colored MPs started to decrease, maybe due to degradation, suggesting their longer permanence in the water. Degradation finds its highest peak in summer / late summer 2019, when highest values of small MPs links with highest values of concentration (MPs m⁻³), also with new introduction.
of white foams, possibly from food and beverage consumption and relative disposal at sea (summer as peak of tourism, Agovino et al., 2021). Transparent films find their highest peak in January 2020, which may derive from photodegradation and long residence time from the previous summer. It cannot be excluded, though, that the pattern derives from random variations within samples.

![Figure 4.1.4. Principal Component Analysis (PCA) on 10 variables (indicated on the plot). The two components described 60.3% of the total variability. Colors describe the different time points as in the legend: MP18A = January 2018; MP18B = July 2018; MP19A = August 2019; MP19B = September 2019; MP20A = January 2020; Contrib are the contributions of each variable. The thicker the lines, the higher the contribution of the parameter to total variability.](image)

Polymer identification with FTIR-ATR-polymer determination was possible to assess for 2018 samples (1881 MP pieces from 5 net trawls, 5 stations, two sampling dates). Unfortunately, due to delay in access to analytical facilities due to COVID-19 outbreak, the others could not be analysed on time for this thesis. Polyethylene (PE) was the most abundant plastic polymer, accounting for 57.5% (± SD 1.5%) of total pieces analysed, followed by Poly (ethylene-propylene-diene) (PEPD - 14.2% ± SD9.2%), Polypropylene (PP 10.1% ± SD1.7%) and others (including cellophane). While PE and PP showed similar percentages
in January and July, PEPD and cellophane were more abundant in July, where the latter accounted for 100% of the “others” (Table 4.1.2).

Table 4.1.2. Chemical composition of the total samples from January and July 2018 samplings. Data are %. PE is Polyethylene; PP is Polypropylene; PEPD is Poly(Ethylene:Propylene:Diene); PS is Polystyrene.

<table>
<thead>
<tr>
<th>(%)</th>
<th>January 2018</th>
<th>July 2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>73.3</td>
<td>58.5</td>
</tr>
<tr>
<td>PP</td>
<td>11.7</td>
<td>11.3</td>
</tr>
<tr>
<td>PEPD</td>
<td>10.0</td>
<td>20.8</td>
</tr>
<tr>
<td>PS</td>
<td>3.3</td>
<td>1.9</td>
</tr>
<tr>
<td>CELLOPHANE</td>
<td>1.7</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Regarding the Bay of Pozzuoli (BoP), MP concentrations ranged between 0.53 to 12.81 MP m\(^{-3}\). As in GoN, the highest concentration of MPs was found both in summer/late summer periods, in July 2018 and October 2019, and the lowest in winter (February 2019) (Table 4.1.3). Size fraction dominance did not change throughout the seasons, indeed 81.4% of total MPs were large (1000-5000 µm), while 17.1% were smaller 1000-300 µm) in size. Only 1.5% were smaller than 333 µm. (Figure 4.1.5).

Table 4.1.3. MP concentration related to the different stations of the Bay of Pozzuoli (BoP) (st.ab1, ab2 and ab5) at the different samplings (with total average and standard deviation). Data are in MP m\(^{-3}\). No samplings were carried out for station ab1 in February 2019 and ab5 for July 2018.

<table>
<thead>
<tr>
<th></th>
<th>Jun-18</th>
<th>Jul-19</th>
<th>Oct-19</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ab1</td>
<td>1.41</td>
<td>-</td>
<td>3.71</td>
<td>4.96</td>
</tr>
<tr>
<td>ab2</td>
<td>10.37</td>
<td>0.53</td>
<td>4.89</td>
<td>12.81</td>
</tr>
<tr>
<td>ab5</td>
<td>-</td>
<td>1.69</td>
<td>3.14</td>
<td>3.41</td>
</tr>
<tr>
<td>Average</td>
<td>5.89</td>
<td>1.11</td>
<td>3.92</td>
<td>7.06</td>
</tr>
<tr>
<td>Dev.St</td>
<td>6.34</td>
<td>0.82</td>
<td>0.89</td>
<td>5.04</td>
</tr>
</tbody>
</table>
Sixty-point-seven percent (60.7%) of all the MPs found through the year were fragments, followed by foams (18.3%) and films (14.8%, Figure 4.1.6). Other MP types were filaments (6.1%), pellets (0.1%) and granules (0.1%). All the secondary MPs (fragments, foams, films and filaments) were always present in the different samplings, while granules and pellets, belonging to primary MPs, were found only in July 2018 and 2019. Winter time showed higher relative abundance of fragments and also more films than foams, which, in turn, were more abundant in summer / autumn, probably deriving from fish packaging being disposed more frequently during the summer touristic season.
White MPs were more abundant in both July samplings, matching the two peaks of foams (Figure 4.1.7). Transparent MPs were more abundant during winter, while more colored MPs were reported in late summer / autumn, probably marking recent introduction of plastics in the sea, which have not yet been exposed to significant photo-oxidation by UV light and consequent discoloration.
High proportions of white foams could derive from elevated consumption of food and beverages in the summer period, as these are used in food and beverage packaging. In the same period there is also a dominance of filaments, possibly related to active fishery activities in the area. So, in these conditions there could be an accumulation of MPs, with newly introduced waste at sea, supported by high presence of colored MPs. During winter, there are more transparent MPs and slightly smaller pieces than summer. This might be because during the months of accumulation and irradiance of UV light, these MPs undergo photodegradation by UVb light and mechanical forces at sea, resulting in smaller pieces with less bright colours. A Principal Component Analysis (PCA) (Figure 4.1.8) confirms that in July, higher abundance of large MPs and mainly white foams are found, as polystyrene most probably deriving from food packaging (fish and seafood, abundant in the area). In winter (February), instead, small MPs (300-1000 µm), mainly transparent fragments dominate the MPs, suggesting degradation and weathering from mechanical/chemical factors. Finally, in late summer (very beginning of October) filaments, mostly colored, are dominant, possibly from fish nets and seafood containment activities products. Unfortunately, no analysis related to FTIR polymer identification are available for this thesis.
4.1.1 Discussion

Data showed here are in agreement with a rising concern regarding the variability that comes with the assessment of MP pollution when it finds different environmental conditions in particular areas (Ruiz-Orejon et al., 2019; Pereiro et al., 2019). The potential influence of the nearshore dynamics in the spatio-temporal understanding of floating plastic and its potential effects on the retention of plastics is a current knowledge gap (Compa et al., 2020). Hence sampling campaign data and marine circulation data are fundamental in the microplastic pollution study, as outlined in other studies (Pini et al., 2018). In particular, MP distribution is strongly influenced by hydrodynamic forcings (winds, tides and currents) and human impacts, which in turn are strictly linked to seasons. The coast of Campania region, not only is interested by relevant recreational and touristic activities, especially during warmer seasons (Agovino et al., 2021), but also site of active hydrodynamic features.
(Cianelli et al., 2021). GoN and BoP showed that MPs concentration were more abundant in summer than in winter times for both areas. Most common MPs in this area were large (1-5 mm) fragments probably formed by washed particles from the beach and/or due to touristic activities. A difference was related to other common MP types. For GoN area, films were more abundant probably related to a higher use of shopping bags, as this area is inhabited by more than 3M people (Agovino et al., 2021). On the other hand, for BoP, known fishing and aquaculture area, foams were most commonly found probably related to the fragmentation of boxes to transport seafood and fish. These results were possible thanks to continuous samplings of a 3 years-monitoring of the area, highlighting the limits of single-spot surveys especially in coastal areas.

4.2 Temporal variability of the plastisphere

Seventy-five MPs pieces were retrieved for parallel plastisphere characterization in the GoN. Fourty-nine Polypropylene (PE), 13 Polypropylene (PP), 5 Polystyrene (PS), 2 Ethylene vinyl acetate (EVA), 2 Poly (Ethylene:Propylene:Diene) (PEPD), 2 Waxes (WAX), 1 Polyurethane (PU), 1 Cellophane. Almost all (97.3%) of the polymers recognized in this work were positively buoyant (PE, PP, expanded PS, EVA, PEPD, Wax) while only PU and Cellophane were negatively buoyant. PE was the only polymer found in all samples. Prokaryotes were the most abundant microbe member with an average of $0.9 \times 10^4 \pm 0.5 \times 10^4$ SD ind mm$^{-2}$ (Table 4.2.1). As for temporal variability, January 2020 was the date with lowest abundance of prokaryotes attached ($0.4 \times 10^4$ prokaryotes mm$^{-2}$), while August 2019 showed the highest abundance ($1.4 \times 10^4$ ind mm$^{-2}$, Table 4.2.1).
Table 4.2.1. Prokaryotic and diatom density at different sampling dates of the Gulf of Napoli (with total average and standard deviation). Data are prokaryotes mm$^{-2}$ and diatoms mm$^{-2}$.

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes mm$^{-2}$</th>
<th>Diatoms mm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan-18</td>
<td>$1.0 \times 10^4$</td>
<td>$1.3 \times 10^2$</td>
</tr>
<tr>
<td>Jul-18</td>
<td>$1.1 \times 10^4$</td>
<td>$2.5 \times 10^2$</td>
</tr>
<tr>
<td>Aug-19</td>
<td>$1.4 \times 10^4$</td>
<td>$1.7 \times 10^2$</td>
</tr>
<tr>
<td>Sep-19</td>
<td>$1.0 \times 10^4$</td>
<td>$0.4 \times 10^2$</td>
</tr>
<tr>
<td>Jan-20</td>
<td>$0.4 \times 10^4$</td>
<td>$4.0 \times 10^2$</td>
</tr>
<tr>
<td>Average ± dev.st</td>
<td>$0.9 \times 10^4 \pm 0.5 \times 10^4$</td>
<td>$2.0 \times 10^2 \pm 1.4 \times 10^2$</td>
</tr>
</tbody>
</table>

The lowest prokaryotic diversity in January 2020 was associated with high silicates and low phosphates values (Table S1). On the contrary, the highest prokaryotic density was observed at st. 1 in July 2018, coincident with low Total Inorganic Nitrogen (TIN) and phosphates. This might be due the fact that at low nutrient concentrations attachment to surfaces in many prokaryotic species appears to increase, because of a higher transcription factor in such extreme conditions which triggers a transition from surface-attached cells into microcolonies (Stanley and Lazzazzera, 2004; Oberbeckmann et al., 2018). This suggests that MP are a favourable habitat to grow when environmental conditions are limiting. The prokaryotes found were divided in the following morphotypes: bacilli $<$2 µm, cocci $<$1 µm, coccobacilli $<$2 µm, bacilli $>$2 µm, cocci $>$1 µm and coccobacilli $>$2 µm, Figure 4.2.1.
Bacilli <2 µm was the most common prokaryotic morphotype found (Frequency of Observation, FO 46%), followed by cocci <1 µm (FO 44%). Another relevant contributor was coccobacilli <2 µm (FO 6%), and all together contributed for more than 95% of all the prokaryotic morphotypes found. As for temporal trends, August 2019 had the highest diversity of morphotypes while January had the lowest (Figure 4.2.2). The lowest prokaryotic diversity was found in January 2018 together with high silicates and low phosphates values (Table S1). On the contrary, the highest prokaryotic density was observed at st. 1 in July 2018, matched with low TIN and low phosphates. This latter trend took place also at st. 2 in September 2019. This confirms previous work, like Oberbeckmann et al. (2018) who reported higher level of differentiation of prokaryotes attached to MPs for low levels of nutrients. The results presented here support the hypothesis that differences in community composition is ruled by resource availability rather than by substrate characteristics.
As for diatoms, the second most abundant member of the microbial plastisphere in this area, an average of \(2.0 \times 10^2 \pm 1.4 \times 10^2\) diatoms attached were counted (Table 4.2.1).

The diatoms found were classified in several groups (Figure 4.2.3), which are the following: *Navicula* <20 µm, *Navicula* >20 µm, *Amphora*, *Nitzschia longissima*, unknown centrics, rectangular-shaped (10-20 µm in size), *Cocconeis*, *Nitzschia sigmoidea* group, *Chaetoceros*, *Diploneis*, *Achnantes*, *Mastogloia*, *Synedra*, *Skeletonema* and *Cylindrotheca*.
Both Navicula morphotypes (< and > 20 µm), Nitzschia longissima, Cocconeis and Amphora were always present at all stations and sampling dates. On average, the most abundant groups were: Navicula <20 µm and > 20 µm, unknown centrics, Nitzschia sigmoidea, Nitzschia longissima, Amphora, and rectangular-shaped. The first two most abundant groups were the two Navicula groups (FO 57.8% for Navicula <20 µm also the most abundant in all the stations; FO 13.6% for Navicula <20 µm). Navicula <20 µm was also the diatom group most abundant for all the seasons except September 2019, when N. longissima was the most abundant (Figure 4.2.4). In terms of diversity, diatom groups were more in summer dates (August and September 2019 showed 12 diatom groups) than winter (January 2018 showed 9 diatom groups). As for season specificity, diatom groups like Mastogloia and N. sigmoidea group were only showed in summer dates, while Cocconeis was found only in winter dates.
The dominance related to *Navicula* morphotype was also confirmed by qualitative sequencing results related to the re-assign of the plastidic sequences. Indeed, together with Chaetoceraceae, Naviculaceae family was present 4 out of 5 samplings (Table 4.2.2). Cymbellaceae was the family present at all samplings, which morphologically is similar to *Amphora* morphotype hence can be a proof of the presence of this diatom morphotype. Other taxa relevant to this analysis were the presence of *Thalassiosira* genus and Thalassionemataceae family which could be a corroboration to the morphotypes unknown centrics and rectangular-shaped found in the SEM microphotographs. A taxon that was found in sequencing analysis but not in SEM counting was the eukaryotic Prymnesiaceae (present in 3 out of 5 samplings). As for temporal variability results, from this analysis it was found that in winter samples *Thalassiosira* and Cymbellaceae were always present, while in summer Cymbellaceae, Chaetoceraceae and Naviculaceae were observed.
Table 4.2.2. Qualitative results list of most common eukaryotic taxa identified from plastidic OTUs. Note that in the list some sampling times, like September 2019 and January 2020, showed more diversity of re-assigned eukaryotes, so their list is longer than other sampling times.

<table>
<thead>
<tr>
<th>Diatoms</th>
<th>Jan-18</th>
<th>Jul-18</th>
<th>Aug-19</th>
<th>Sep-19</th>
<th>Jan-20</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thalassiosira sp.</em></td>
<td>Cymbellaceae</td>
<td>Cymbellaceae</td>
<td>Cymbellaceae</td>
<td>Cymbellaceae</td>
<td></td>
</tr>
<tr>
<td>Cymbellales</td>
<td>Chaetoceraceae</td>
<td>Chaetoceraceae</td>
<td>Chaetoceraceae</td>
<td>Naviculaceae</td>
<td></td>
</tr>
<tr>
<td>Cymatosirales</td>
<td>Naviculaceae</td>
<td>Naviculaceae</td>
<td>Naviculaceae</td>
<td>Thalassionemataceae</td>
<td></td>
</tr>
<tr>
<td>Triceratiales (Odontella sinensis)</td>
<td>-</td>
<td>Thalassionemataceae</td>
<td>Thalassionemataceae</td>
<td>Chetocerotaceae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Thalassiosira oceanica</td>
<td>Bacteriastrum hyalinum</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Triceratiae</td>
<td>Thalassiosira oceanica</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Stephanodiscaceae</td>
<td>Melosiraceae</td>
</tr>
<tr>
<td>Other Eukaryotes</td>
<td>-</td>
<td>Phaeocystales (Phaeocystis spp.)</td>
<td>Prymnesiaceae (Phaeocystis sp.)</td>
<td>-</td>
<td>Prasinococcales</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Prymnesiaceae</td>
</tr>
</tbody>
</table>

In order to better characterize the prokaryotes composing the microbial plastisphere, 74 MPs were used to extract DNA and perform 16S rRNA sequencing. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities was performed in order to highlight patterns in gradients related to community assessments (Ramette et al., 2007). The NMDS revealed that the global pattern of prokaryotic diversity was not explained by a spatial trend but rather by a temporal one, since dates clustered together all stations from the same sampling date (Figure 4.2.5). In addition, marked differences between different years were noted, possibly related to different meteorological conditions or specific biological adaptations.
Figure 4.2.5. Temporal non-metric multidimensional scaling (NMDS) representation of Bray-Curtis dissimilarity matrix of the attached prokaryotic communities (stress 9.5 e-05).

In terms of year comparisons, 2018 and 2019 showed similar alpha diversity indexes, while 2020 lower values for both indexes (Table 4.2.3). As for seasonal differences, summer dates (July 2018 and September 2019) showed the highest number of OTUs and the higher diversity (Figures 4.2.6), while winter samples showed lower alpha diversity indexes (Table 4.2.3). This showing a higher richness and diversity in summer times than winter ones for the MP-attached communities.
Table 4.2.3. Alpha diversity indexes (Observed OTUs and Shannon) of the different years (MP18-19-20), with averages per year.

<table>
<thead>
<tr>
<th></th>
<th>Observed</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP18A</td>
<td>191.3</td>
<td>4.6</td>
</tr>
<tr>
<td>MP18B</td>
<td>243</td>
<td>4.8</td>
</tr>
<tr>
<td>av. MP18</td>
<td>217.2</td>
<td>4.7</td>
</tr>
<tr>
<td>MP19A</td>
<td>193.9</td>
<td>4.7</td>
</tr>
<tr>
<td>MP19B</td>
<td>212.6</td>
<td>4.9</td>
</tr>
<tr>
<td>av. MP19</td>
<td>203.3</td>
<td>4.8</td>
</tr>
<tr>
<td>MP20A</td>
<td>171.9</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Figure 4.2.6. Boxplots showing alpha diversity indexes related to different samplings throughout the years 2018-2020; A) Observed OTUs; B) Shannon Index.

As for annual variability, the same most abundant phyla were present in 2018 and 2019, with slight differences in percentages, like higher contribution of Bacteroidetes and Cyanobacteria in 2018 and of Proteobacteria and Planctomycetes in 2019. As for 2020, noteworthy were the contribution of Firmicutes and Euryarchaeota and the very low percentages of Actinobacteria and Verrucomicrobia (Figure 4.2.7). All but one of the most abundant families related to the attached communities, accounting for more than 2%, were...
shared among 2018 and 2019. The only difference was the presence of Cyclobacteriaceae in 2018 and Moraxellaceae in 2019. As for 2020, the only families shared among the other two datasets were Flavobacteriaceae and Rhodobacteraceae, which are known members of the microbial Plastisphere. Relevant of the latter dataset were families like Burkholderiaceae (Gammaproteobacteria), two Firmicutes families (Veillonellaceae and Ruminococcaceae) and an archaeal one (Methanospirillaceae), as in Figure 4.2.8.

![Taxonomical classification for different polymer associated communities throughout the years 2018-2020 at phylum level (>0.5%). Mp18 is 2018; mp19 is 2019; mp20 is 2020.](image)

Figure 4.2.8. Taxonomical classification for different polymer associated communities throughout the years 2018-2020 at family level (>2%). Mp18 is 2018; mp19 is 2019; mp20 is 2020.

In order to investigate the high spatial variability, OTUs were displayed in Venn diagrams. No OTUs were shared between the years, and the highest percentages of the Venn diagrams were all related to exclusive OTUs. The only shared OTUs higher than 0 (0.9%) were among the years 2019 and 2020 (Figure 4.2.9 A). As PE was the only polymer shared among all the seasons retrieved, the PE core microbiome was analyzed for shared and unique OTUs.
throughout the years. Similar results for PE are showed in Figure 4.2.9 B, with only 6 OTUs shared between 2019 and 2020.

Figure 4.2.9. Venn diagrams showing shared and exclusive OTUs among different polymer associated communities (A) and PE (B) at the different sampling dates. Numbers shown are actual OTUs and percentage of dataset contribution.
A total of 46 samples were analysed in order to characterize the microbial plastisphere found on the MPs at the Bay of Pozzuoli. Seventeen samples were analysed for July 2018, 8 for February 2019, 6 for July and 14 for October 2019. 19 Polypropylene (PE), 15 Polypropylene (PP), 7 Polystyrene (PS), 2 Poly-vinyl alcohol (PVA), 2 Waxes (WAX), 1 Polyamide Nylon 6 (PA6). Almost all (93.5%) of the polymers recognized in this work were positively buoyant (PE, PP, expanded PS, Wax) while only PA6 and PVA were negatively buoyant. PE, PP and PS were the only polymers found in all samples. As for the BoP samples, prokaryotic concentrations on MPs were very similar in July 2018, February 19 and July 19. Maximum concentrations were observed in July 2018 and lowest values were observed in October 2019 (Table 4.2.4). In July 2018 also the number of prokaryotic morphotypes was the highest (5, and the relative proportions of different morphotypes varied over time). Bacilli <2 micron and coci 1 micron were the morphotypes more abundant (Figure 4.2.10).

As for diatoms, the highest concentrations were observed in February 2019 (Table 4.2.4). Three diatom groups were found in all samples: Navicula <20 µm, Cocconeis and Amphora (Figure 4.2.11). February samples showed a clear dominance of Cocconeis morphotype with over 4.0 x 10^2 ind mm^-2 and the highest number of diatom groups were observed in July 2018 (8), while the lowest was observed in October 2019 with only 4 diatom groups.

Figure 4.2.10. Prokaryotic morphotypes found attached to MPs: A) bacilli (<2 µm); B) coci (>1 µm); C) coccobacilli <2 µm; D) bacilli >2 µm; E) coci 1 µm. Scale bar size is 2 µm.
Figure 4.2.11. SEM microphotographs of diatom groups found. A) Rectangular-shaped; B) *Navicula* <20 µm; C) *Amphora*; D) *Navicula* >20 µm; E) Unknown centric; F) *Nitzschia longissima*; G) *Cocconeis*. The scale bar is indicated on each photo.

Table 4.2.4. Individuals per mm² (prokaryotes and diatoms) for each sampling period together with % contribution of different prokaryotic morphotypes and diatom groups.

<table>
<thead>
<tr>
<th></th>
<th>Jul-18</th>
<th>Feb-19</th>
<th>Jul-19</th>
<th>Oct-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prokaryotes (ind mm²)</td>
<td>5000</td>
<td>4000</td>
<td>4000</td>
<td>1000</td>
</tr>
<tr>
<td>Diatoms (ind mm²)</td>
<td>120</td>
<td>450</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>Bacilli &lt;2 µm %</td>
<td>47</td>
<td>52</td>
<td>64</td>
<td>55</td>
</tr>
<tr>
<td>Cocci 1 µm %</td>
<td>44</td>
<td>48</td>
<td>36</td>
<td>45</td>
</tr>
<tr>
<td>Coccobacilli &lt;2 µm %</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacilli &gt;2 µm %</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cocci &gt;1 µm %</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Navicula</em> &lt;20 µm %</td>
<td>77</td>
<td>4</td>
<td>12</td>
<td>87</td>
</tr>
<tr>
<td><em>Navicula</em> &gt;20 µm %</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown centrics %</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Amphora</em> %</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Rectangular-shaped %</td>
<td>3</td>
<td>0</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td><em>Nitzschia longissima</em> %</td>
<td>2</td>
<td>0</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td><em>Cocconeis</em> %</td>
<td>1</td>
<td>96</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>
As for prokaryotes, no temporal trend was found, even though the highest number of prokaryotic morphotypes (5) was found in July 2018, whereas only 3 and 2 morphotypes have been found in other seasons. The highest concentrations of diatoms were found in the winter, confirming previous observations for these microbes. From the bioinformatic analysis of sequences reassigned using plastidic 16S rRNA data, *Amphora* is confirmed as ubiquitous in the microbial plastisphere. This genus is very similar morphologically to Cymbellaceae, which is one of the families belonging to the OTUs found (Table 4.2.5). The other ones were Chaetoceraceae and *Phaeocystis* sp., both not classified as abundant from SEM microphotographs. Other OTUs abundant in these samples were assigned to Thalassionemataceae, and were found in all the samples except in October 2019. This could be matching the group “rectangular-shaped small” identified by SEM. All the four matching OTUs (Cymbellaceae, Chaetoceraceae, Thalassionemataceae and *Phaeocystis* sp.) were found together in both July samples, suggesting that these diatoms thrive particularly well in summer on MPs.
Table 4.2.5. Qualitative list of most common eukaryotic OTU distribution in the BoP from PHYTOREF assignment. Note that some of the sampling times, like October 2019, showed more diversity of re-assigned eukaryotes, so their list is longer than other sampling times.

<table>
<thead>
<tr>
<th></th>
<th>July 2018</th>
<th>February 2019</th>
<th>July 2019</th>
<th>October 2019</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DIATOMS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cymbellaceae</td>
<td>Thalassionemaceae</td>
<td>Chaetoceraceae</td>
<td>Chaetoceraceae</td>
<td></td>
</tr>
<tr>
<td>Thalassionemaceae</td>
<td>(especially Thalassionema fraunfeldii sp.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chaetoceraceae</td>
<td>Fragilariaeeae</td>
<td>Cymbellaceae</td>
<td>Cymbellaceae</td>
<td></td>
</tr>
<tr>
<td>Naviculaceae</td>
<td>Chaetoceraceae</td>
<td>Triceratiaceae</td>
<td>Triceratiaceae</td>
<td></td>
</tr>
<tr>
<td>Thalassionemaceae</td>
<td>Cymbellaceae</td>
<td>Thalassionemataceae</td>
<td>Fragilariaeae</td>
<td></td>
</tr>
<tr>
<td>Coscinodiscophycbeae</td>
<td>Stephanodiscaceae</td>
<td>-</td>
<td>Naviculaceae</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Thalassiosira oceanica</td>
</tr>
<tr>
<td><strong>OTHER EUKARYOTES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prymnesiaeae (Phaeocystis sp.)</td>
<td>Phaeocystis sp. (Haptophyta)</td>
<td>Aureombrap sp., Ectocarpus sp., Pinguiochrysidaeae (Ochrophyta)</td>
<td>Pinguiooccus pyrenoidosus and Eucampia zodiacus (Ochrophyta)</td>
<td></td>
</tr>
<tr>
<td>Corallinaceae (Corallina sp.)</td>
<td>Mamiellaceae (Chlorophyta)</td>
<td>Phaeocystis sp., Prymnesiaeae (Haptophyta)</td>
<td>Phaeocystis sp., Chrysochromulinaceae (Haptophyta),</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Ahnfeltiophycidae (Chlorophyta)</td>
<td>Wrangeliaecae (Rhodophyta)</td>
<td>Tetraselmis cordiformis (Chlorophyta)</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bangiopsis subsimplex (Rhodophyta)</td>
<td></td>
</tr>
</tbody>
</table>

A total of 45 samples were analyzed. Temporal variability was the most relevant parameter to take into account (Figure 4.2.12). July-18 samples was the most distinct group (light green), both from a different season (February-19) and from the same season of another year (July-19). Regarding the year 2019, the most distanced group was the winter one which formed a cluster (red). Another cluster, less distinct in terms of temporal variability, was the one of October-19 (pink) which had some outliers from July-19 (dark green) which would account as a summer-19 cluster. The latter samples were the ones with more outliers, both together with October-19 and alone, at the bottom of the graph. Alpha diversity showed agreement with these results from NMDS (Table 4.2.6), indeed similar values were shared among summer-19 samples. While highest indexes were observed in July-18 and lowest in February-19.
Figure 4.2.12. Non-metric multidimensional scaling (NMDS) representation of Bray-Curtis dissimilarity matrix of prokaryotic OTUs attached to MPs (stress 8.6e-05)
Table 4.2.6. Alpha diversity: Shannon index and Observed OTUs of the different MPs associated communities

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Observed OTUs</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 2018</td>
<td>294.4</td>
<td>5.1</td>
</tr>
<tr>
<td>February 2019</td>
<td>144.5</td>
<td>4.4</td>
</tr>
<tr>
<td>July 2019</td>
<td>160.0</td>
<td>4.4</td>
</tr>
<tr>
<td>October 2019</td>
<td>161.0</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Among all samplings Proteobacteria were always the most abundant phylum (Figure 4.2.13). Within this phylum, Rhodobacteraceae (5.4-9.8%) and Vibrionaceae (2.8-8.0%) were always abundant. Other relevant families were Sphingomonadaceae, Alteromonadaceae and Pseudoalteromonadaceae. The highest percentage of this phylum was found in October 2019 (46.3%), together with the highest Vibronaceae (Figure 4.2.14). The second most abundant phylum was Bacteroidetes (22.5-39%) with Saprospiraceae (2.8-15.6%), Flavobacteriaceae (6.2-13.5%) and Cyclobacteriaceae (0.9-2.9%) as the most abundant families. The highest percentage of Bacteroidetes was found in July 2018 (39.0%), with similar values in October 2019 and a noteworthy drop in February and July 2019 (22.5-23.4%). The latter event coincided with an increase in Firmicutes (22.7% in February and 16.5% in July 2019). At the same time, very low percentages of Firmicutes were found in the other two samplings: 3.9% in October 2019 and even 0.6% in July 2018. Ruminococcaceae was the most abundant family of this phylum, always found and reaching also 16.1% in the winter. A phylum which did not show significant changes between samplings was Planctomycetes, which showed a minimum of 6.4% in July 2019 and a maximum of 9% in July 2018. Its most relevant family was Pirellulaceae, found abundant in the summer and less abundant in the autumn. Cyanobacteria accounted for 0.5% during winter and peaked at 2.4% in July 2019.
Figure 4.2.13. Taxonomical classification for different samplings of the MPs associated communities at the phylum level (>0.5%). Abb19-1 is February 2019; abb19-4 is July 2019; abb19-5 is October 2019; abb18-2 is July 2018.
Figure 4.2.14. Taxonomical classification for different samplings of the MPs associated communities at the family level (>2%). Abb19-1 is February 2019; abb19-4 is July 2019; abb19-5 is October 2019; abb18-2 is July 2018.
In order to check for a further temporal variability, among all seasons were identified all the OTUs shared and unique among the attached community. No shared OTUs were found among all the time points, even between the two July samples of 2018 and 2019 (Figure 4.2.15).

![Venn diagram showing shared and exclusive OTUs among different samplings for the MP-attached community. Numbers shown are actual OTUs and percentages of total. Abb19-1 is February 2019; abb19-4 is July 2019; abb19-5 is October 2019; abb18-2 is July 2018.]

**Figure 4.2.15.** Venn diagram showing shared and exclusive OTUs among different samplings for the MP-attached community. Numbers shown are actual OTUs and percentages of total. Abb19-1 is February 2019; abb19-4 is July 2019; abb19-5 is October 2019; abb18-2 is July 2018.

### 4.2.1 Discussion

As also pointed out by Oberbeckmann et al. (2019), Pinnell and Turner (2020), and Amaral-Zettler et al. (2021), in order to understand community structure, recruiting and microbial ecology features related to MPs, more research should be focused on variability along spatial and temporal gradients. Molecular results presented in this thesis showed that there is a clear signal related to the temporal variability linked to the attached community structure in both Gulf of Napoli and Bay of Pozzuoli. This is likely related to the origin of the samples collected, as they were sampled from the environment, and not, as the majority of studies of plastisphere, from incubation experiments. Different environmental conditions in different seasons would recruit different community attached. This is not the case for higher
taxonomic levels. Indeed, Proteobacteria and Bacteroidetes were always found as the most common phyla, but rather at family or lower level. Not even for morphological features, for examples related to bacilli and cocci which were always the most common prokaryotic morphotypes, which together with diatoms, form the most abundant members of the plastisphere as visible from SEM microphotographs. The latter analysis shows that the assessment of morphological features is in agreement with molecular results. Indeed, the majority of the diatom morphotypes have been found also in plastidic sequences. This is the case for Naviculaceae, Cymbellaceae and Thalassionemataceae. Another agreement between SEM and molecular results is the higher density of prokaryotes showed in summer together with higher OTUs found in the same season attached to MPs. On the other hand, diatoms density was higher in winter times, for both areas studied. In summary, environmental samples show that plastisphere communities are strongly linked to the season of sampling and, in turn, to the environmental conditions, even though similarities are found.

4.2.2 Conclusions

In conclusion, the data presented highlight the importance of repeated sampling to assess MP pollution and its attached community in coastal areas because of complex circulation and multiple terrestrial discharges. MP variability in the Gulf of Napoli and Bay of Pozzuoli is very high, both in terms of total concentrations and community composition of attached microbes. The plastisphere community data presented here underline again the importance of temporal variability and environmental parameters shaping the microbial community, as reported by other studies (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2020). Further studies should be aimed at functional measurements of microbial activity of MPs, so to better understand their role as new habitats and also their potential threat to the environment and the final consumers (Wang et al., 2021).
4.3 Spatial variability on MPs distribution and characteristics

As mentioned above, a high spatial variability is observed for both MP concentrations and plastisphere community composition. In this subchapter data from different areas are presented, from coastal areas of the Campania region to the Northern Adriatic Sea and from Azores to Sicily.

4.3.1 Gulf of Napoli

Microplastic concentrations in the Gulf of Napoli, assessed as mentioned earlier twice a year in 2018 and 2019 and once in 2020, ranged around one order of magnitude, from 1.12 to 12.08 MP m\(^{-3}\), from the same station, st.1 (Portici), with 5.69 MPs m\(^{-3}\); Table 4.3.1.1, which showed the highest concentration, as expected from its close vicinity to the town of Portici, inhabited by an annual average of 53,400 people and placed in an area of over 3M people living in the Metropolitan city of Napoli (2018-2020, ISTAT; protezionecivile.gov.it). This confirms previous observations that MPs are more concentrated in close vicinity to urban settlements (Mezzelani et al., 2018). The second highest concentrations were at st. 2, which, although it lies 2 miles off the coast, is frequently impacted by runoffs from the large city of Napoli and its terrestrial urban discharges (D’Alcalà et al., 2004). The less impacted was st.3 which is relatively protected from the dominant water circulation of the Gulf and its discharges (Cianelli et al., 2012). All three stations show dominance of large MPs as the most abundant (Figure 4.3.1.1A). Fragments was the MP type most abundant for all three stations (Figure 4.3.1.1B). St. 1 was the station with highest contribution of the latter MP type (68.8%), followed by st.2 and 3. Films and filaments, on the contrary, were more abundant in the latter stations. Transparent was the most common colour of MPs found in the area (47.8%), followed by coloured (36.7%) and white (15.5%) (Figure 4.3.1.1C). This trend was found at all the sampling dates and especially at st. 1 which showed averagely higher percentages of transparent MPs, which were dominant at the other stations as well (47.5% st.2; 44.5% st.3).
Table 4.3.1.1. MP concentrations (MPs m$^{-3}$) at the different stations and sampling dates. No sampling was carried out in St.3 in July 2018.

<table>
<thead>
<tr>
<th></th>
<th>St.1</th>
<th>St.2</th>
<th>St.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan-18</td>
<td>1.12</td>
<td>1.78</td>
<td>1.27</td>
</tr>
<tr>
<td>Jul-18</td>
<td>4.66</td>
<td>1.60</td>
<td>-</td>
</tr>
<tr>
<td>Aug-19</td>
<td>9.46</td>
<td>8.39</td>
<td>1.29</td>
</tr>
<tr>
<td>Sep-19</td>
<td>12.08</td>
<td>9.89</td>
<td>5.32</td>
</tr>
<tr>
<td>Jan-20</td>
<td>1.13</td>
<td>1.42</td>
<td>1.78</td>
</tr>
<tr>
<td>average</td>
<td>5.69</td>
<td>4.61</td>
<td>2.41</td>
</tr>
<tr>
<td>dev.st</td>
<td>4.94</td>
<td>4.16</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Figure 4.3.1.1. Percentage distribution for the three stations studied of A) Size fraction; B) MP type; C) MP colour.
4.3.2 Bay of Pozzuoli (BoP)

As for the Bay of Pozzuoli, MP concentrations ranged between 0.53 to 12.81 MPs m$^{-3}$, one order of magnitude at the same station, Ab2, which showed the highest and the lowest concentration of the area, indeed showed also the highest standard deviation related to the three stations for four times throughout 2018 and 2019. On average, Ab2 showed the highest concentration of the area (Table 4.3.2.1), followed by ab1 and ab5. This area is characterized to have an annual average of 79,580 inhabitants (2018-2020 (ISTAT)) and over 500,000 people living in the area (Zona Flegrea protezionecivile.gov.it).

Table 4.3.2.1. MP concentrations (MPs m$^{-3}$) at the different stations and sampling dates (with total average and standard deviation). No sampling was carried out in AB5 in July 2018 and in station AB1 in February 2019.

<table>
<thead>
<tr>
<th>MP m$^{-3}$</th>
<th>AB1</th>
<th>AB2</th>
<th>AB5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jul-18</td>
<td>1.41</td>
<td>10.37</td>
<td>-</td>
</tr>
<tr>
<td>Feb-19</td>
<td>-</td>
<td>0.53</td>
<td>1.69</td>
</tr>
<tr>
<td>Jul-19</td>
<td>3.71</td>
<td>4.89</td>
<td>3.14</td>
</tr>
<tr>
<td>Oct-19</td>
<td>4.96</td>
<td>12.81</td>
<td>3.41</td>
</tr>
<tr>
<td>average</td>
<td>3.36</td>
<td>7.15</td>
<td>2.75</td>
</tr>
<tr>
<td>dev.st</td>
<td>1.80</td>
<td>5.52</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Large MPs were always the most abundant size fraction found regarding all the stations (Figure 4.3.2.1A). Their contribution was often exceeding 80%, only twice was less than 80% for AB2 and AB5 in February 2019 and July 2018, respectively. As for the contribution of the MPs smaller than 333 µm, only in one sampling were found to be higher than 1% in contribution, AB5 in July 2018, proof of an efficient sampling with the manta net.
Fragments was the MP type which accounted always for the highest contribution for all the stations. On average films was the second MP type per contribution (Figure 4.3.2.1B), but as for individual stations, foams were more abundant in AB1 and AB2. Pellets have been never found in AB5, while granules have never been found in the Gulf of Pozzuoli. No colour dominated at the different stations. Indeed, all the average contributions were 33.3% for colored, transparent and white MPs, as shown in figure 4.3.2.1C. Colored MPs were mostly found at AB1, transparent at AB2 and white at AB5. Among all the stations AB5 was the one with more uncertainty of color dominance, as was the one with highest standard deviation for these values.

4.3.3 The Cilento and Vallo di Diano Marine Protected Area

Going south of the Campania region, the Cilento bay has been assessed for MP horizontal distribution in May 2018. MP concentrations ranged between 0.285 and 1.105 MP m$^{-3}$, with the lowest concentration at st. 4 (and similar st.3 and 6) and the highest at st. 5 (with similar values in st.1 and 2, Table 4.3.3.1). This MPs concentration is not considered high at coastal stations (De Lucia et al., 2013; Collignon et al., 2012; Mezzelani et al., 2018). Most common
MPs were small size (300-1000 µm) fragments decreasing from the coast to offshore (st.2-3-4-5) (Table 4.3.3.1), suggesting a degradation process of larger litter. No MPs smaller than 333 µm were found. Five MP types have been found (fragments, films, filaments, foams, pellets), with no observation of granules. At all stations fragments dominated (86.1% average), with films accounting for a significant part at stations 2 and 5 (36.9%), supporting the observation that all MPs resulted from fragmentation of larger plastics. MP diversity types changed with distance from the coast: St.1 and 2 presented all 5 MPs types. Pellets (the only primary MPs observed in this area) and foams were only present at st.1 and 2, possibly indicating a local runoff which was not dispersed further. Moreover, st.5 presented fragments, films and filaments, st.6, instead, only fragments and films, considered these coastal stations less impacted by runoff in comparison with st.1 and 2. Most MP found were colored (55.5 %) followed by transparent (34.1%) and white (10.3%), Table 4.3.3.1. Stations 2 and 5 had more transparent, less colored and almost no white MPs. Stations 3 and 4 showed a dominance of colored, very low transparent and white MPs.

Table 4.3.3.1. Microplastic concentrations (MP m⁻³) and percentages by size, shape and colour at the different stations

<table>
<thead>
<tr>
<th></th>
<th>st.1</th>
<th>st.2</th>
<th>st.3</th>
<th>st.4</th>
<th>st.5</th>
<th>st.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP m⁻³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1000 &lt;5000 µm (%)</td>
<td>0.929</td>
<td>1.002</td>
<td>0.433</td>
<td>0.285</td>
<td>1.015</td>
<td>0.431</td>
</tr>
<tr>
<td>&gt;300 &lt;1000 µm (%)</td>
<td>65.3</td>
<td>27.2</td>
<td>21.6</td>
<td>24</td>
<td>41</td>
<td>60</td>
</tr>
<tr>
<td>&lt;300 µm (%)</td>
<td>34.7</td>
<td>72.8</td>
<td>78.4</td>
<td>76</td>
<td>59</td>
<td>40</td>
</tr>
<tr>
<td>Fragments (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Films (%)</td>
<td>83.1</td>
<td>59.1</td>
<td>89.5</td>
<td>92</td>
<td>57</td>
<td>80</td>
</tr>
<tr>
<td>Foams (%)</td>
<td>11</td>
<td>35.7</td>
<td>0</td>
<td>0</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td>Filaments (%)</td>
<td>0.8</td>
<td>2.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pellets (%)</td>
<td>4.2</td>
<td>1.7</td>
<td>10.5</td>
<td>8</td>
<td>5.1</td>
<td>0</td>
</tr>
<tr>
<td>Granules (%)</td>
<td>0.8</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colored (%)</td>
<td>35.6</td>
<td>33.3</td>
<td>83.8</td>
<td>88</td>
<td>39.2</td>
<td>53.3</td>
</tr>
<tr>
<td>Transparent (%)</td>
<td>54.2</td>
<td>53.5</td>
<td>8.1</td>
<td>0</td>
<td>55.7</td>
<td>33.3</td>
</tr>
<tr>
<td>White (%)</td>
<td>10.2</td>
<td>13.2</td>
<td>8.1</td>
<td>12</td>
<td>5.1</td>
<td>13.3</td>
</tr>
</tbody>
</table>
4.3.4 Sarno river

To conclude with the Campania region, a very relevant area of probable influence of MP in the Campania region, was assessed. This is the case of the Sarno river, as it is known that the majority of MPs come from land-based origins, which before ending up in the oceans come from the rivers. MP concentrations at Downstream Station (SD), last station of the river before ending up into the sea, were 2048 MP m$^{-3}$, ca. 2000 times more than at the other two stations (1.2 at Sea Station (SS) and 0.8 MP m$^{-3}$ at Upstream Station (SU), at the river spring, Table 4.3.4.1). While at SU no single plastic type dominated, at SS fragments accounted for 43.6% of total, while at SD foams dominated (69.5%), with the dominance of MPs ranging between 1000 and 5000 µm. No primary MPs types were found in riverine samples, while pellets were found at SS, although for only 0.7% of the total. As for color, river samples showed a dominance of transparent MPs, especially at SD (83.3%), while distribution was more homogeneous at SS (Table 4.3.4.1).

Table 4.3.4.1. MPs characteristics at different stations (concentration, size fraction, type and colour). Stations abbreviations are: SS = Sarno Sea; SD = Sarno Downstream; SU = Sarno Upstream.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>SD</th>
<th>SU</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP m$^{-3}$</td>
<td>1.2</td>
<td>2048.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Fragments (%)</td>
<td>43.6</td>
<td>20.3</td>
<td>22.2</td>
</tr>
<tr>
<td>Films (%)</td>
<td>11.4</td>
<td>6.8</td>
<td>33.3</td>
</tr>
<tr>
<td>Foams (%)</td>
<td>32.2</td>
<td>69.5</td>
<td>22.2</td>
</tr>
<tr>
<td>Filaments (%)</td>
<td>12.1</td>
<td>3.4</td>
<td>22.2</td>
</tr>
<tr>
<td>Pellets (%)</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>&gt;1000 &lt;5000 µm (%)</td>
<td>87.9</td>
<td>89.8</td>
<td>77.8</td>
</tr>
<tr>
<td>&gt;300 &lt;1000 µm (%)</td>
<td>12.1</td>
<td>10.2</td>
<td>22.2</td>
</tr>
<tr>
<td>&lt;300 µm (%)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Colored (%)</td>
<td>34.9</td>
<td>13.3</td>
<td>22.2</td>
</tr>
<tr>
<td>Transparent (%)</td>
<td>27.5</td>
<td>83.3</td>
<td>55.6</td>
</tr>
<tr>
<td>White (%)</td>
<td>37.6</td>
<td>3.3</td>
<td>22.2</td>
</tr>
</tbody>
</table>
4.3.5 North-Adriatic Sea

Away from the Campania region, during the winter of 2019, MP horizontal distribution was assessed in the North-Adriatic, in an area interested by river Po and Venice lagoon. A total of 202 total pieces of MPs were counted. Concentrations ranged from 0.067 (PTAA) to 0.530 MPs m\(^{-3}\) (N1, Table 4.3.5.1). Regarding the transect close to the river Po delta, the highest concentration was found at the furthest station from the delta (N1) and was the double in comparison to the ones close to the coast (N5 and S1). Station in between the furthest and the closest stations, showed the lowest concentration in the Gulf (N3). Exact same situation was found for the stations related to Venice lagoon. VE02 showed the highest concentration together with C10, while PTAA showed the lowest in the transect. Paloma showed concentration similar to the ones closer to the river delta. The most abundant MP type was filaments (45.3 %), followed by fragments (43.7%). Filaments were abundant especially at stations N1, VE02, N3 and C10, while fragments were abundant N5, PTAA and S1Post. The abundance of filaments in estuaries has often been linked to effluent from wastewater treatment, particularly from fibres shed during the washing of textiles (Napper et al., 2016), but also from industrial origin (Cole et al., 2011). Unfortunately, no chemical characterization of filaments is available in this study, so that it is not possible to trace their origin. This is because only pieces related to the plastisphere analysis were characterized for their polymer and no filaments were found large enough to be analysed for this analysis. Films and pellets were also present, but at very low percentages (8.7% and 1.9%, respectively). Pellets were present only at stt. PAL and N3P, the latter sampled after the storm. The failure to detect pellets may suggest an efficient reduction by industry, which are their main contributors (Govender et al., 2020). However, we cannot exclude the possibility that pellets are found in streams and rivers and somehow filtered before entering the sea. No films were observed at stt. N3 (before the storm) and PTAA. No foams were detected at any station (Table 4.3.5.1). Most MPs were coloured (57.4%), while transparent ones were 34.8% of the total, and the remaining 5.9% were white MPs, found only at coastal stt. (N5, VE02, PAL and N3P). High amounts of coloured MPs may suggest a higher load of newly introduced plastics, as opposed to transparent and white ones, which are attributable to degradation and discoloration due to physico-chemical processes (Govender et al., 2020). As predators appear to preferentially ingest coloured MPs due to resemblance to living prey (Boerger et al., 2010), the low presence of white MPs may suggest high consumption rates at the time of sampling. Even if only two stations were assessed before and after the storm,
it seems that the storm did not affect MPs distribution radically, but slightly higher concentrations (0.15 MPs m\(^{-3}\)) were observed before as compared to after the storm (0.13 MPs m\(^{-3}\)). The after-storm scenario showed higher concentrations at stt. N1 to N5, and lower at those south of the Po River delta. S1 stayed similar in both concentration and type distribution, with only slightly more coloured MPs, while at N3 more MPs types were observed after the storm with additional films and pellets, less filaments and the same percentage of fragments but white MPs appeared, while coloured MPs stayed the same and the transparent ones diminished (Table 4.3.5.1).

Table 4.3.5.1. MP characteristics (total MPs counted; MPs m\(^{-3}\); % Fragments; % films; % foams; % filaments; % pellets; % colored; % transparent; % white) according to station and date.

<table>
<thead>
<tr>
<th>STATION (surface)</th>
<th>Sampling day</th>
<th>total MPs</th>
<th>Mps m(^{-3})</th>
<th>Fragments</th>
<th>Films</th>
<th>Foams</th>
<th>Filaments</th>
<th>Pellets</th>
<th>Colored</th>
<th>Transparent</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>21/02/2019</td>
<td>54</td>
<td>0.53</td>
<td>3.70</td>
<td>3.70</td>
<td>0.00</td>
<td>92.60</td>
<td>0.00</td>
<td>94.40</td>
<td>5.60</td>
<td>0.00</td>
</tr>
<tr>
<td>N3</td>
<td>21/02/2019</td>
<td>7</td>
<td>0.07</td>
<td>42.90</td>
<td>0.00</td>
<td>0.00</td>
<td>57.10</td>
<td>0.00</td>
<td>57.10</td>
<td>42.90</td>
<td>0.00</td>
</tr>
<tr>
<td>N5</td>
<td>21/02/2019</td>
<td>32</td>
<td>0.24</td>
<td>65.60</td>
<td>9.40</td>
<td>0.00</td>
<td>25.00</td>
<td>0.00</td>
<td>25.00</td>
<td>62.50</td>
<td>12.50</td>
</tr>
<tr>
<td>S1</td>
<td>21/02/2019</td>
<td>25</td>
<td>0.23</td>
<td>36.00</td>
<td>20.00</td>
<td>0.00</td>
<td>40.00</td>
<td>0.00</td>
<td>56.00</td>
<td>44.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C10</td>
<td>22/02/2019</td>
<td>17</td>
<td>0.18</td>
<td>35.30</td>
<td>11.80</td>
<td>0.00</td>
<td>52.90</td>
<td>0.00</td>
<td>82.40</td>
<td>17.60</td>
<td>0.00</td>
</tr>
<tr>
<td>PTAA</td>
<td>22/02/2019</td>
<td>3</td>
<td>0.04</td>
<td>66.70</td>
<td>0.00</td>
<td>0.00</td>
<td>33.30</td>
<td>0.00</td>
<td>66.70</td>
<td>33.30</td>
<td>0.00</td>
</tr>
<tr>
<td>VE02</td>
<td>22/02/2019</td>
<td>12</td>
<td>0.16</td>
<td>25.00</td>
<td>8.30</td>
<td>0.00</td>
<td>66.70</td>
<td>0.00</td>
<td>58.30</td>
<td>33.30</td>
<td>8.30</td>
</tr>
<tr>
<td>PAL</td>
<td>25/02/2019</td>
<td>20</td>
<td>0.21</td>
<td>55.00</td>
<td>10.00</td>
<td>0.00</td>
<td>30.00</td>
<td>5.00</td>
<td>50.00</td>
<td>45.00</td>
<td>5.00</td>
</tr>
<tr>
<td>N3 post</td>
<td>26/02/2019</td>
<td>21</td>
<td>0.17</td>
<td>42.86</td>
<td>14.29</td>
<td>0.00</td>
<td>28.57</td>
<td>14.29</td>
<td>27.04762</td>
<td>9.52</td>
<td>33.33</td>
</tr>
<tr>
<td>S1 post</td>
<td>26/02/2019</td>
<td>11</td>
<td>0.08</td>
<td>63.64</td>
<td>9.09</td>
<td>0.00</td>
<td>27.27</td>
<td>0.00</td>
<td>56.54545</td>
<td>34.55</td>
<td>0.00</td>
</tr>
</tbody>
</table>

According to the two areas identified, Not River Influenced (NRI) area, where the salinity values were higher than 36.5 for salinity, showed higher concentration of MPs, especially coloured filaments, while River Influenced (RI), area with salinity values less than 36.5, showed more transparent and white fragments (Table 4.3.5.2). Films, foams and pellets were equally distributed in the two areas. All together, these results suggest that in NRI areas more recently introduced MPs are present, maybe from domestic and/or industrial activities (Avio et al., 2020) while RI stt. would be more interested by fragmentation of older marine litter by meteorological weathering including UV (Andrady et al., 2011), indicating that they have travelled a long way before being washed out at sea.
Table 4.3.5.2. MP characteristics (total MPs counted; MPs m⁻³; % Fragments; % films; % foams; % filaments; % pellets; % colored; % transparent; % white) according to different areas NRI (Not-river influenced) and RI (River-influenced).

<table>
<thead>
<tr>
<th>area</th>
<th>total MPs</th>
<th>MPs m⁻³</th>
<th>Fragments</th>
<th>Films</th>
<th>Foams</th>
<th>Filaments</th>
<th>Pellets</th>
<th>Colored</th>
<th>Transparent</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRI (n=3)</td>
<td>91</td>
<td>0.31</td>
<td>31.33</td>
<td>8.50</td>
<td>0.00</td>
<td>58.50</td>
<td>1.67</td>
<td>75.60</td>
<td>22.73</td>
<td>1.67</td>
</tr>
<tr>
<td>RI (n=7)</td>
<td>111</td>
<td>0.14</td>
<td>48.96</td>
<td>8.73</td>
<td>0.00</td>
<td>39.71</td>
<td>2.04</td>
<td>49.53</td>
<td>40.01</td>
<td>7.73</td>
</tr>
</tbody>
</table>

4.3.6 Discussion

Horizontal distribution of MPs was assessed at coastal areas in order to look for features related to spatial variability. As for MP concentrations, the Campania region showed higher values in places closer to urban areas (e.g. Portici and Bagnoli towns) and linked to river influence (Sarno Downstream). In particular the latter showed 2000 times the concentration usually found in the Gulf. Large MPs were the ones more usually found at sea surface (MPs ranging between 1-5 mm). This was not the case only for Cilento area, where small MPs (0.3 – 1 mm) was the size fraction more abundant. Fragments were surely the most common MP type found around the Gulf of Napoli, a part from Sarno river area which did not show any MP type dominance. Films were found abundant both for GoN and Cilento bay, while foams were the second most abundant MP type in Pozzuoli bay. For color comparison of MPs, results were similar in different areas. Colored and transparents MPs were more abundant both in GoN and in Cilento bay, while no straight dominance was found both for Pozzuoli bay and Sarno river. Not in agreement with Sarno river results, North Adriatic MP concentration were higher in areas not linked with river Po delta and Venice lagoon, while further away from the coast like in furthest stations linked to the Po river transect (N1 and N3) and Paloma, close to the city of Trieste. The latter in agree with Gulf of Napoli results linking MP pollution to vicinity to urban areas. As for the results of the Gulf of Napoli, fragments were found as one of the most abundant MP type in the North Adriatic. In the latter area, though, the most abundant MP type was filaments, linking this area to aquaculture activities which are very relevant in the area. As for MP concentration research, also for
plastisphere studies there are different discriminants which shape the different communities. One is surely the biogeography, as showed elsewhere (Amaral-Zettler et al., 2015) is relevant. Lifestyle is another, as most of the plastisphere studies showed (Erni-Cassola et al., 2020; Oberbeckmann et al., 2020). Environmental conditions (Oberbeckmann et al., 2019) is another discriminant. In this subchapter all these discriminants will be touched in different MP samplings reported in the Mediterranean Sea.

Another discriminant, added in this thesis, is the relevance of continuous studies of the plastisphere which will give to the scientific community more information related to this field.
4.4 Spatial variability on plastisphere research

4.4.1 Plastisphere of Gulf of Napoli samples

Seventy-five MPs pieces were retrieved for parallel plastisphere characterization in the Gulf of Napoli. 49 Polypropylene (PE), 13 Polypropylene (PP), 5 Polystyrene (PS), 2 Ethylene vinyl acetate (EVA), 2 Poly(Ethylene:Propylene:Diene) (PEPD), 2 Waxes (WAX), 1 Polyurethane (PU), 1 Cellophane. 97.3% of the polymers recognized in this work were positively buoyant (PE, PP, expanded PS, EVA, PEPD, Wax) while only PU and Cellophane were negatively buoyant.

Prokaryotes and diatoms were the two most abundant members of the microbial plastisphere in the area, confirming previous observations (Dussud et al., 2018; Dudek et al., 2020). No big difference was retrieved st.1 and st.3 in terms of prokaryotic density, while st.2 samples showed almost the double of density in comparison with the other stations (Table 4.4.1.1). As for diatoms, no significant difference between the stations was found. Indeed, all the stations showed average values of around 200 diatoms mm$^{-2}$. Bacilli and cocci showed the same dominance in terms of prokaryotic diversity, indeed all the stations sampled showed both these morphotypes as the most abundant (Figure 4.4.1.1A). As for the minor contributors, st.1 showed more cocci >1 micron, st.2 more bacilli bigger than 2 microns while st.3 more coccobacilli. As for diatoms, st.1 and 3 showed the same dominance of *Navicula* and *Amphora* (Figure 4.4.1.1B), while at st. 2, despite the dominance of the two *Navicula* groups, relevant percentages of *Nitzschia longissima* were observed (14.6% ± 23.2% on average), with a peak in September 2019 (55%), as shown in the previous subchapter.

Table 4.4.1.1. Prokaryotes and diatoms density (with relative standard deviation) measured in ind. mm$^{-2}$ at the different stations of the Gulf of Napoli.

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes (ind mm$^{-2}$) - average</th>
<th>dev.st</th>
<th>diatoms (ind mm$^{-2}$) - average</th>
<th>dev.st</th>
</tr>
</thead>
<tbody>
<tr>
<td>St.1</td>
<td>7320</td>
<td>4755</td>
<td>214</td>
<td>194</td>
</tr>
<tr>
<td>St.2</td>
<td>11400</td>
<td>2302</td>
<td>226</td>
<td>199</td>
</tr>
<tr>
<td>St.3</td>
<td>7750</td>
<td>4645</td>
<td>210</td>
<td>139</td>
</tr>
</tbody>
</table>
Navicula (< and > 20 µm), Nitzschia longissima, Cocconeis and Amphora were always present at all stations and sampling dates. On average, the most abundant groups were: Navicula <20 µm and > 20 µm, unknown centrics, Nitzschia sigmoidea, Nitzschia longissima, Amphora, and rectangular-shaped. The first two most abundant groups were the two Navicula groups (over 70% for each station, Figure 4.4.1.1B). Navicula <20 µm was also the diatom group most abundant for all the seasons except September 2019, when N. longissima was the most abundant. For st. 2, despite the dominance Navicula <20 µm (53.4 ± 20.7% on average), Nitzschia longissima also showed significant percentages (14.1 ± 23.2% on average), especially with a peak in September 2019 (55.2%). As minor contributors, noteworthy were Navicula >20 µm (9.8 %± 6.4%), Figure 4.4.1.1B.

Figure 4.4.1.1. Contribution (%) of Prokaryotic (A) and Diatom (B) morphotypes at the different stations through the years studied.

Proof of the relevance of the eukaryotic counterpart was assessed by sequencing results related to the re-assign of the plastidic sequences. Navicula and Amphora dominance was confirmed by the presence of Cymbellaceae, morphologically similar to Amphora morphotype, and Naviculaceae present at all the stations assessed. Other taxa relevant to this analysis were the presence of Thalassiosira genus and Thalassionemataceae family which could be a corroboratation to the morphotypes unknown centrics and rectangular-shaped found
in the SEM microphotographs. A taxon that was found in sequencing analysis but not in SEM counting was the eukaryotic Prymnesiaceae. In terms of prokaryotic richness and diversity, st.1 and 3 showed the highest number of OTUs and higher diversity, with st.1 having higher numbers of Observed OTUs and st.3 more diversity having a higher Shannon index (Table 4.4.1.2). These two stations mentioned were the closest to the coast, possibly linking number of OTUs and diversity index to the distance from the coast.

Table 4.4.1.2. Alpha diversity indexes (Observed OTUs and Shannon) at the different stations studied.

<table>
<thead>
<tr>
<th></th>
<th>Observed</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>St.1</td>
<td>230.63</td>
<td>4.77</td>
</tr>
<tr>
<td>St.2</td>
<td>189.50</td>
<td>4.67</td>
</tr>
<tr>
<td>St.3</td>
<td>222.73</td>
<td>4.84</td>
</tr>
</tbody>
</table>

Throughout the 3 years assessed, 95% of total prokaryotes were represented by Proteobacteria, Bacteroidetes, Firmicutes, and Planctomycetes (Figure 4.4.1.2) regarding the MP-attached communities. St.1 and 3 showed higher percentages of Proteobacteria, in comparison with Bacteroidetes, while st.2 almost identical among the two (41.8% Proteobacteria; 41.6% Bacteroidetes), (Figure 4.4.1.2). At the family level (Figure 4.4.1.3), three out of four shared families among the most abundant ones (>2%), belonged to Bacteroidetes: Flavobacteriaceae, Saprospiraceae and Cyclobacteriaceae. The other family contributing 2% of the dataset and shared among all the other stations was Rhodobacteraceae (Proteobacteria). including families Burkholderiaceae and Alteromonadaceae (shared among st.1 and 3) and Vibrionaceae, shared among st.1 and 2. Considering the minor contributors, Planctomycetes was higher in st.2 and 3 than in st.1, especially for the high abundance (>2%) of Pirellulaceae family in st.2, while the duo Firmicutes-Planctomycetes was almost identical for st.1. Noteworthy was also Cyanobacteria higher than Firmicutes in st.2, this in particular for Cyanobiaceae family in st.2 and 3.
Figure 4.4.1.2. Taxonomical classification for plastic-associated communities related to stations at the phylum level (>0.5%). PORTICI is st.1; MC is st.2; GAIOLA is st.3.
Figure 4.4.1.3. Taxonomical classification for plastic-associated communities related to stations at the family level (>2%). PORTICI is st.1; MC is st.2; GAIOLA is st.3.

Shared and exclusive OTUs are shown in Figure 4.4.1.4. Coastal stations like st.1 (Portici) and 3 (Gaiola) were the ones sharing fewer OTUs among each other than among any other station together. Less shared OTUs would mean a higher spatial variability for the MP samples, in comparison with more unique OTUs related individually to each station.
4.4.2 Plastisphere of Bay of Pozzuoli samples

A total of 46 samples were analysed in order to characterize the microbial plastisphere found on the MPs at the Bay of Pozzuoli. 17 samples were analysed for July 2018, 8 for February 6 for July and 14 for October 2019. 19 Polypropylene (PE), 15 Polypropylene (PP), 7 Polystyrene (PS), 2 Poly-vinyl alcohol (PVA), 2 Waxes (WAX), 1 Polyamide Nylon 6 (PA6). 93.5% of the polymers recognized in this work were positively buoyant (PE, PP, expanded PS, Wax) while only PA6 and PVA were negatively buoyant. PE, PP and PS were the only polymers found in all samples.

The most abundant members of the plastisphere were prokaryotes and diatoms. On average the density accounted for $3.1 \times 10^4$ prokaryotes mm$^{-2}$ and $2.0 \times 10^2$ diatoms mm$^{-2}$. AB2 samples showed higher prokaryotic density (Table 4.4.2.1) in comparison to the other two stations. Diatoms density was particularly relevant among stations, indeed AB5 showed 5 times the density of the other two stations (Table 4.4.2.1). Bacilli and cocci were the most common and abundant members of prokaryotic morphotypes (Figure 4.4.2.1A). Bacilli slightly contributed more than the other morphotype, especially in ab1 and ab2 stations, while ab5 showed a perfect balance among the two.
Table 4.4.2.1. Prokaryotes and diatoms density (with relative standard deviation) measured in ind. mm$^{-2}$ at the different stations of the Bay of Pozzuoli.

<table>
<thead>
<tr>
<th></th>
<th>Prokaryotes</th>
<th>dev.st</th>
<th>Diatoms</th>
<th>dev.st</th>
</tr>
</thead>
<tbody>
<tr>
<td>ab1</td>
<td>2437</td>
<td>1865</td>
<td>79</td>
<td>120</td>
</tr>
<tr>
<td>ab2</td>
<td>3828</td>
<td>2187</td>
<td>81</td>
<td>27</td>
</tr>
<tr>
<td>ab5</td>
<td>2955</td>
<td>2157</td>
<td>431</td>
<td>594</td>
</tr>
</tbody>
</table>

Figure 4.4.2.1. Contribution (%) of Prokaryotic (A) and Diatom (B) morphotypes at the different stations through the years studied.

As for diatoms, *Navicula* and *Cocconeis* were the most abundant morphotypes found in the BoP. Ab1 showed a dominance of *Navicula* <20 with almost 90% of contribution (Figure 4.4.2.1B). The other two stations showed as well high contribution of the latter morphotype (both around 50%). *Cocconeis* showed a high contribution in ab5 (around 50%) and more than 10% in ab2. *N. longissima* and rectangular-shaped were found commonly in station ab2. From the bioinformatic analysis of sequences reassigned using plastidic 16S rRNA data, *Amphora* is confirmed as ubiquitous in the microbial plastisphere. This genus is very similar morphologically to Cymbellaceae, which is one of
the families belonging to the OTUs found. The other ones were Chaetoceraceae and *Phaeocystis* sp., both not classified as abundant from SEM microphotographs. Other OTUs abundant in these samples were assigned to Thalassionemataceae, and were found in all the samples except in October 2019. This could be matching the group “rectangular-shaped small” identified by SEM. As for prokaryotic sequencing results, no significant spatial results were found for samples from the Bay of Pozzuoli for the samples collected, as mentioned before in the previous chapter. Ab2 showed higher Observed OTUs and more diversity in comparison with the coastal attached samples, but not a significant difference (Table 4.4.2.2).

Table 4.4.2.2. Alpha diversity indexes (Observed OTUs and Shannon diversity index) at the different stations studied.

<table>
<thead>
<tr>
<th>Station</th>
<th>Observed</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1</td>
<td>189.64</td>
<td>4.71</td>
</tr>
<tr>
<td>AB2</td>
<td>239.41</td>
<td>4.93</td>
</tr>
<tr>
<td>AB5</td>
<td>168.90</td>
<td>4.65</td>
</tr>
</tbody>
</table>

The three phyla most abundant of the attached communities were the following: Proteobacteria, Bacteroidetes, Firmicutes and Planctomycetes. All three stations showed similar percentages of Proteobacteria, while AB2 showed the highest percentage of Bacteroidetes, as the two most abundant phyla (Figure 4.4.2.2). AB1 showed an important percentage of Firmicutes, even though AB5 showed the highest contribution of the latter phylum. AB1 and AB2 showed similar contribution of Planctomycetes. As for families, Flavobacteriaceae, Vibrionaceae, Saprospiraceae and Rhodobacteriaceae were the ubiquitous families in the top 5 always present in the 3 stations analysed, with Saprospiraceae preferring AB1 and AB5, while Flavobacteriaceae for AB2 (Figure 4.4.2.3). Other families shared among stations were Cyclobacteriaceae, Pseudoalteromonadaceae and Hyphomonadaceae. Ruminococcaceae and Alteromonadaceae were mostly present in station AB5 but also in AB1, as Veillonellaceae. Pirellulaceae, instead, was mostly present at stations AB1 and AB2.
Figure 4.4.2.2. Taxonomical classification for plastic-associated communities related to stations at the phylum level (>0.5%).
Figure 4.4.2.3. Taxonomical classification for plastic-associated communities related to stations at the family level (>2%).

As a support of sequencing results being spatial distribution not significant for their microbial plastisphere characterization, more OTUs were shared among ab1 and ab5 (81 OTUs) and ab2 and ab5 (55 OTUs), than from coastal ones (ab1-ab2) (50 OTUs, Figure 4.4.2.4).
4.4.3 Plastisphere in the Cilento area

Only 6 fragments were large enough to be cut in three for subsequent polymer characterization, SEM and DNA sequencing. 5 were positively identified as plastics, 3 belonged to PE and 2 to PP, both being buoyant polymers. These pieces were also characterized for their prokaryotic attached communities using SEM and 16S rRNA sequencing methods described in Chapter 2.
Every plastic piece analysed showed approximately 35.0% coverage of the surface by microbes (Table 4.4.3.1). St.6 showed the highest value with 50% of the surface covered, followed by st. 3 (41.7%), while st.2 the lowest, with averagely less than 25% surface covered. This may reflect different residence times of MPs at different sites, maybe differentially influenced by currents bringing MPs from the coast. In SEM analysis, the microbial plastisphere was numerically dominated by prokaryotes ($0.8 \times 10^4 \pm 1.3 \times 10^4$ ind mm$^{-2}$ on average). They were divided in 6 morphotypes: cocci $<1$ µm, cocci $>1$ µm, bacilli $<2$ µm, bacilli $>2$ µm, coccobacilli ($<2$ µm) and 4-5 µm cocci chains (Figure 4.4.3.1). Diatoms, the second most abundant members of the plastisphere community, were $13.4 \times 10^2 \pm 4.8 \times 10^2$ ind mm$^{-2}$ with a dominance of rectangular-shaped diatoms smaller than 10 µm and Navicula $<20$ µm, accounting, together, for 99% of total diatoms. 6 diatom groups were recognised: rectangular-shaped $<10$ µm, Navicula $<20$ µm, *Navicula* $>20$µm, *Cocconeis*, *Synedra* and *Nitzschia* sp. (circa 30 µm) (Figure 4.4.3.2).

Figure 4.4.3.1. Prokaryotic morphotypes found in the plastisphere: A-B) cocci chains (embedded in the biofilm); C) bacilli $<2$ µm; D-E-F) cocci. Scale bar size is 2 µm.
Figure 4.4.3.2. SEM microphotograph of diatom groups found. A) *Navicula* < 20 µm; B) *Navicula* 20 µm; C) *Synedra*; D) group of rectangular-shaped diatoms < 10 µm.
Table 4.4.3.1. Total coverage, average abundance (ind mm\(^{-2}\)) and Frequency of observation (times observed divided by total samples), of prokaryotes and diatoms, total and by category.

<table>
<thead>
<tr>
<th></th>
<th>Average Cilento (5 samples)</th>
<th>FO % (5 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage (%)</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Prokaryotes (total)</td>
<td>7745.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Bacilli &lt; 2 µm</td>
<td>2868.2</td>
<td>37.0</td>
</tr>
<tr>
<td>Cocci 1 µm</td>
<td>3003.9</td>
<td>38.8</td>
</tr>
<tr>
<td>Coccobacilli (1-2 µm)</td>
<td>45.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Bacilli &gt;2 µm</td>
<td>58.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Cocci &gt; 1 µm</td>
<td>833.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Cocci chains</td>
<td>936.7</td>
<td>12.1</td>
</tr>
<tr>
<td>Diatoms (total)</td>
<td>1345.5</td>
<td>100.0</td>
</tr>
<tr>
<td><em>Navicula</em> &lt;20 µm</td>
<td>150.9</td>
<td>11.2</td>
</tr>
<tr>
<td><em>Navicula</em> &gt;20 µm</td>
<td>2.4</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Cocconeis</em></td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Rectangular shape 10 µm</td>
<td>1188.3</td>
<td>88.3</td>
</tr>
<tr>
<td><em>Synedra</em></td>
<td>1.4</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Nitzschia</em> genus 30 µm</td>
<td>1.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>

As for spatial variability, St.2 presented the highest prokaryotic density (1.1 \(\times\) 10\(^4\) ± 2.0 \(\times\) 10\(^3\) ind mm\(^{-2}\), Table 4.4.3.2) with a dominance of cocci 1 µm, bacilli <2 µm and cocci chains (ca 94% altogether). This is surprising because st.2 gave the lowest coverage (see above), but it must be considered that the coverage assessment was performed on 500x SEM microphotographs, providing only general assessment of the matrix covering the pieces.

The highest diatom densities were found at st. 3 (28.5 \(\times\) 10\(^2\) ± 11.0 \(\times\) 10 \(^2\) ind mm\(^{-2}\)) with rectangular shape 10 µm and *Navicula* <20 µm accounting for 95% of the total. This matched with the lowest value of Chl a, which could probably be evidence of a preference of diatoms to live attached to MPs, as Chl a was measured in the surrounding environments (Table S2).
In order to get more information about the eukaryotic community of the plastisphere, the chloroplastic sequences from the sequencing analysis have been re-assigned using PHYTOREF database (Decelle et al., 2015). Most (68%) of the re-assigned OTUs belonged to Bacillariophyta (diatoms), confirming SEM observations (data not shown). Bacillariophyceae in general but also Cymbellaceae, Naviculaceae, Fragilariaceae, Thalassionemataceae and Chetocerotaceae. Other OTUs related to Ochrophyta were Pinguiophyceae. From Hacrobia instead, there were OTUs related to Chrysochromulinae. Naviculaceae was one of the most abundant family in these samples, followed by Thalassionemataceae. Considering this high presence, its morphology and the high presence of “rectangular-shaped” diatoms found in this area, perhaps we could speculate that the latter diatom group could be classified under this family name. As for the other families, they were

<table>
<thead>
<tr>
<th></th>
<th>st2 (2 samples)</th>
<th>st3 (2 samples)</th>
<th>st6 (1 sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage (%)</td>
<td>23.0</td>
<td>41.8</td>
<td>50</td>
</tr>
<tr>
<td>Prokaryotes (total)</td>
<td>10772</td>
<td>4522</td>
<td>8139.5</td>
</tr>
<tr>
<td>Bacilli &lt; 2 µm</td>
<td>3649.9 (31.3)</td>
<td>2325.6 (48.6)</td>
<td>2390.2 (29.4)</td>
</tr>
<tr>
<td>Coci 1 µm</td>
<td>4764.2 (53.0)</td>
<td>1679.6 (35.9)</td>
<td>2131.8 (26.2)</td>
</tr>
<tr>
<td>Coccobacilli (1-2 µm)</td>
<td>113 (3.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bacilli &gt;2 µm</td>
<td>0 (0)</td>
<td>64.6 (2)</td>
<td>161.5 (2)</td>
</tr>
<tr>
<td>Coci &gt; 1 µm</td>
<td>468.3 (2.6)</td>
<td>0 (0)</td>
<td>3230 (39.7)</td>
</tr>
<tr>
<td>Coci chains</td>
<td>1776.5 (9.8)</td>
<td>452.2 (13.5)</td>
<td>226.1 (2.8)</td>
</tr>
<tr>
<td>Diatoms (total)</td>
<td>461.8</td>
<td>2846.8</td>
<td>110.4</td>
</tr>
<tr>
<td>Navicula &lt;20 µm</td>
<td>228.2 (41.2)</td>
<td>125.6 (4.9)</td>
<td>46.7 (42.3)</td>
</tr>
<tr>
<td>Navicula &gt;20 µm</td>
<td>1.8 (1.5)</td>
<td>0 (0)</td>
<td>8.5 (7.7)</td>
</tr>
<tr>
<td>Cocconeis</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4.2 (3.8)</td>
</tr>
<tr>
<td>Rectangular shape 10 µm</td>
<td>228.2 (54.2)</td>
<td>2721.2 (95.1)</td>
<td>42.5 (38.5)</td>
</tr>
<tr>
<td>Synedra</td>
<td>3.5 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nitzschia genus 30 µm</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>8.5 (7.7)</td>
</tr>
</tbody>
</table>
present in this qualitative assessment but absent in our SEM microphotographs. For instance, no individuals belonging to Ochrophyta or Hacrobia, present in the sequences, were found from the microscopic analysis. Thirteen samples were sequenced for DNA: 5 from microplastics and 8 from seawater (free prokaryotes). A total of 196,241 sequences have been retrieved for a total of 2866 OTUs. 62,623 sequences have been retrieved from attached prokaryotes for a total of 1613 OTUs; 133,618 sequences have been retrieved from free prokaryotes for a total of 1284 OTUs. Samples were normalized for 9382 sequences, the minimum resulted number of sequences. MPs and seawater samples presented saturating rarefaction curves (Figure 4.4.3.3) indicating sufficient depth of sequencing to account for most of the taxa amplified in both microplastic and seawater matrices.

Figure 4.4.3.3. MPs and free-prokaryotic samples’ rarefaction curves. Reads per sample on x axis; OTUs numbers on the y axis. The plateau showed by all the curves indicates a sufficient depth of sequencing.

Cluster analysis plotting the Bray-Curtis distances showed a distinction among free and attached communities. In particular, noteworthy was a small subgroup related to two PE
samples (mp 3 – 4 from st. 2 and 3) (Figure 4.4.3.4 A). The Venn diagram (Figure 4.4.3.4 B) showed indeed just 1.1% of the OTUs shared among the two communities (total OTUs=2866). Alpha diversity was estimated using 2 indexes, Observed OTUs and Shannon (community diversity). In general, the attached communities were richer and more diverse (Figure 4.4.3.5), in particular PP was richer and more diverse than PE (Figure 4.4.3.6).

Figure 4.4.3.4 A) Cluster analysis calculated by Bray-Curtis dissimilarities; 6.B) Venn diagrams of the OTUs shared among free (Free AMP) and attached (MP AMP) prokaryotes.
OTUs were mostly classified as Proteobacteria, Bacteroidetes and Cyanobacteria, but also Planctomycetes and Firmicutes, especially for attached communities (Figure 4.4.3.7). The
free community was mostly represented by Proteobacteria (66.5%) especially *SAR11 clades* (23.1% Clade I, 12.7% *SAR116* and 4.6% *SAR 86_Clade*), Bacteroidetes (26.4%) especially Flavobacteriaceae 19% and Balneolaceae 4.1% and Cyanobacteria (4.0%). PP community presented more diverse phyla than the other two communities, Proteobacteria (39.5%), Bacteroidetes (32.2%), but also Firmicutes (7.7%), Cyanobacteria (6.6%) and Planctomycetes (6.1%). PE community was mostly based on Proteobacteria (44.8%), Bacteroidetes (27.6%), Cyanobacteria (15.8%) and Planctomycetes (7.5%). As for family level, all the most abundant (>2.0%) from the attached communities were all shared, but distributed in different percentages. This is the case of Rhodobacteraceae (PP 12.9%; PE 17.6%), Flavobacteriaceae (13.3% PP; 13.0% PE), Phormidesmiaceae (3.2% PP; 9.1% PE) and Pirellulaceae (2.0% PP; 4.7% PE) (Figure 4.4.3.8). The two samples from st. 2 (PE) and single sample from st. 6 (PP) differing for their alpha diversity, had the same most abundant prokaryotic families (Flavobacteriaceae, Cyclobacteriaceae, Saprospiraceae).

![Taxonomical classification for plastic-associated (MP) and free (FB) communities related to stations at the phylum level (>0.5%).](image-url)
These were characterized by genera already found in other works related to MP-attached communities. This was the case for *Lewinella* and *Erythrobacter*, both associated with PP. *Lewinella* has been isolated from plastic surfaces (Oberbeckmann et al., 2016), while *Erythrobacter* has been related to use PAHs in its pathways (Oberbeckmann et al., 2017), thus confirming the accumulation of toxic compounds by MPs. PE from st. 2, on the contrary, was characterized by uncultured genera from Cyclobacteriaceae, Flavobacteriaceae and Rhodobacteriaceae, but also from *Filomicrobium* and *Blastopirellula*. Both have been found in Zettler et al., 2013, the latter also in the work cited above Oberbeckmann et al., 2017, while the former was also found by Wang and colleagues (2020) in the intestinal bacteria of Medaka fish after a MPs exposure.
4.4.4 Plastisphere of Sarno river area samples

21 pieces analysed were positively identified as plastic polymers (Table 4.4.4.1), while for three pieces identification was not possible.

Table 4.4.4.1. Polymer composition of selected MP pieces and their further analysis. SS = Sarno Sea; SD = Sarno Downstream; SU = Sarno Upstream. IR is Polyisoprene; PE is Polyethylene; PP is Polypropylene; PS is Polystyrene; ALK is Alkyd; WAX is wax.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Station</th>
<th>Polymer</th>
<th>SEM</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SS</td>
<td>IR</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>SS</td>
<td>PE</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>SS</td>
<td>PE</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>SS</td>
<td>PE</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>SS</td>
<td>PP</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>SS</td>
<td>PE</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>SS</td>
<td>PE</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>SS</td>
<td>PP</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>SS</td>
<td>PE</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>SS</td>
<td>PE</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>SD</td>
<td>WAX</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>SD</td>
<td>WAX</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>SD</td>
<td>WAX</td>
<td>Yes</td>
<td>No</td>
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<td>15</td>
<td>SD</td>
<td>WAX</td>
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<td>16</td>
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<td>17</td>
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<td>18</td>
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<td>PS</td>
<td>Yes</td>
<td>No</td>
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<td>19</td>
<td>SU</td>
<td>ALK</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>20</td>
<td>SU</td>
<td>ALK</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>21</td>
<td>SU</td>
<td>WAX</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>22</td>
<td>SU</td>
<td>WAX</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Sequencing failed for many of these MPs, due to insufficient DNA quality. Unfortunately, this could not be remediated for re-sequencing, due to COVID-19 restrictions. All MPs selected for plastisphere characterization were colonized by biofilm. On average, river samples were more covered than sea samples, with a decreasing gradient from the spring to
the estuary area. SU samples were covered at 30 ± 15%, SD samples at 25 ± 5% and SS samples at 20 ± 10% (Table 4.4.4.2).

Table 4.4.4.2. Average abundance for coverage, diatoms and prokaryotic divided for stations. Stations abbreviations are: SS = Sarno Sea; SD = Sarno Downstream; SU = Sarno Upstream.

<table>
<thead>
<tr>
<th></th>
<th>SS (5 samples)</th>
<th>SD (4 samples)</th>
<th>SU (3 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage (%)</td>
<td>20 +- 10</td>
<td>25 + - 5</td>
<td>30 + - 15</td>
</tr>
<tr>
<td>Prokaryotes</td>
<td>4961.2</td>
<td>10941.5</td>
<td>24171.0</td>
</tr>
<tr>
<td>Bacilli &lt;2 µm</td>
<td>3346.3</td>
<td>3407.6</td>
<td>21156.3</td>
</tr>
<tr>
<td>Cocci 1 µm</td>
<td>1563.3</td>
<td>1582.7</td>
<td>3014.6</td>
</tr>
<tr>
<td>Coccobacilli</td>
<td>51.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cocci &gt; 1 µm</td>
<td>0.0</td>
<td>5943.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Bacilli &gt;2 µm</td>
<td>0.0</td>
<td>8.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Diatoms</td>
<td>52.4</td>
<td>52.2</td>
<td>83.7</td>
</tr>
<tr>
<td>Unknown centrics</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Navicula &lt;20 µm</td>
<td>33.3</td>
<td>42.5</td>
<td>76.7</td>
</tr>
<tr>
<td>Cocconeis</td>
<td>14.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Amphora</td>
<td>3.5</td>
<td>2.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Navicula &gt;20 µm</td>
<td>0.0</td>
<td>0.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Rectangular-shaped &lt;10 µm</td>
<td>0.0</td>
<td>0.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Mastogloia</td>
<td>0.0</td>
<td>4.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Synedra</td>
<td>0.0</td>
<td>0.9</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Prokaryotes and diatoms were the most abundant members of the microbial plastisphere. Prokaryotes were more abundant in SU samples, twice the abundance as at SD and five times more abundant than at SS. SD showed more prokaryotic morphotypes (4) than the other two stations (SS, 3; SU, 2). The morphotypes found were: Bacilli <2 µm, Coccobacilli 1-2 µm, Cocci >1 µm, Bacilli >2 µm and Cocci 1 µm (Figure 4.4.4.1). Bacilli <2 µm and cocci 1 µm were found at all stations, while coccobacilli, SS samples showed on average 6000 ind mm$^{-2}$ against 0 for the other stations (Table 4.4.4.2).
Figure 4.4.4.1. Prokaryotic morphotypes attached to MPs: A) Bacilli (<2 µm); B) Coccobacilli 1-2 µm (blue arrow) C) Cocci (>1 µm); D) Bacilli >2 µm; E) Cocci 1 µm. Scale bars are 2 µm.

As for diatoms, SU samples showed higher abundances than the other stations (1.5 times), with very similar values at the other two stations. SD samples showed the highest number of groups found, 6 against 4 of the other stations. Diatoms were represented by *Mastogloia, Cocconeis, Synedra, Amphora, Navicula* < and >20 µm, Rectangular-shaped < 10 µm and unknown centrics (Figure 4.4.4.2). Unknown centrics and *Cocconeis* were present only at SS, while *Navicula* >20 µm and rectangular-shaped <10 µm only at SD and SU. *Mastogloia* and *Synedra* were only present at SD. *Navicula* <20 µm showed higher abundances at SU.
The eukaryotic component, assessed from plastid reassignment using PhytoRef, confirmed that Bacillariophyta (diatoms) were the dominant microbes, supporting also SEM observations. In detail, Cymbellaceae and Naviculaceae were the most represented, also supporting the morphological assignment of *Amphora*, belonging to Cymbellaceae. OTUs from higher plants (Solenaceae, Myrtaceae and Pinaceae) were also detected, marking the agricultural area crossed by the river. Only 8 samples provided positive results for 16S amplicon sequencing (Table 4.4.4.3), 5 from MPs (both marine and riverine), 2 for free community (from SS and SD stations) and 1 for “particle attached” (PA), from SD station.

Table 4.4.4.3. Positive sequences retrieved at the different stations. SS is Sarno Sea; SD is Sarno Downstream; SU is Sarno Upstream.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>SD</th>
<th>SU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free community</td>
<td>x</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>Particle-Attached</td>
<td>-</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>MP attached</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
At river stations (SD and SU), free and PA communities were very similar to each other and overlapped in the NMDS (red triangle and circle on the left, Figure 4.4.4.3 A). Both were very different from MP-attached and marine free communities. MP-attached were different from the other fractions, but similar between marine and freshwater. This was confirmed by the highest percentage of shared OTUs between riverine free and PA communities (3000 OTUs, 24% of the total, Figure 4.4.4.3 B). All the other categories showed less than 0.5% of OTUs shared. This confirms what observed by Amaral-Zettler et al., (2021), free and MP-attached riverine prokaryotic communities are different to each other similarly to what observed in the sea.

Figure 4.4.4.3. (A) Non-metric multidimensional scaling representation of Bray-Curtis dissimilarity matrix of the different prokaryotic communities (stress 9.5 e-05). (B) Venn diagrams showing shared and exclusive OTUs between the different communities. RIV_MP is riverine MP-attached; RIV_FREE is free living riverine communities; SEA_FREE is free living marine communities; SEA_MP is marine MP-attached and RIV_PA is riverine particle-attached.

Alpha diversity of river samples was higher for free and PA communities than for MP-attached ones (Figure 4.4.4.4), suggesting that MPs were a rather selective habitat at the
river, or that prokaryotes were not able to attach efficiently, maybe due to the high energy present in the environment, due to strong altitudinal gradients.

Most abundant prokaryotic phyla were Proteobacteria, Firmicutes, Bacteroidetes and Planctomycetes. Differences were noted between the different lifestyle communities. Proteobacteria were abundant (>40%) among all the different lifestyle communities (free, PA and MP-attached). Whereas Firmicutes were relatively more abundant in free and PA communities, especially in the river (Figure 4.4.4.5 and 4.4.4.7). Bacteroidetes were more abundant in the marine communities (both MP and free, >20%, Figure 4.4.4.6) than in the riverine ones, but in the river was more abundant as MP-attached than free or PA-attached. This suggests that the even if they live better in the sea, they find adaptative advantages to be attached to MP even in freshwater. Planctomycetes were always > 10% in MP-attached community but one order of magnitude less in the free and PA communities. Similarly, Euryarchaeota were, on average, more abundant in MP-attached than in free and PA. The latter phylum is very much rare in Plastisphere communities works (in particular in Agostini et al., 2021 regarding MPs found at deep-sea environments), remarking the selective and rare biosphere nature of this peculiar community. MP-associated prokaryotes were taxonomically more similar to each other than to the other communities. In particular, they
showed high abundance of Proteobacteria and Bacteroidetes families like Rhodobacteraceae, Flavobacteriaceae, known components of the plastisphere (Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015; 2021) but also Rhodocyclaceae and Rubinisphaeraceae. Some families were also shared with the PA community (like Rhodobacteraceae) which were also composed by Lactobacillaceae, Ruminococcaceae and Pseudomonadaceae (Figure 4.4.4.5), which indicates a preference for attachment. Ruminococcaceae and Pseudomonadaceae were also abundant in river free community (Figure 4.4.4.7), where Burkholderiaceae, Lactobacillaceae, Bacteroidaceae and uncultured family from Chitinhophagales were the most abundant families. These were also abundant in MP-associated communities, suggesting a recruitment from the surrounding seawater.

In summary, MP-associated community appear to reflect the free-living community from which they are recruited, mainly from Proteobacteria. Some other phyla prefer to grow in different conditions: Firmicutes in the river and Bacteroidetes in the sea. In addition, the MP-attached community shows members of the so-called “rare biosphere” (Sogin, 2006), like Planctomycetes and Euryarchaeota which evidently find a competitive advantage from attachment. Amaral-Zettler et al., (2021) found other families dominating in mediterranean river samples, like Comamonadaceae and Enterobacteriaceae. These also reported in other studies on riverine MPs (McCormick et al., 2014; 2016), however, they were not abundant in our MP river samples, possibly due to seasonal differences (this study was in January, theirs in the summer) or also to biogeography factors, similar to what reported for the sea (Amaral-Zettler et al., 2015). Another family associated to MP river communities by Amaral-Zettler et al (2021) is Pseudomonadaceae, which in the present study was detected, but in the PA fraction. This may suggest a preference for attachment independent from the substrate, at least in this particular case.
Figure 4.4.4.5. Taxonomical classification for plastic-associated (MP), free (FB) and particle-attached (PA) communities related to upstream station (SU) at the family level (>2%).
Figure 4.4.4.6. Taxonomical classification for plastic-associated (MP) and free (FB) communities related to sea station (SS) at the family level (>2%).
Figure 4.4.4.7. Taxonomical classification for plastic-associated (MP), free (FB) and particle-attached (PA) communities related to downstream station (SD) at the family level (>2%).

**4.4.5 Plastisphere of the North-Adriatic area samples**

Of the 28 single items isolated, 22 were confirmed to be MPs: 9 being Polyethylene (PE), 3 Polystyrene (PS), 2 Alkyds (ALK), 2 Indurent materials (dental manufacturing), 2 Polypropylene (PP), 1 Polyamide (PA), 1 Poly-ethyl-acrylate (PEA) and 1 Resin (R). PE, PS and PP are positively buoyant while PA, Alkyd Resins, Indurent and PEA are negatively buoyant because their density is higher than seawater density. Of these, 64% are known to be positively buoyant (PE, PS and PP) and 36% negatively (IND, PA, AL, RES and PEA). Of these isolated MPs items, 16 were used for both SEM and DNA extraction, 16 for SEM only and 17 for DNA sequencing only. Fourteen polymers have been positively recognized as plastics in the pre-storm situation and 8 polymers for the post storm situation. In the pre-
storm situation 7 PE, 3 ALK, 1 PP, 1 PS, 1 IND and 1 PA were found. In the post-storm situation 2 PE, 2 PS, 1 PP, 1 IND, 1 RES, 1 PEA were found. Very similar percentages of positively buoyant polymers were found before (64%) and after (62%) the storm. MPs found in the area had on average a coverage index of 30 ± 17.5 % (defined in Chapter 2, Materials and Methods), therefore less than 50% of the MPs area was covered by a biofilm, on average. Microorganisms found attached on MPs were mainly Prokaryotes (Bacteria and Archaea) and diatoms. Average densities of prokaryotes and diatoms were 944 mm⁻² (SD ± 1244) and 191 (SD ± 455), respectively (Table 4.4.5.1 and 4.4.5.2). Prokaryotic morphotypes were cocci (1 µm in size), bacilli <2 µm, bacilli > 2 µm, and coccobacilli (1 µm in size) (Figure 4.4.5.1). The prokaryotic community was dominated by cocci (70.4%) and bacilli <2 µm (27.4%, Table 18). Cocci and bacilli <2 µm were the only prokaryotic morphotypes present at all stations, Coccobacilli were absent at N5, C10 and N3 post (while present at N3 before the storm). Bacilli >2 µm were only present at C10 and PAL, both belonging to the NRI area, highlighting a rather preference of more saline environments. Cocci were found in higher densities as compared to a site between the Hawaii islands and the coast of California (169 ± 39 mm⁻², Carson et al., 2013), while less bacilli were found comparing it with the same study (1664 ± 243 individuals mm⁻²). The same trend was found as for frequency of observation (FO%), times observed divided by total number of samples, when compared to a study from the coast of Australia (Reisser et al., 2014). These differences may be due to the different environments favouring adaptation of different prokaryotic groups. These differences in the prokaryotic groups probably derive from the known differences in oceanographic conditions and plankton communities in different areas, as reported in Amaral-Zettler et al., 2021.

Table 4.4.5.1. Average coverage, density (ind mm⁻²) and frequency of observation (%) for prokaryotes and their morphotypes. n= 16

<table>
<thead>
<tr>
<th>Coverage (%)</th>
<th>Prokaryotes (total)</th>
<th>cocci 1 µm</th>
<th>bacilli &lt;2 µm</th>
<th>coccobacilli 1 µm</th>
<th>bacilli &gt;2 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>average n=16 samples</td>
<td>30 ± 17.5</td>
<td>944 ± 1244</td>
<td>665 ± 984</td>
<td>259 ± 305</td>
<td>12 ± 23</td>
</tr>
<tr>
<td>FO % (16 samples)</td>
<td>-</td>
<td>100.0</td>
<td>70.4</td>
<td>27.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Table 4.4.5.2. Average coverage, density (ind mm$^{-2}$) and frequency of observation (%) for diatoms and their morphotypes. n= 16

<table>
<thead>
<tr>
<th></th>
<th>Coverage index (total)</th>
<th>Diatoms &lt;20 µm</th>
<th>Navicula &gt;20 µm</th>
<th>Navicula &gt;20 µm</th>
<th>Skeletonoema</th>
<th>Cocconeis</th>
<th>Diploneis</th>
<th>Chaetoceros Rectangular-shaped</th>
<th>N. sigmoidea</th>
<th>Amphora</th>
<th>Other unknown centrics</th>
<th>Synedra</th>
<th>N. longissima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>30 ± 17.5</td>
<td>191 ± 455</td>
<td>73 ± 175</td>
<td>6 ± 9</td>
<td>20 ± 37</td>
<td>11 ± 40</td>
<td>1 ± 2</td>
<td>1 ± 3</td>
<td>8 ± 22</td>
<td>8 ± 18</td>
<td>61 ± 236</td>
<td>1 ± 3</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>FO%</td>
<td>-</td>
<td>100.0</td>
<td>38.3</td>
<td>2.9</td>
<td>10.5</td>
<td>5.9</td>
<td>0.3</td>
<td>0.5</td>
<td>4.2</td>
<td>4.1</td>
<td>32.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>
As for diatoms, 12 morphotypes were classified as: *Navicula* <20 μm, *Amphora*, *Skeletonema*, *Cocconeis*, Rectangular-shaped, *Nitzschia sigmoidea* group, *Navicula* >20 μm, *Chaetoceros*, unknown centrics, *Diploneis*, *Synedra* and *N. longissima*. (Figures 4.4.5.2 and 4.4.5.3). The most abundant, like *Navicula* (both size) and *Skeletonema*, were mostly present: the first two groups were always found except at C10, similar trend for *Skeletonema*, always found except at N5. Pennate diatoms are the most common diatoms found on MPs (Zettler et al., 2013; Carson et al., 2013; Reisser et al., 2014; Dudek et al., 2020). In particular the *Navicula* genus has been already found in several other works as a prominent member of the microbial plastisphere (Zettler et al., 2013; Carson et al., 2013; Reisser et al., 2014; Dudek et al., 2020), while *Skeletonema* genus has been found only in another work in the Black Sea (Basak Esensoy et al., 2020). All the other groups were mostly found at the Po transect, suggesting a preference of environments more characterized by less salinity and with more nutrient availability. Other genera like *Amphora*, *Nitzschia*, *Cocconeis* and *Diploneis* (Reisser et al., 2014; Dudek et al., 2020; Basak Esensoy et al., 2020) are quite common members of the microbial plastisphere in different environments, which suggests no biogeography from this group of colonizers. Total diatoms had densities of 191 diatoms mm⁻² (± 455 SD) and were mainly represented by *Navicula* <20 μm (38.3%), *Amphora* (32.2%) and *Skeletonema* (10.6%). Other diatom groups accounted for lower percentages (Table 19). Less diatoms mm⁻² were found in comparison with Carson et al., (2013) work (pennate diatoms 1097 ± 154 mm⁻²), while more diatom density was found in comparison with Dudek et al., (2020). After 6 weeks of incubation, MPs found by Dudek et al., were covered by 0.9 ± 0.2 diatoms mm⁻². This might suggest a high time of residency from the
MPs found in the Adriatic Sea and/or better conditions to thrive by the diatoms on MPs. In terms of FO%, our observation showed higher contribution of *Navicula* (38% vs 9%) and *Amphora* (32% vs 13%) in comparison with Reisser et al., 2014 work, while less contribution by *Nitzschia* (4% vs 43%).

![SEM microphotograph of diatom groups found. A) Synedra-like; B) Navicula >20 µm; C) Rectangular-shaped; D) Skeletonema; E) Amphora and Cocconeis. Scale bar is indicated on each photo.](image)

Figure 4.4.5.2. SEM microphotograph of diatom groups found. A) Synedra-like; B) *Navicula* >20 µm; C) Rectangular-shaped; D) *Skeletonema*; E) *Amphora* and *Cocconeis*. Scale bar is indicated on each photo.
Figure 4.4.5.3. SEM microphotograph of diatom groups found. A) *Navicula* <20 μm; B) Unknown centric group; C) *Diploneis*; D) *Chaetoceros*; E) *Nitzschia sigmoidea* group; F) *Nitzschia longissima*. Scale bar is indicated on each photo.

As for environmental conditions influencing the communities, in particular river influence in this case, River-influenced (RI) samples, belonging to stations characterized by salinity lower than 36.5, showed higher coverage (35 vs 22.5 %, Table 20), higher prokaryotic (2x) and diatom (5x) densities. As for prokaryotes, cocci density was three times higher than on Not-river-influenced (NRI) samples, belonging to stations characterized by salinity higher than 36.5, (>75% of prokaryotic morphotypes contribution, Table 4.4.5.3), while similar densities were found for both coccobacilli and bacilli <2 μm. Noteworthy was the absence of bacilli >2 μm in RI as opposed to NRI, possibly indicate a preference for higher salinities.

As for diatoms, RI showed a dominance of *Amphora* (ca 100 times higher than NRI, Table 7) and *Navicula* <20 μm (10x higher than NRI), both accounting for 38.0% and 35.7% of the diatom community (Table 4.4.5.4). RI samples showed all the diatom groups, while NRI only four: *Skeletonema* (highest contribution of the area and higher density than RI), *Navicula* <20 μm and unknown centrics.
Table 4.4.5.3. Average coverage (%), density (ind mm$^{-2}$) and frequency of observation (FO, %, in brackets) for prokaryotes and their morphotypes divided by area. NRI means Not-influenced by rivers; RI means River-Influenced areas.

<table>
<thead>
<tr>
<th></th>
<th>RI (n=10)</th>
<th>NRI (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage (%)</td>
<td>35 ± 17.5</td>
<td>22.5 ± 10</td>
</tr>
<tr>
<td>Prokaryotes</td>
<td>1183 ± 1477</td>
<td>544 ± 636</td>
</tr>
<tr>
<td>Bacilli &lt;2 µm</td>
<td>278 ± 358 (23.5)</td>
<td>226 ± 216 (41.6)</td>
</tr>
<tr>
<td>Cocci</td>
<td>899 ± 1165 (75.9)</td>
<td>275 ± 409 (50.5)</td>
</tr>
<tr>
<td>Coccobacilli</td>
<td>6 ± 20 (0.5)</td>
<td>22 ± 26 (4.0)</td>
</tr>
<tr>
<td>Bacilli &gt;2 µm</td>
<td>0 (0)</td>
<td>22 ± 39 (4.0)</td>
</tr>
</tbody>
</table>
Table 4.4.5.4. Average coverage, density (ind mm\(^2\)) and frequency of observation (FO, %) for diatoms and their morphotypes divided by area. NRI means Not-influenced by rivers; RI means River-Influenced areas.

<table>
<thead>
<tr>
<th></th>
<th>RI</th>
<th>NRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage (a.u.)</td>
<td>35 ± 17.5</td>
<td>22.5 ± 10</td>
</tr>
<tr>
<td>diatoms mm(^2)</td>
<td>274 ± 566</td>
<td>51 ± 79</td>
</tr>
<tr>
<td><em>Navicula</em> &lt;20 µm</td>
<td>104 ± 205</td>
<td>10 ± 14 (19.8)</td>
</tr>
<tr>
<td></td>
<td>(38.0)</td>
<td></td>
</tr>
<tr>
<td><em>N. sigmoidea</em></td>
<td>12 ± 22 (4.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Cocconeis</em></td>
<td>18 ± 50 (6.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Skeletonema</em></td>
<td>16 ± 18 (6.0)</td>
<td>29 ± 59 (57.0)</td>
</tr>
<tr>
<td><em>Diploneis</em></td>
<td>1 ± 2 (0.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Chaetoceros</em></td>
<td>2 ± 4 (0.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rectangular-shaped</td>
<td>13 ± 28 (4.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Navicula</em> &gt;20 µm</td>
<td>3 ± 3 (1.1)</td>
<td>10 ± 13</td>
</tr>
<tr>
<td><em>Amphora</em></td>
<td>98 ± 298 (35.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other unknown centrics</td>
<td>0 ± 1 (0.1)</td>
<td>2 ± 4</td>
</tr>
<tr>
<td><em>Synedra</em></td>
<td>1 ± 2 (0.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>N. longissima</em></td>
<td>0 ± 1 (0.1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
As sequencing was targeted towards 16S for bacterial and archaeal detection, chloroplast sequences from eukaryotes were taken aside before progressing with bioinformatics analysis. Although, these sequences were successively reassigned using PhytoRef (Decelle et al., 2015) for a qualitative analysis of phototrophic eukaryotic microbes. Most of the reassigned OTUs belonged to Bacillariophyta (diatoms), confirming the SEM visualization. Family Bacillariophyceae dominated but also Cymbellaceae, Fragilariaceae, Thalassionemataceae, Chaetocerotaceae and the species *Thalassiosira oceanica* were present. Other OTUs could be assigned to Chlorophyta (*Tetraselmis convolutae*), Prymnesiaceae and Mamiellaceae families and to Hacrobia (Chrysochromulinaeae and Phaeocystaceae). These results match with SEM observations, as Chaetoceraceae were also present in the SEM images. The Thalassionemataceae and Cymbellaceae sequences could be attributed to the “rectangular-shaped” and *Amphora* morphotypes, respectively. The *Thalassiosira oceanica* sequences could be attributed to the unknown centrics (as a matter of fact, Figure 2B closely resembles a *Thalassiosira*, although the photograph does not allow a proper taxonomic determination). The other OTUs were referred to as fragile-structured species, in terms of morphology, which possibly have been damaged during SEM preparation. Amplicon sequencing targeting the V4V5 region of the 16S rRNA gene resulted in 17 positively sequenced samples, providing 74,802 sequences for a total of 2,990 OTUs, achieving a sufficient depth of sequencing in all samples, as shown by rarefaction curves (Figure 4.4.5.4 A). As for 16S amplicon sequencing results, no clear pattern appeared to influence prokaryotic colonization on plastics (Figure 4.4.5.4 B), therefore stations were grouped based on salinity values and considered as either NRI or RI (see above).

In general, Proteobacteria and Bacteroidetes accounted for 78.7% of the whole dataset, followed by Firmicutes (8.6%), Actinobacteria (4.5%), Planctomycetes (2.9%) and Cyanobacteria (0.9%). (Figure 4.4.5.5).
Figures 4.4.5.4. A) MP samples rarefaction curves. Reads per sample on x axis; OTUs numbers on the y axis. The plateau showed by all the curves indicates a sufficient depth of sequencing; B) Non-metric multidimensional scaling representation of Bray-Curtis dissimilarity matrix of prokaryotic OTUs attached to MPs (stress 0.25).

Figure 4.4.5.5. Taxonomic classification for areas (NRI=Not-river influenced; RI = river influenced) at the phylum level (>0.5%).

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Bacteroidetes were similar (42.9 vs 41.2%) for the two areas, especially represented by Flavobacteriaceae family, found as the most abundant family for both areas (Figure 4.4.5.6 and 4.4.5.7). This family was rather abundant (over 30%) in PS and ALK samples in the RI area (Figure 4.4.5.6), suggesting a preference for low salinity-high nutrients. Flavobacteriaceae have been described as keystone taxa in biofilms that feed off exudates and some have the ability to prey on diatoms via lysis (Amin et al., 2012). Proteobacteria, instead, were higher in RI areas (39.3 vs 33.2%). This phylum in particular is usually represented by early colonizers (De Tender et al., 2017). Rhodobacteraceae and Desulfobulbaceae mostly accounted for the difference in Proteobacteria. The former family was rather abundant (14.8%) in PS samples in the RI area, meanwhile Desulfobulbaceae were very abundant (>40%) attached to IND in the same area. Minor contributors such as Firmicutes and Actinobacteria were more abundant in NRI than RI area. Bacillaceae and Staphylococcaceae accounted for the difference in Firmicutes, while Propionibacteraceae for the one in Actinobacteria (NRI) (Figure 4.4.5.7). Bacillaceae were similarly abundant (23.9% for both) attached to PP and IND, while Staphylococcaceae were found especially associated with IND and ALK. Propionibacteraceae (Actinobacteria) were one of the few families shared among all the polymers found in the area. This would suggest that higher percentages of dominant phyla like Bacteroidetes and Proteobacteria prefer environments with higher nutrients and chlorophyll, while minor contributors, putative core plastisphere members, rather prefer to thrive on MPs because they might offer hotspots of nutrients.
Figure 4.4.5.6. Taxonomical classification for RI area (RI=River influenced) at family level (>2%) for different polymers.
This was confirmed by higher alpha diversities (Observed OTUs and Shannon) on RI samples than on NRI ones (Table 4.4.5.5), especially on PS and ALK samples (Figures 4.4.5.8 A and B). Even though Firmicutes is rather a proxy of freshwater environment (DeVos et al., 2009), NRI area showed higher abundance in Firmicutes, maybe indicating their former freshwater origin. This was confirmed by more abundant and more unique OTUs on RI samples (vs NRI) (Figures 4.4.5.9). As for the other phyla, Planctomycetes,
Cyanobacteria and Acidobacteria were found in similar percentages, suggesting a rather generalist lifestyle, independent from the environment in which they have been found. Cyanobiaceae (Cyanobacteria) was found >2% in both NRI and RI areas, attached to the same polymer, ALK. No clear pattern was found to be statistically significant, not even for taxa characterizing different environments. For these results a Linear Discriminant analysis (LefSe) was performed but gave no statistically significant results (data not shown).

Table 4.4.5.5. Alpha diversity indexes (Observed OTUs and Shannon) in Non-River Influenced (NRI) or River-Influenced (RI) areas (see above)

<table>
<thead>
<tr>
<th>Station</th>
<th>Observed</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRI</td>
<td>146.4</td>
<td>4.5</td>
</tr>
<tr>
<td>RI</td>
<td>160.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Figures 4.4.5.8. Average, median and SD of alpha diversity indices on different polymers in the two areas identified A) Observed; B) Shannon.

One-hundred and seventy-five OTUs were found shared among both areas, accounting for only 7.2% of the whole dataset (Figure 4.4.5.9), confirming that salinity is a strong determinant of the community composition. River-Influenced areas provided better
environment for different OTUs as 58.2% of them were unique for this area, while 34.7% were unique for Not-river influenced area.

![Venn diagram showing shared and exclusive OTUs for areas (NRI=Not river influenced; RI = river influenced).]

**Figure 4.4.5.9.** Venn diagram showing shared and exclusive OTUs for areas (NRI=Not river influenced; RI = river influenced).

### 4.4.6 Plastisphere of the samples collected during the Pelagia transit cruise

MP items were classified as reported in Chapter 2 and diatoms and prokaryotes grouped by morphotype, as visualized in SEM images. Their respective densities on MPs were estimated following the protocol described in Chapter 2. As for this subchapter, the discussion exclusively will be about the morphological characterization of the plastisphere community carried out with SEM analysis, MP counting, quantification and DNA sequencing were not analysed for this particular sampling campaign. Dissimilarity matrices, Nonmetric Multidimensional Scaling (NMDS) and Redundancy analysis (RDA) were generated using R (R Core Team 2017, [https://www.R-project.org/](https://www.R-project.org/)). The Shannon Index was calculated as in Krebs (1999).
The different water masses present at the surface during the cruise were clearly identified in the T-S plot (Figure 4.4.6.1). The ENACWu (Eastern North Atlantic Central Water upper layer, Bashmachnikov et al., 2015) was characterized by lower salinity and temperature values as compared to Med surface water masses. The BMAW (Balearic Modified Atlantic Water) and the TMAW (Tyrhenian Modified Atlantic Water, Knoll et al., 2017) were characterized by relatively higher salinity and temperatures. Both ENACWu and both MAWs) show a high variability due to surface instability as also related to mesoscale structures, but in general both temperature and salinity showed an increasing trend going eastward, as also observed by other authors (Hainbucher et al., 2014). Station 11, in the Gibraltar area (G) showed the lowest values of both temperature and salinity, clearly indicating a very active area where waters of different origin mix and interact, also through upwelling. Other clusters considered in the Mediterranean included Stations 13 and 14, situated in front of the Alboran Sea (AS), and stations from 15 to 18, from the Balearic Islands to Sardinia, separated for geographical reasons. Stations from 19 to 22, with higher salinity (37.8 – 38.4 S) and temperature (28.6 – 29.0 °C) values, towards the center of the Mediterranean Sea were also considered. These clusters are located within eddies (AS), or other hydrological features, and also show the evolution of the ENACWu during its course along the Mediterranean Sea, evolving into the MAW.

Figure 4.4.6.1. Potential Temperature (θ)-Salinity (S)-diagram (ENACWu: blue; G: yellow; BMAW: green; TMAW: orange; AS: violet).
Based on the water mass classification the manta stations have been subdivided into 5 groups and denominated based on their geographical location and/or water mass: from 1-10 ENACWu; 11 Gibraltar (G); 13-14 Alboran Sea Front (AS); 15-18 Balearic MAW (BMAW); 19-22 Tyrrhenian MAW (TMAW). A total of 53 MP from 19 out of the 21 manta trawls were processed, (Table 4.4.6.1), of which 52 could be analysed by SEM (one was damaged during preparation and could not be processed). No MP were found at station 10 and 11. Of these 52 pieces, 32 were fragments, 10 were films, 9 were filaments and 1 was foam (Table 4.4.6.1), all belonging to secondary MP, therefore originating from fragmentation of larger items, which is known be the majority of MPs found in the ocean (Cozàr et al., 2014). All 52 items were covered by a biofilm (average coverage index 30% ± 17.5SD. Prokaryotes were observed on 100% of the MP items, while diatoms on 49 (94% of the total). Based on counts, we estimated on average, 3808 ±3917SD prokaryotes per mm\(^2\) and 158 ±249SD diatoms per mm\(^2\).
Table 4.4.6.1. List of MP items with their characterization, coverage index (a.u.) and density (ind. mm$^{-2}$) of diatoms and prokaryotes. Data are Means ± SD, n=6 fields of view.

<table>
<thead>
<tr>
<th>Station</th>
<th>Date</th>
<th>Sample</th>
<th>Type</th>
<th>Coverage Index</th>
<th>Diatoms (ind. mm$^{-2}$)</th>
<th>Prokaryotes (ind. mm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27/07/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>0.8 ± 0.3</td>
<td>7 ± 11</td>
<td>9302 ± 7281</td>
</tr>
<tr>
<td>1</td>
<td>27/07/2018</td>
<td>M3</td>
<td>FILM</td>
<td>0.8 ± 0.3</td>
<td>0 ± 0</td>
<td>65 ± 100</td>
</tr>
<tr>
<td>2</td>
<td>27/07/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>1.2 ± 0.4</td>
<td>4 ± 9</td>
<td>4425 ± 5607</td>
</tr>
<tr>
<td>2</td>
<td>27/07/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>0.3 ± 0.3</td>
<td>4 ± 9</td>
<td>194 ± 324</td>
</tr>
<tr>
<td>2</td>
<td>27/07/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>0.6 ± 0.2</td>
<td>71 ± 44</td>
<td>5103 ± 3829</td>
</tr>
<tr>
<td>3</td>
<td>28/07/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>1.3 ± 0.4</td>
<td>492 ± 623</td>
<td>1066 ± 1620</td>
</tr>
<tr>
<td>3</td>
<td>28/07/2018</td>
<td>M2</td>
<td>FILAMENT</td>
<td>0.8 ± 0.3</td>
<td>665 ± 344</td>
<td>2164 ± 1889</td>
</tr>
<tr>
<td>3</td>
<td>28/07/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>0.8 ± 0.3</td>
<td>35 ± 48</td>
<td>194 ± 123</td>
</tr>
<tr>
<td>4</td>
<td>28/07/2018</td>
<td>M1</td>
<td>FILAMENT</td>
<td>2.0 ± 0.8</td>
<td>28 ± 35</td>
<td>388 ± 534</td>
</tr>
<tr>
<td>5</td>
<td>29/07/2018</td>
<td>M1</td>
<td>FILAMENT</td>
<td>2.2 ± 0.6</td>
<td>85 ± 84</td>
<td>388 ± 649</td>
</tr>
<tr>
<td>5</td>
<td>29/07/2018</td>
<td>M2</td>
<td>FILM</td>
<td>1.3 ± 0.3</td>
<td>166 ± 151</td>
<td>2099 ± 1993</td>
</tr>
<tr>
<td>5</td>
<td>29/07/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>0.7 ± 0.3</td>
<td>32 ±36</td>
<td>8624 ± 5133</td>
</tr>
<tr>
<td>6</td>
<td>29/07/2018</td>
<td>M1</td>
<td>FILM</td>
<td>0.9 ± 0.4</td>
<td>4 ± 8</td>
<td>4037 ± 5214</td>
</tr>
<tr>
<td>6</td>
<td>29/07/2018</td>
<td>M2</td>
<td>FILAMENT</td>
<td>1.5 ± 0.6</td>
<td>7 ± 10</td>
<td>969 ± 1082</td>
</tr>
<tr>
<td>6</td>
<td>29/07/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>1.9 ± 0.7</td>
<td>14 ± 16</td>
<td>581 ± 849</td>
</tr>
<tr>
<td>7</td>
<td>30/07/2018</td>
<td>M2</td>
<td>FOAM</td>
<td>1.8 ± 0.4</td>
<td>110 ± 51</td>
<td>2778 ± 2308</td>
</tr>
<tr>
<td>7</td>
<td>30/07/2018</td>
<td>M3</td>
<td>FILM</td>
<td>0.9 ± 0.7</td>
<td>0 ± 0</td>
<td>452 ± 381</td>
</tr>
<tr>
<td>8</td>
<td>30/07/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>0.8 ± 0.4</td>
<td>7 ± 16</td>
<td>5459 ± 4541</td>
</tr>
<tr>
<td>8</td>
<td>30/07/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>1.7 ± 0.4</td>
<td>25 ± 38</td>
<td>7171 ± 7357</td>
</tr>
<tr>
<td>8</td>
<td>30/07/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>2.3 ± 0.4</td>
<td>927 ± 419</td>
<td>9819 ± 9254</td>
</tr>
<tr>
<td>9</td>
<td>31/07/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>2.3 ± 0.9</td>
<td>0 ± 0</td>
<td>1712 ± 1240</td>
</tr>
<tr>
<td>9</td>
<td>31/07/2018</td>
<td>M2</td>
<td>FILAMENT</td>
<td>0.8 ± 0.4</td>
<td>42 ± 68</td>
<td>226 ± 396</td>
</tr>
<tr>
<td>10</td>
<td>01/08/2018</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>AS</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>02/08/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>0.9 ± 0.4</td>
<td>64 ± 21</td>
<td>12435 ± 6946</td>
</tr>
<tr>
<td>14</td>
<td>02/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>1.4 ± 0.2</td>
<td>21 ± 47</td>
<td>1938 ± 2509</td>
</tr>
<tr>
<td>14</td>
<td>02/08/2018</td>
<td>M3</td>
<td>FILAMENT</td>
<td>0.6 ± 0.5</td>
<td>7 ± 10</td>
<td>1066 ± 558</td>
</tr>
<tr>
<td>BMAW</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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153
<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>Type</th>
<th>Colonization</th>
<th>MP Weight</th>
<th>Total Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>03/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>0.9 ± 0.4</td>
<td>400 ± 285</td>
<td>1163 ± 442</td>
</tr>
<tr>
<td>03/08/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>1.3 ± 0.5</td>
<td>88 ± 79</td>
<td>9916 ± 4587</td>
</tr>
<tr>
<td>03/08/2018</td>
<td>M1</td>
<td>FILM</td>
<td>1.0 ± 0.3</td>
<td>53 ± 36</td>
<td>17022 ± 18309</td>
</tr>
<tr>
<td>03/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>1.5 ± 0.0</td>
<td>74 ± 62</td>
<td>5749 ± 3984</td>
</tr>
<tr>
<td>03/08/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>1.0 ± 0.4</td>
<td>25 ± 26</td>
<td>2810 ± 2021</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>0.2 ± 0.3</td>
<td>7 ± 10</td>
<td>1001 ± 937</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M2</td>
<td>FILM</td>
<td>0.6 ± 0.2</td>
<td>11 ± 16</td>
<td>258 ± 200</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M3</td>
<td>FILAMENT</td>
<td>0.4 ± 0.4</td>
<td>0 ± 0</td>
<td>97 ± 237</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M4</td>
<td>FRAGMENT</td>
<td>1.3 ± 0.3</td>
<td>142 ± 118</td>
<td>7429 ± 7209</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>1.3 ± 0.3</td>
<td>1267 ± 1027</td>
<td>5685 ± 4302</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>1.6 ± 0.4</td>
<td>442 ± 458</td>
<td>1583 ± 2251</td>
</tr>
<tr>
<td>04/08/2018</td>
<td>M3</td>
<td>FILAMENT</td>
<td>1.4 ± 0.7</td>
<td>7 ± 16</td>
<td>194 ± 388</td>
</tr>
<tr>
<td>05/08/2018</td>
<td>M1</td>
<td>FRAGMENT</td>
<td>0.7 ± 0.4</td>
<td>152 ± 127</td>
<td>8463 ± 3735</td>
</tr>
<tr>
<td>05/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>2.4 ± 0.4</td>
<td>280 ± 307</td>
<td>2552 ± 1669</td>
</tr>
<tr>
<td>05/08/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>3.0 ± 0.5</td>
<td>149 ± 118</td>
<td>5200 ± 2180</td>
</tr>
<tr>
<td>05/08/2018</td>
<td>M1</td>
<td>FILM</td>
<td>1.3 ± 0.6</td>
<td>85 ± 49</td>
<td>10594 ± 9275</td>
</tr>
<tr>
<td>05/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>1.1 ± 0.2</td>
<td>375 ± 187</td>
<td>2842 ± 3809</td>
</tr>
<tr>
<td>05/08/2018</td>
<td>M3</td>
<td>FRAGMENT</td>
<td>2.3 ± 0.8</td>
<td>156 ± 96</td>
<td>969 ± 1150</td>
</tr>
<tr>
<td>06/08/2018</td>
<td>M1</td>
<td>FILM</td>
<td>1.5 ± 0.5</td>
<td>166 ± 176</td>
<td>937 ± 1021</td>
</tr>
<tr>
<td>06/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>0.8 ± 0.3</td>
<td>57 ± 57</td>
<td>5523 ± 6175</td>
</tr>
<tr>
<td>06/08/2018</td>
<td>M3</td>
<td>FILM</td>
<td>1.0 ± 0.8</td>
<td>113 ± 234</td>
<td>6492 ± 7713</td>
</tr>
<tr>
<td>07/08/2018</td>
<td>M1</td>
<td>FILM</td>
<td>0.3 ± 0.3</td>
<td>4 ± 8</td>
<td>517 ± 596</td>
</tr>
<tr>
<td>07/08/2018</td>
<td>M2</td>
<td>FRAGMENT</td>
<td>3.1 ± 0.4</td>
<td>368 ± 90</td>
<td>3133 ± 2431</td>
</tr>
<tr>
<td>07/08/2018</td>
<td>M3</td>
<td>FILAMENT</td>
<td>1.3 ± 0.4</td>
<td>513 ± 318</td>
<td>549 ± 512</td>
</tr>
<tr>
<td>07/08/2018</td>
<td>M4</td>
<td>FRAGMENT</td>
<td>1.6 ± 0.6</td>
<td>326 ± 191</td>
<td>10368 ± 11948</td>
</tr>
</tbody>
</table>

No plastic item was completely covered by a biofilm, maybe because when fully covered, MP sink and exit the surface layer of the water column. This hypothesis needs supporting data since density measurements are scarce in the literature and even less data were available on changes in buoyancy when MP are colonized. On the other hand, full biofilm growth may make MP more palatable to grazers which may egest them after removing the biofilm, making them able to float at the surface again. Highest values of colonization were observed.
at stations lying outside of identified mesoscale turbulence, in particular stations 19 and 22 (TMAW) and lowest inside the mesoscale turbulence zone (AS) (Table 4.4.6.2). This suggests that stable conditions were offering more favorable habitat in terms of surface to colonize, hydrodynamic conditions for the biofilm to grow, and possibly represented a hot spot for light and nutrients, especially, found in lower concentrations in the surrounding environment as reported above (Sioukou-Fangou et al., 2010; Reisser et al., 2014).

Table 4.4.6.2. Coverage index, diatom and prokaryotic densities (ind mm$^{-2}$) in the different areas sampled. In Gibraltar (G) no MPs were found. Data are means ± Standard Error.

<table>
<thead>
<tr>
<th></th>
<th>Coverage (%)</th>
<th>Diatoms (ind mm$^{-2}$)</th>
<th>Prokaryotes (ind mm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NACW</td>
<td>32.5 ± 10</td>
<td>106 ± 147</td>
<td>2801 ± 2262</td>
</tr>
<tr>
<td>G</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AS</td>
<td>20 ± 7.5</td>
<td>72 ± 58</td>
<td>3688 ± 2063</td>
</tr>
<tr>
<td>BMAW</td>
<td>25 ± 7.5</td>
<td>106 ± 71</td>
<td>4369 ± 2919</td>
</tr>
<tr>
<td>TMAW</td>
<td>35 ± 12.5</td>
<td>203 ± 78</td>
<td>4541 ± 747</td>
</tr>
</tbody>
</table>

In order to identify possible relevant factors on biofilm amount (coverage index) or composition (total prokaryotic and diatom densities) an ANOVA test was performed on a Bray-Curtis dissimilarity matrix and only Salinity, Silicates and 19’BF (proxy of nanoflagellates (Prymnesiophytes and Chrysophytes, Blain et al., 2001) were significant (Table 4.4.6.3).
Table 4.4.6.3. p values of ANOVA test run. * indicates significant p values (< 0.1). 19-hexanoyloxyfucoxanthin (19 HF) and 19-butanoxyloxyfucoxanthin (19 BF).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO$_2$</td>
<td>0.020 *</td>
</tr>
<tr>
<td>S</td>
<td>0.023 *</td>
</tr>
<tr>
<td>19’BF</td>
<td>0.067 *</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.102</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.106</td>
</tr>
<tr>
<td>PO$_4$</td>
<td>0.12</td>
</tr>
<tr>
<td>Divinyl chla</td>
<td>0.128</td>
</tr>
<tr>
<td>Diadinoxantin</td>
<td>0.451</td>
</tr>
<tr>
<td>Zeaxantin</td>
<td>0.463</td>
</tr>
<tr>
<td>Peridinidine</td>
<td>0.487</td>
</tr>
<tr>
<td>βcarotene</td>
<td>0.541</td>
</tr>
<tr>
<td>NH$_4$</td>
<td>0.574</td>
</tr>
<tr>
<td>NO$_3$</td>
<td>0.617</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>0.707</td>
</tr>
<tr>
<td>Fucoxantin</td>
<td>0.748</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.844</td>
</tr>
<tr>
<td>19’HF</td>
<td>0.968</td>
</tr>
</tbody>
</table>

Based on these results, these three parameters were used as explanatory variables for a Redundancy analysis (RDA), while the Coverage index, prokaryotes and diatoms per mm$^2$ were used as response variables (Figure 4.4.6.2). The RDA indicated a significant (p=0.032, $r^2$=0.10) direct correlation between prokaryotic density and salinity, which confirms previous results by Oberbeckmann et al., (2019), which reported that salinity shapes different 156
prokaryotic communities: OTUs belonging to microplastics-attached communities found in similar conditions of salinity seemed to cluster together, highlighting that this parameter plays an important role in the community composition. Here, we support this hypothesis with abundance data from our counts. Another significant direct correlation was observed between diatom abundance and silicates from which diatom depend for their frustule formation. A negative correlation was instead, found between 19’BF, prokaryotic density and coverage index (Figure 4.4.6.2).

Figure 4.4.6.2. Redundancy Analysis (RDA) plot obtained using 19.BF, S and SiO$_2$ as explanatory variables and Coverage, Bacteria (=Prokaryotes) and Diatom densities as response variables.
In agreement with this last analysis, MPs found in ENACWu and TMAW, characterized by highest salinity and silicates, showed a higher coverage index, than MPs from other areas (32.5 ±10% SD and 35±12.5 % SD, respectively) (Table 4.4.6.2). In particular, TMAW samples showed highest density of diatoms, 203 ± 78 ind mm⁻². Alboran Sea showed the lowest abundances for both values (20 ± 7.5 % coverage index; 72 ± 58 diatoms mm⁻²), as related to low salinity and silicate values for the area. As for prokaryotic densities, these were higher in both Mediterranean water masses, especially in TMAW where found highest densities were found, 4541 ± 747 SD ind mm⁻². Similar prokaryotic density was observed in BMAW (4369 ± 2919), while lower ones were found in the AS (3688 ± 2063) and ENACWu (2801 ± 2262SD ind mm⁻², Table 4.4.6.2). Prokaryotes and diatoms were the most abundant organisms observed attached on MPs. The prokaryotic morphotypes could be classified into 9 categories: bacilli <2 μm, bacilli >3 μm, cocci <1 μm, cocci >1 μm, coccobacilli <2 μm, coccobacilli >2 μm, stalked prokaryotes, cocci chains and folded-structured prokaryotes (Figure 4.4.6.3). From SEM microphotographs we found that bacilli <2 μm, cocci <1 μm and coccobacilli <2 μm were the most abundant prokaryotic morphotypes, observed respectively on 96%, 90% and 87% of the MPs. Other prokaryotic morphotypes were >1 μm cocci (6% of microplastics), folded-structured prokaryotes (2%), stalked coccobacilli (2%), folded-structured prokaryotes (4%), coccobacilli >2 μm (4%) and >bacilli 3 μm (4%). All MP samples were dominated by <2 μm prokaryotic morphotypes (bacilli, coccobacilli and cocci), accounting overall for 1747 ± 1920, 1022 ± 1373 and 945 ± 1456 total prokaryotic densities (ind mm⁻²). All the other prokaryotic morphotypes accounted on average for < 15 ind mm⁻² (Table 4.4.6.4).
Figure 4.4.6.3. Plastic-attached prokaryotic morphotypes A) cocci chains; B) stalked-coccobacilli; C) cocci <1 µm (blue arrow), cocci >1 µm (red arrow); D) coccobacilli <2 µm; E) bacilli>3 µm; F) coccobacilli > 2 µm; G) folded-structured prokaryotes (blue arrow); H) bacilli <2 µm. Scale bar is 2 µm.

Table 4.4.6.4. Most abundant microbial plastisphere microbial morphotypes. Densities (individual per mm²) and Frequency of observation (FO%, percentage of total samples (52)).

<table>
<thead>
<tr>
<th></th>
<th>Bacilli &lt;2 µm</th>
<th>Cocci &lt;1 µm</th>
<th>Coccobacilli &lt;2 µm</th>
<th>Folded-structured prokaryotes</th>
<th>Cocci &gt;1 µm</th>
<th>Stalked-coccobacilli</th>
<th>Cocci chains</th>
<th>Coccobacilli &gt;2 µm</th>
<th>Bacilli &gt;3 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>density</td>
<td>1747 ± 1920</td>
<td>945 ± 1486</td>
<td>1022 ± 1373</td>
<td>1 ± 9</td>
<td>7 ± 31</td>
<td>2 ± 10</td>
<td>2 ± 49</td>
<td>7 ± 49</td>
<td>15 ± 103</td>
</tr>
<tr>
<td>FO%</td>
<td>96</td>
<td>90</td>
<td>87</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

In general, distribution of prokaryotic types was very similar in the different areas. The most frequent morphotypes, prokaryotes <2 µm, were also the more abundant (Table 4.4.6.4). These prokaryotes dominated four out of five water masses identified, as in Gibraltar no MPs were found (Figure 4.4.6.4). In the ENACWu and TMAW 6 and 7 out of the 9 morphotypes were observed. The other two areas showed 4 prokaryotic morphotypes (AS) and 5 (BMAW). In particular, <2 µm bacilli showed a decreasing trend on MP from the ENACWu to the TMAW, reaching the lowest in the TMAW. In the AS, cocci <1 µm and coccobacilli <2 µm showed the lowest and the highest percentages, respectively. Among the less represented prokaryotic groups, stalked coccobacilli, coccobacilli >2 µm and bacilli >3 µm were the ones most commonly observed. The last two prokaryotic groups were larger in size than the usual prokaryotic groups found (<2 µm), which might suggest a trophically
more favourable environment to grow (both were found at Mediterranean stations). The presence of stalked coccobacilli could suggest a high-energy environment needing stronger attachment to MP surface.

Figure 4.4.6.4. Percentage contribution of the different prokaryotic morphotypes identified. No MPs were found in G.

As for diatoms, 16 morphotypes were observed (Figure 4.4.6.5): (A) *Nitzschia longissima* (B) *Nitzschia sigmoidea* group, (C) *Synedra*, (D) Rectangular-shaped 10-20 µm, (E) *Achnantes* sp. >20 µm, (F) *Mastogloia*, (G) Rectangular-shaped >20 µm, (H) *Diploneis*, (I) Unknown naviculoids, (J) Unknown centrics, (K) *Achnantes* sp. <20 µm, (L) Rectangular-shaped <10 µm, (M) *Navicula* >20 µm, (N) *Navicula* <20 µm, (O) general pennates and (P) *Amphora*. The most common morphotype, *Navicula* <20 µm, was present in 45 out of 52 samples (87% of the all samples), followed by *Navicula* >20 µm (35%), *Amphora* (33%) and rectangular-shaped diatoms <10 µm in size, present in 29% of the samples (Figure 4.4.6.5). Less common diatoms were *Mastogloia* and *N. longissima* (10%), *Diploneis* and rectangular-shaped 10-20 µms (8%), *N. sigmoidea* group and *Synedra* (6%), unknown pennates and rectangular-shaped > 20 µm (4%). Finally, the least abundant were *Achnantes*, unknown centrics, unknown naviculoids, accounting for 2% of the frequency. Abundance values (diatoms mm\(^{-2}\)) showed dominance of *Navicula* <20 µm (100 ± 172 ind mm\(^{-2}\)), one order of magnitude more than any other diatom group classified. Other groups relevant were
rectangular-shaped <10 µm (11 ± 49 ind mm⁻²), *Amphora* (6 ± 14) and *Navicula* >20 µm (5 ± 10). All the other groups showed less than 5 ind mm⁻² of MPs (Table 4.4.6.5).

Figure 4.4.6.5. Examples of MP-attached diatoms as grouped into the 16 categories: A) *Nitzschia longissima*; B) *Nitzschia sigmoidea* group C) *Synedra* sp. D) Rectangular-shaped 10-20 µm E) Achnantes sp. >20 µm F) *Mastogloia* sp. G) Rectangular-shaped >20 µm H) *Diploneis* I) Unknown naviculoids J) Unknown centrics K) *Achnantes* sp. <20 µm L) Rectangular-shaped <10 µm M) *Navicula* <20 µm N) *Navicula* >20 µm O) general pennates (mixed) P) *Amphora*. Scale bar is 10 µm.
Table 4.4.6.5. Abundance (ind per mm$^2$) and Frequency of Observation (FO%) for the different diatom morphotypes found.

<table>
<thead>
<tr>
<th></th>
<th>Density</th>
<th>FO%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Navicula &lt;20 µm</td>
<td>100 ± 172</td>
<td>87</td>
</tr>
<tr>
<td>Rectangular-shaped &lt;10 µm</td>
<td>11 ± 49</td>
<td>29</td>
</tr>
<tr>
<td>General Pennate</td>
<td>1 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>Rectangular-shaped 10-20 µm</td>
<td>2 ± 9</td>
<td>8</td>
</tr>
<tr>
<td>Navicula &gt;20 µm</td>
<td>5 ± 10</td>
<td>35</td>
</tr>
<tr>
<td>Amphora</td>
<td>6 ± 14</td>
<td>33</td>
</tr>
<tr>
<td>Nitzchia group sigmoidae 25-30 µm</td>
<td>1 ± 2</td>
<td>6</td>
</tr>
<tr>
<td>Rectangular-shaped &gt;20 µm</td>
<td>2 ± 11</td>
<td>4</td>
</tr>
<tr>
<td>Diploneis</td>
<td>1 ± 3</td>
<td>8</td>
</tr>
<tr>
<td>N. longissima</td>
<td>1 ± 4</td>
<td>10</td>
</tr>
<tr>
<td>Centric diatoms</td>
<td>0 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>Unknown naviculoids 10 µm</td>
<td>0 ± 0</td>
<td>2</td>
</tr>
<tr>
<td>Achnantes &gt;20 µm</td>
<td>1 ± 4</td>
<td>2</td>
</tr>
<tr>
<td>Achnantes &lt;20 µm</td>
<td>0 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>Mastogloia about 20 µm</td>
<td>2 ± 8</td>
<td>10</td>
</tr>
<tr>
<td>Synedra-like</td>
<td>3 ± 17</td>
<td>6</td>
</tr>
</tbody>
</table>

Five groups were present at 19 out of 21 stations, representing more than 80% of all diatoms: Navicula (<20 and >20 µm), Rectangular-shaped <10 µm, Amphora and N. longissima. At the remaining 2 stations (2 and 5) unknown pennates and rectangular-shaped >10 µm accounted for 80% of total diatoms. Navicula <20 µm was the only diatom group always present at all the stations sampled, contributing 65% on average and were more abundant in Mediterranean samples and more specifically at stations 15 and 18, where they contributed 95 and 97% respectively. Amphora contributed on average 10% of the total diatoms found on MP and were slightly more abundant in Mediterranean samples where showed their peak at station 17 (49%), and were completely absent from the first three stations of the NACW. Navicula >20 µm contributed on average 8%, and was mostly present at the NACW stations, especially at station 4 where showed its highest contribution (50%). Rectangular-shaped
diatoms <10 µm contributed on average 7%, and were more abundant in ENACWu, with the highest contribution at station 1 (50%). Finally, *N. longissima* showed an average contribution of 3%, was very commonly found in the Mediterranean but it showed its peak at station 9 (Figure 4.4.6.6). In the ENACWu, the dominant diatom was the rectangular-shaped diatoms <10 µm (18.1%), and, together with rectangular-shaped >20 µm diatom group, accounted for 23% of the total diatom contribution (Figure 4.4.6.7). *Navicula* <20 µm found its lowest peak in this area (64% of total diatoms). In the AS only five morphotypes were observed, with *Diploneis* accounting for 0.8% of total diatoms. It is noteworthy that *Amphora* showed its highest contribution (18%) here, where the lowest density of diatoms and prokaryotes were observed, confirming that this genus grow well on MPs (Reisser et al., 2013; Dudek et al., 2020). In the BMAW *Navicula* <20 µm showed its highest (85%) contribution and *Navicula* >20 µm the lowest (1%), and *N. longissima* showed the highest contribution (2.9%). Finally, in the TMAW the highest number of diatom groups were observed (12), with a peak of *Navicula* >20 µm (7.3%) and the lowest contribution of *Amphora* (%) and the complete absence of rectangular-shaped <10 µm. *Mastogloia* and *Synedra* together contributed for 8.3% of total diatoms.

Figure 4.4.6.6 Percent contribution of the different diatom groups identified. Numbers under each column are manta trawls.
Figure 4.4.6.7. Average percentage contribution of the different diatom groups in the different areas sampled. No MPs were found in Gibraltar area, so it is not showed.

In order to explore spatial distribution of the morphotypes of prokaryotes and diatoms attached to MPs, an NMDS plot was generated using Kulczynski distances (figure 4.4.6.8). The plot shows a scattering of points, indicating that no spatial segregation was present in the samples, therefore no such spatial variability in terms of morphotypes density was found at the different areas classified. This means that somehow the microbial community living on the different MPs collected were colonized by similar communities all around, highlighting no such biogeography discriminant in terms of community structure in terms of SEM data. As shown above, the discriminant in this subschapter would be the environmental conditions (e. g. silicates and salinity in this case) which in this case would drive the density of the microbial community attached to MPs.
Figure 4.4.6.8. Multidimensional scaling representation of the Kulczynski distances. Data from different areas sampled are color-coded: BMAW (Balearic Modified Atlantic Water) = red; A-S FR (Alboran sea front) = green; NACW (North Atlantic Central Water) = blue; TMAW (Thyrrenean Modified Atlantic Water) = purple. Stress = 0.19. Gibraltar is absent because no MPs were found in this area.
4.4.7 Discussion

Spatial variability has been found in previous studies to be one of the discriminants linked to shape the plastisphere communities (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2014; Amaral-Zettler et al., 2021). Together with the latter, also environmental conditions seemed to be relevant in this cause, especially nutrients and salinity (Oberbeckmann et al., 2014; 2016; Amaral-Zettler et al., 2021). As mentioned above, the relevance of the continuous monitoring of microplastic research has been reported in few reports (Ruiz-Orejon et al., 2019; Compa et al., 2020), while no studies have assessed this parameter in terms of plastisphere research. Lifestyle was found to be another relevant discriminant once comparing the communities living attached to substrates and freely living were based on different members (Zettler et al., 2013; Dussud et al., 2018; Erni-Cassola et al., 2019). Less confirmation has been found comparing different attached communities of different substrate (as glass, wood, faecal pellets, etc. Oberbeckmann et al., 2016; Dussud et al., 2018). In this subchapter, it has been highlighted the relevance of all the mentioned parameters influencing the plastisphere adding up another, which in turn include all of the ones cited, the importance of continuous study for plastisphere, especially for coastal areas. The data presented highlight the importance of repeated sampling to assess MP pollution and its attached community in coastal areas because of complex circulation and multiple terrestrial discharges, especially because in the literature is very uncommon to find some kind of long-term assessments, especially from environmental random MPs retrieved from manta nets. This supports the observation that only one sampling is not enough to assess such relevant and unfortunately actual threat, especially in complex and varied coastal area. Variability in the Gulf of Napoli and similar coastal areas is very high, both in terms of total concentrations and community composition of attached microbes. The plastisphere community data presented here underlined again the importance of temporal variability and environmental parameters shaping the microbial community, as reported by other studies (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2020). From the data presented in this chapter it appears that no significant spatial variability stands - but rather temporal, as found in the previous chapter- in terms of prokaryotic community structure of the plastisphere.

Spatial variability has been found rather related to quantitative assessments of the members of the plastisphere. Indeed, different density have been found related to different areas studied. Even though, not so many works have assessed the microbial density on MPs. It
was noticed that prokaryotic and diatom morphotypes were similar to the ones found in other works (Carson et al., 2013; Reisser et al., 2014; Dudek et al., 2020; Amaral-Zettler et al., 2021). Comparison with other works of the microbial density.

In this chapter it has been shown how microbial density attached to MPs may change as changing sample sites as in the latters it may be reported different environmental conditions. In general, environmental factors (in the case of the Pelagia cruise case salinity and silicates) appear to shape the biofilm in quantity and quality, even though their relative importance changes with the season and the region, confirming previous observations as bacilli and cocci while diatoms were dominated by pennates of benthic origin. Commonly found were *Navicula, Amphora, Nitzschia* (Oberbeckmann et al., 2019; Amaral-Zettler et al., 2020). Highest values of colonization were observed at stations lying outside of identified mesoscale turbulence, in particular stations 19 and 22 (TMAW) and lowest inside the mesoscale turbulence zone (AS). This suggests that stable conditions were offering more favorable habitat in terms of surface to colonize, hydrodynamic conditions for the biofilm to grow, and possibly represented a hot spot for light and nutrients, especially, found in lower concentrations in the surrounding environment as reported above (Reisser et al., 2014).

MP-associated prokaryotes were taxonomically more similar to each other than to the other communities. In particular, they showed high abundance of Proteobacteria and Bacteroidetes families like Rhodobacteraceae, Flavobacteriaceae, known components of the plastisphere (Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015; 2021), especially Flavobacteriaceae which has been described as keystone taxa in biofilms that feed off exudates and some have the ability to prey on diatoms via lysis (Amin et al., 2012). Of course, also other families have been reported to be shared in this chapter, like Vibrionaceae, Saprospiraceae, Hyphomonadaceae, Ruminococcaceae, ecc. found in numerous other works.

Difference in lifestyle have been found. During this chapter there were proofs of difference between attached and free-living communities, numerously confirmed in other works. Despite the limited size of the dataset, the results presented in this subchapter point to the existence of a difference between MP-associated community and PA (particle-attached) community. In a very infant branch of research, the ones studying the plastisphere in different environments like riverine and marine, PA-associated community appear to reflect the free-living community from which they are recruited, while the MP-associated one appeared to be different from the two cited communities. This may suggest a preference for attachment independent from the substrate, at least in this particular case. Difference among riverine and marine MP-associated communities were found, which could help us
understand also where pathogens and toxic microbes can “hitchhike” on MPs, as reported in the literature (Frere et al., 2018).

Regarding to alpha diversity, results in disagreement have been found among Sarno river area and Cilento bay. From one hand, plastisphere community seemed more abundant and diverse with in turn the free-living community being more compact and selected. The viceversa happened for Sarno area. This has been found also often in this branch of research, there is no agreement about it (Oberbeckmann et al., 2019; Amaral-Zettler et al., 2021).

To conclude, all of the different stations studied in the same area, showed % of OTUs shared among each other very low among MP-attached communities. This highlights also a difference among plastisphere communities within the same area, forming an inter-spatial variability. This, together with the results of the previous chapter, the temporal variability, shed light on an even bigger difference among plastisphere communities of the same and different areas, underlining the recruiting of this community to be related to all these parameters cited, together.
CHAPTER 5.

The relevance of “core plastisphere”
As infant as plastisphere research is, a relevant effort is being addressed at the different discriminants in order to understand community structure, with biogeography and polymer-specificity leading the options (Oberbeckmann et al., 2014; 2019; Amaral-Zettler et al., 2015; 2021). These are usually proven to be right for high taxonomic levels e.g. phyla, but going deeper into the taxonomic classification level, some bacterial families and genera are reported to be consistently common as obligate-MP related. In particular, Rhodobacteraceae, Flavobacteriaceae, Vibrionaceae have been often reported attached to MPs in many different sites (Dussud et al., 2018; Dudek et al., 2020; Murano et al., 2021). Genera known for their ability to degrade carbon long chains and/or pathogenicity (e.g. Vibrio, Pseudomonas, Alcanivorax (Zadjelovic et al., 2019). These genera are found to be similar at different time, spatial and polymer-wise scales, suggesting they belong what is called a “core microbiome”, a strict group of prokaryotes which would prefer to be attached to such inorganic particles, MPs in this case. The latter strict group of prokaryotes has been found to be based not on the abundant and common taxa but rather from the rare ones (Kirstein et al., 2019). These findings have been found especially during incubation experiments, but this chapter reports similar results from the field. Apart from diatoms, already discussed in the previous chapters, the plastisphere “core” prokaryotic communities appear to be represented by a reduced number of prokaryotic taxa which thanks to the sequencing analysis can be analysed in the following subchapter in detail.
5.1 Core Plastisphere in the Gulf of Napoli

For parallel characterization of the plastisphere in the Gulf of Napoli, 75 MPs pieces were retrieved. The Gulf of Napoli showed in their 5 samplings carried out that rather a temporal core microbiome, there was a rather spatial one. Indeed, as for the Venn diagrams showing the shared and exclusive OTUs for the different years assessed, no OTUs were shared between the years, and the highest percentages of the Venn diagrams were all related to exclusive OTUs. The only shared OTUs higher than 0 (0.9%) were among the years 2019 and 2020 (Figure 5.1.1A). Similar results for PE are showed in Figure 5.1.1 B, with only 6 OTUs shared between 2019 and 2020.
Figure 5.1.1. Venn diagrams showing shared and exclusive OTUs among different polymer associated communities (A) and PE (B) at the different sampling dates. Numbers shown are actual OTUs and percentage of dataset contribution.

On the other hand, 221 OTUs (Figure 5.1.2) shared among all the samples found in the Gulf belonged to 54 taxa (Table 5.1.1). Apart from typical taxa found already in previous studies attached to MPs like genera belonging to families like Flavobacteriaceae, Saprospiraceae, Rhodobacteraceae etc, some of the genera shared among all the stations like *Pseudoalteromonas*, *Oleiphilus* and *Acinetobacter* are known plastic degraders or degraders of long chain organic carbons. Genera like *Vibrio*, some of which can be pathogenic, were
also noted, though these are commonly found in the marine environment as both attached and free-living, and most are not pathogenic (Wright et al., 2021).

Figure 5.1.2. Venn diagram showing shared and exclusive OTUs among different stations monitored throughout the years. Portici is st.1; MC is st.2; Gaiola is st.3.

Table 5.1.1. Two-hundred and twenty-two shared OTUs belonged to 54 taxa among the different samples of attached microbial communities in different stations throughout the years monitored

<p>| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Paludibacteraceae;D_5__uncultured;D_6__uncultured bacterium |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__U29-B03;D_6__uncultured bacterium |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Tannerellaceae;D_5__Macellibacteroides |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__Saprspiraceae;D_5__Lewinella;D_6__uncultured organism |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__uncultured;D_5__uncultured organism;D_6__uncultured organism |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhdna;D_6__Ekhdna lutea |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Crocinitomicaceae;D_5__Fluviicola |</p>
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<tr>
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<tr>
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<td><strong>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae;D_5__Acidovorax</strong></td>
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<td><strong>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Rhodocyclaceae;D_5__Thauera</strong></td>
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<td><strong>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirales;D_4__Saccharosporillaceae;D_5__Oleispira</strong></td>
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<td><strong>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</strong></td>
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5.2 Core Plastisphere in the Bay of Pozzuoli

Very similar trend was found for the Bay of Pozzuoli. Indeed, in the total of 46 samples collected at the Bay of Pozzuoli, no shared OTUs were found among all the time points, even between the two July samples of 2018 and 2019 (Figure 5.2.1).

![Venn diagram showing shared and exclusive OTUs among different samplings for the MP-attached prokaryotes. Numbers shown are actual OTUs and percentages of total. ABB18_2 is July 2018; ABB19_1 is February 2019; ABB19_4 is July 2019 and ABB19_5 is October 2019.](image)

On the other hand, 28 OTUs were found to be shared among the different stations throughout the year (Figure 5.2.2). Eighteen belonging to Proteobacteria, 5 to Bacteroidetes, 2 to Firmicutes, 1 to Actinobacteria, Planctomycetes and Verrucomicrobia. The majority of Proteobacteria belonged to Gammaproteobacteria, more in particular to genus *Vibrio* (Table 5.2.1). The latter could not be more in detail for the species and, in turn, for the pathogenicity. All the other genera have been already found in plastisphere work both because genus known to carry pathogenic species like *Alteromonas*, *Cutibacterium*, *Staphylococcus*, this one as we did not run any further counter-analysis we cannot be sure of contamination, *Acinetobacter* and *Lewinella* (Table 5.2.1; Kirstein et al., 2019; Oberbeckmann et al., 2016; Dudek et al., 2020; Scales et al., 2021).
Figure 5.2.2. Venn diagram showing shared and exclusive OTUs among different stations monitored throughout the years.
Table 5.2.1. Twenty-eight shared OTUs among the different samples of attached microbial communities in different stations throughout the years monitored.

<table>
<thead>
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<th>OTUs</th>
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<tbody>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Propionibacterales;D_4__Propionibacteriaceae;D_5__Cutibacterium;D_6__uncultured bacterium</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__Sa prospiraceae;D_5__uncultured</td>
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<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__Sa prospiraceae;D_5__uncultured</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Propionibacterales;D_4__Propionibacteriaceae;D_5__Cutibacterium;D_6__uncultured bacterium</td>
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<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Vibrio</td>
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<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonad ales;D_4__Moraxellaceae;D_5__Acinetobacter</td>
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5.3. Core Plastisphere in the Cilento Area

As for the Cilento Bay, free and attached communities were tested in order to check for peculiar taxa which mostly characterize their community with LefSe statistical test. Thirteen samples were sequenced for DNA: 5 from microplastics and 8 from seawater (free prokaryotes). Biomarkers related to free community belonged to NS9 group (Bacteroidetes) and SAR11 clade (Proteobacteria). Among others, biomarkers related to MP community belonged to *Clostridia* (Firmicutes), *Pleurocapsa* and Syneccochales (Cyanobacteria), *Blastocatella* (Acidobacteria), Propriionilbacteriaceae (Actinobacteria), Chitinophagales and Amoebophilaceae (Bacteroidetes), Phycispharaceae and Pirellulales (Planctomycetes), *Marivita*, Sphingomonadaceae and Sphingomonadaceae (Proteobacteria) (Figure 5.3.1).

In order to find biomarkers specific for polymer communities, LefSe test was carried out among PE, PP and free communities. This test is showed here because was the only area where it showed significant results in terms of peculiar MP-attached taxa. Significant biomarkers were found only for PE community: one belonging to *Pleurocapsa* genus (Cyanobacteria) and one related to *Marivita* genus (Proteobacteria) (Table 5.3.1).
Figure 5.3.1. LefSe results shown as a dendrogram. Green taxa are biomarkers of the attached (MP) communities; Red taxa are biomarkers for the free (FREE) prokaryotes.

Table 5.3.1. LefSe results showing different values tested for PE, PP and free communities. Linear Discriminant Analysis score (LDA), Wilcoxon sum and Kruskal-Wallis score tests are shown.

<table>
<thead>
<tr>
<th></th>
<th>LDA</th>
<th>Polymer</th>
<th>Wilcoxon sum</th>
<th>Kruskal-Wallis</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_5__PleurocapsaPCC_7319</td>
<td>4.963434</td>
<td>PE</td>
<td>4.697626</td>
<td>0.003805</td>
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<tr>
<td>D_6__unculturedbacterium</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D_5__Marivita</td>
<td>3.931385</td>
<td>PE</td>
<td>4.687105</td>
<td>0.010404</td>
</tr>
<tr>
<td>D_6__unculturedbacterium</td>
<td></td>
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Both these genera have been associated with microplastics in previous studies: *Pleurocapsa* on PP (Zettler et al., 2013; Dussud et al., 2018), *Marivita* on PVC (Wang et al., 2020) and PET (Lu et al., 2019). No OTUs resulted to be specific for PE as compared to PP, but 6 OTUs were found both in free and attached communities (Table 5.3.2).
Table 5.3.2. Taxa found to be shared among free and attached communities.

<table>
<thead>
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<th>Taxa</th>
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<tr>
<td>D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Propionibacteriales;D_4__Propionibacteriaceae;D_5__Cutibacterium;D_6__Cutibacterium acnes</td>
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<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae1</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae2</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__NS4 marine group</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Staphylococcaceae;D_5__Staphylococcus</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria</td>
</tr>
</tbody>
</table>

One-hundred and four OTUs were shared among the two groups (PP and PE-attached, Figure 5.3.2), belonging to 37 taxa. Most of these taxa belonged to families like Rhodobacteraceae, Sphingomonadaceae, Saprospiraceae, Flavobacteriaceae and Rhizobiaceae and also order like Bacilli. Among these taxa there were also genera reported to drive antibiotic resistance like *Brevundimonas* (Laganà et al., 2018), utilize polycyclic aromatic hydrocarbons (PAH), like *Erythrobacter* (Oberbeckmann et al., 2017).
As for potential pathogens, *Staphylococcus* unclassified was the only genus contributing 1% of the sequences and it was found in both free and attached prokaryotes. As no further checking analyses was performed, it could not be told if contamination happened. *Vibrio* spp. never contributed even 0.1%, and also was found both in free and attached prokaryotes.

### 5.4 Core Plastisphere of the Sarno river area

Regarding the Sarno river case study, only 8 samples provided positive results for 16S amplicon sequencing (Table 5.4.1), 5 from MPs (both marine and riverine), 2 for free community (from SS and SD stations) and 1 for “particle attached” (PA), from SD station. In order to check if specific taxa could be considered as biomarker of one or the other community, a LefSe test was carried out (Segata et al., 2011). 718 OTUs were found to be specific of the free lifestyle (LDA score between 8.20 and 5.10, data not shown) and 105 for PA (LDA score ranging between 3.79 and 5.62, data not shown), suggesting a separation between the two. Instead, no OTUs clearly marked the MP-attached prokaryotes, suggesting that these are represented by opportunistic generalists.
Based on the observation that a difference was observed between prokaryotes with different lifestyles and considered that the difference between free and attached prokaryotes is already reported in the literature (see Introduction), we compared PA with MP communities, with the aim of elucidating the possible role of different substrates in the prokaryotic attachment process. Here it is considered that the particles investigated are a mix of inert and organic substrate, while MP were clearly identified as plastics. Interestingly, these two did not share any OTU (Figure 5.4.1), but the two MP communities, from the river and the sea shared 26 OTUs. This suggests that plastics represent a distinguished substrate to attach to, and possibly the 26 OTUs could be considered as a core of taxa of MP-attached prokaryotes, independently from the environment examined. The 26 shared OTUs belonged to 4% of the total prokaryotic sequences, highlighting the relevance of the so-called “rare biosphere” within the microbial plastisphere (Kirstein et al., 2019). This core was represented by 13 Proteobacteria, 8 Bacteroidetes, 4 Planctomycetes and 1 Firmicutes (Table 5.4.1). As a matter of fact, some of these genera have been reported as MP-associated, such as Oleiphilus, Prevotella and Lutibacter, and more generally families Rhodobacteraceae, Saprospiraceae and Cyclobacteriaceae (Oberbeckmann et al., 2019; Kesy et al., 2019; Wright et al., 2020; Amaral-Zettler et al., 2021). This again following the suggestion mentioned in Amaral-Zettler et al., (2021), where more emphasis should be pointed out in investigate on how plastisphere community changes moving from rivers to the sea. From these data we can say that the “core” plastisphere community moving from rivers to the sea is selective and only 26 OTUs could survive to both environments (around 4% of the whole community), highlighting the relevance of the “rare biosphere” in such communities attached (Kirstein et al., 2019).
Figure 5.4.1. Shared and exclusive OTUs between attached and free prokaryotes as related to their environments. RIV_MP is riverine MP-attached; RIV_FREE is free living riverine communities; SEA_FREE is free living marine communities; SEA_MP is marine MP-attached and RIV_PA is riverine particle-attached.

Table 5.4.1. Twenty-six shared OTUs belonged between the different samples of MP-attached microbial communities in different environments: riverine and marine.

| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 9 | D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 9;D_6__uncultured bacterium |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;D_6__uncultured bacterium |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__SB-5;D_5__uncultured organism;D_6__uncultured organism |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__Saprospiraceae;D_5__uncultured;D_6__uncultured bacterium |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Lutibacter |
| D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__NS9 marine group;D_5__uncultured bacterium;D_6__uncultured bacterium |
| D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Selenomonas;D_6__uncultured bacterium |
D_0__Bacteria;D_1__Planctomycetes;D_2__Phycisphaerae;D_3__Tepidisphaerales;D_4__WD2101 soil group;D_5__uncultured bacterium;D_6__uncultured bacterium

D_0__Bacteria;D_1__Planctomycetes;D_2__Planctomycetacia;D_3__Pirellulales;D_4__Pirellulaceae;D_5__Pir4 lineage;D_6__uncultured bacterium

D_0__Bacteria;D_1__Planctomycetes;D_2__Planctomycetacia;D_3__Planctomycetales;D_4__Rubinisphaeraceae;D_5__SH-PL14;D_6__uncultured bacterium

D_0__Bacteria;D_1__Planctomycetes;D_2__Planctomycetacia;D_3__Planctomycetales;D_4__Rubinisphaeraceae;D_5__uncultured;D_6__uncultured bacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfobacterales;D_4__Desulfobacteraceae

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae;D_5__Rhodoferax

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Methylphilaceae;D_5__Methylotenera;D_6__uncultured bacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Rhodocyclaceae;D_5__Dechloromonas;D_6__uncultured bacterium

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Oleiphilaceae;D_5__Oleiphilus

D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Arenimonas;D_6__uncultured bacterium
5.5 Core Plastisphere of the North-Adriatic area

Of the isolated MPs items from the North-Adriatic area, 17 were used for DNA sequencing. One-hundred and seventy-five OTUs were found shared among both areas classified previously for the North-Adriatic area studied, accounting for only 7.2% of the whole dataset (Figure 5.5.1). The shared OTUs belonged to 42 taxa (Table 5.5.1); 13 of them were found in other works regarding plastisphere communities. This is the case for the most common Flavobacteriaceae, Rhodobacteraceae, Cryomorphaceae and Moraxellaceae families but also less known like Pseudoalteromonadaceae, Xanthomonadaceae and Cyanobiaceae (Zettler et al., 2013; Bryant et al., 2016; Oberbeckmann et al., 2014; Jiang et al., 2018; Dussud et al., 2018; Debroas et al., 2017; McCormick et al., 2014; Tagg et al., 2019). At the genus level Tenacibaculum, Flavobacterium and Planktomarina were observed, as already reported in other plastisphere studies (Zettler et al., 2013; Oberbeckmann et al., 2014; Tagg et al., 2019). Dokdonia and Sulfitobacter were also observed, confirming previous work in the Adriatic area (Basili et al., 2020). The genus Brevibacillus, known to host species able to degrade PE (Nanda and Sahu, 2010; Mohanrasu et al., 2018).

![Figure 5.5.1. Venn diagram showing shared and exclusive OTUs for areas (NRI=Not-river influenced; RI = River-influenced).](image-url)
Table 5.5.1. Taxa shared among areas (NRI=Not river influenced; RI = river influenced)

<table>
<thead>
<tr>
<th>Taxa</th>
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<tbody>
<tr>
<td>175 OTUs belonging to 42 taxa</td>
</tr>
<tr>
<td><strong>D_0__Bacteria;D_1__Actinobacteria;D_2__Acidimicrobiia;D_3__Actinomarinales;D_4__Actinomarinaceae;D_5__Candidatus Actinomarina;D_6__uncultured bacterium</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Actinobacteria;D_2__Actinomarinales;D_3__Actinomarinaceae;D_4__Propionibacteriales;D_5__Cutibacterium;D_6__uncultured bacterium</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__C hitinophagaceae</strong></td>
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<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__C rocinitomicaceae;D_5__Fluvicola</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__C rymorphaceae;D_5__NS10 marine group</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__C rymorphaceae;D_5__uncultured</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__Dokdonia</strong></td>
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<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__Flavobacterium</strong></td>
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<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__Formosa;D_6__uncultured marine bacterium</strong></td>
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<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__Lacinutrix</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__Maribacter</strong></td>
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<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__NS3a marine group</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__F lavobacteriaceae;D_5__NS5 marine group;D_6__uncultured bacterium</strong></td>
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<tr>
<td><strong>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacterales;D_4__Flavobacteriaceae;D_5__Tenacibaculum</strong></td>
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5.6 Discussion and Conclusions

Despite spatio-temporal variability and environmental conditions appearing to be the main discriminants of plastisphere community composition, a small group of prokaryotic taxa appears to be consistently attached to MPs, as also found by other authors (Kirstein et al., 2019; Wright et al., 2021). From previous works it is known that different genera like *Dokdonia*, *Sulfitobacter*, *Tenacibaculum* are often found attached (Basili et al., 2020; Zettler et al., 2013; Oberbeckmann et al., 2014; Tagg et al., 2019). These taxa, most of the times, belong not to the most abundant taxa, e. g. Proteobacteria, Bacteroidetes, but rather to the “minor contributors”, such as Planctomycetes, Firmicutes, Cyanobacteria, the so-called rare biosphere (Sogin, 2006; Kirstein et al., 2019). These taxa harbor genera known to be able to degrade long chain of inorganic polymers and/or found to be pathogenic to animals and even humans. Some examples are *Pseudoalteromonas*, *Oleiphilus* and *Acinetobacter* which are known plastic degraders or degraders of long chain organic carbons (Wright et al., 2021).

Polymer-specificity results have been found in this thesis, even if with not so robust statistics related to small number of replicates. Indeed, as for the Sarno area, have been found taxa which “preferred” to be attached to inorganic habitats like MPs rather than phycospheres like PA-attached. On the other hand, some other taxa that have been found to be statistically relevant to be attached in one work, could be found in the free-living community. This is the case for generalist kind of prokaryotes which do not prefer one lifestyle of living but can be found in both communities, probably related to which environmental conditions is found (e. g. nutrients and/or salinity as mentioned earlier). Some examples are *Cutibacterium*, uncultured genera of Flavobacteriaceae as found in the Cilento area. In the same area new colonizers never found before were observed, like *Candidatus Actinomarina* and *Cutibacterium* (genera belonging to Actinobacteria), *Formosa*, *Lacinutrix*, NS3a group, NS5 marine group, *Ulhibacter* and *Winogradskyella (Flavobacteriaceae)*, *Staphylococcus* and *Anoxybacillus* (Bacillales), *Brevundimonas*, *Lentibacter*, *Litoreibacter* and SAR11 clade Ia (Alphaproteobacteria) and *Glaciecola*, *Marinobacter*, OM43 Clade and SAR92 clade (Gammaproteobacteria), found in the North-Adriatic Sea. Some of these taxa are also known to harbour pathogenic species or strain. Unfortunately, due to drawbacks related to NGS, is not always possible to confirm their pathogenicity.
Chapter 6. Microbial growth on microbeads in an urchin mesocosm experiment
6.1 Introduction

Microplastics are widespread and persistent in the marine ecosystem and often are mistaken as food by marine organisms and ingested by a range of marine biota which includes corals, phytoplankton, zooplankton, sea urchins, lobsters, fish (Galloway et al., 2017; Chatterjee and Sharma, 2019), entering and being amplified along the trophic food web. The impact of MP on marine biota is an issue of concern as it can be lethal to marine life (Andrady et al., 2011). Litter ingestion has been reported worldwide in over 330 species from different habitats and taxa (Giani et al., 2019). Examples of reported ingestion effects on corals, includes the blockage of the mesenterial tissue which leads to reduction in feeding capability and lowering in energy reserves (Reichert et al., 2017; Tang et al., 2018). In fish ingestion of MP causes histopathological modifications in the intestine, resulting in the increase in number of globet cells and alterations in the structure of serosa (Chatterjee and Sharma, 2019).

Figure 6.1. Biological and chemical interactions of microplastics in the marine environment (from Mammo et al., 2020).

Plastics, in general, and more specifically MPs, can offer colonization surfaces to microorganism and adsorption substrate to several chemicals (De Tender et al., 2015; Rummel et al., 2017; Miao et al., 2019a). Furthermore, the association of MPs with 194
chemicals and microbes could increase their threat to living organisms (Figure 6.1, from Mammo et al., 2020), as discussed in Chapter 1, especially the possible presence of pathogenic “hitchhikers” inside the plastic-associated community. Different types of toxic chemicals have been reported to be adsorbed on MPs, the majority of these are either metals (like copper, cadmium, arsenic, lead and chromium) or persistent organic pollutants (POPs), which include polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), the insecticide dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane isomers (HCHs). Some of the factors which affect adsorption of chemical onto MPs include its type, size, pH and salinity of the environment and plastic aging. Such MPs may be ingested by different marine species and slowly enter the marine food web causing serious challenges to all forms of marine life (Mammo et al., 2020). In a study done on Mediterranean fin whale (Balaenoptera physalus), high concentration of phthalates was detected in these baleen whales, pointing out the severity of pollution in world ocean (Fossi et al., 2012). One of the key species of Mediterranean habitats is the sea urchin Paracentrotus lividus, known in terms of ecological contribution to ecosystem functioning. By its apostatic diet selection and intense grazing activity, sea urchins control the organization, structure and composition of shallow macroalgal assemblages (Boudouresque and Verlaque, 2020). Their feeding behaviour contributes to plastic fragmentation inducing the release of secondary microplastics in the marine environment to other benthic species (Porter et al., 2019; Cau et al., 2020). Despite these key features, up to now, few data are available on the presence of MPs in wild specimens of the sea urchin P. lividus. Only recently, Feng et al. (2020) have investigated the abundance, distribution and characteristics of plastic debris in wild sea urchin (S. intermedius, T. reevesii, T. hardwickii, H. pulcherrimus) collected along the coastal areas of Northern China. They showed MP accumulation in the gut but also in the coelomic fluid and gonads with abundances strictly related to anus size, shell diameter and gonad index (Feng et al., 2020). During this research work, I was given the opportunity to collaborate with Dr. Anna Palumbo and the PhD student Carola Murano on an experiment aimed at assessing the effects of microplastics on the sea urchin Paracentrotus lividus. An existing study (Murano et al 2020) had explored the effects of polystyrene microbeads (MB) on the immunological system of this model species, indicating the uptake of MBs in the digestive tract, water vascular systems, as well as in the gonads of adult P. lividus specimens. As a follow-up study, an experiment was planned to test the effects on sea urchins of MB either virgin (e. g. as provided by the manufacturer), or colonized by a microbial biofilm. The working hypothesis was that the biofilm colonization modulates or amplifies the effect
of the plastic, triggering a differential immunological response in the exposed animals. Therefore, my aim was to characterize the biofilm attached to MBs and to compare the communities among the different treatments. It is to be noted that the seawater used for the initial incubation for biofilm growth was the same used for the incubation with the sea urchins. Although the prokaryotic community might have changed in the meantime, we can hypothesize that the prokaryotes present are the same all along the experiment.

6.2 Experimental procedures

As already described in Chapter II (Material and Methods), 4 x 10^4 MBs L^{-1} were incubated in a glass Erlenmeyer flask for one week in 1 L of unfiltered natural seawater collected from a coastal site in the Gulf of Napoli (40° 49’13.8” N, 14° 18’09.7” E) in a temperature-controlled culture cabinet chamber (Angelantoni, Italy; temperature: 18±1°C; light 100 µmol; LD 12:12 cycle) on an orbital shaker. After one week, 500 ml of seawater were centrifuged with an Allegra 5r centrifuge (Beckman Coulter, CA, USA) at 3500 rpm speed for 10 minutes. The pellet was then collected on a 47 mm filter of 10 µm pore size to retrieve MBs. The supernatant was filtered onto 0.22 µm, 47 mm diameter, mixed cellulose ester (MCE) filters (Millipore, USA) and processed as indicated above for prokaryotic DNA sequencing (sections DNA Extraction, Sequencing and Bioinformatical analyses, mentioned above). Growth of biofilm (or lack of) was verified by Scanning Electron Microscopy (SEM).

6.3 Results and discussion

6.3.1 Morphological characterization of the microbial plastisphere

MBs from the manufacturer’s stock showed a smooth surface and no presence of any organic material attached (Fig 6.2 A and B). MBs incubated in natural seawater showed a consistent growth on their surface of a microbial community, where mostly prokaryotes were recognized (Figure 6.2C, D).
After 48h of incubation in the sea urchin tanks, all MBs were covered by a biofilm, either initially virgin or pre-colonized (Figure 6.3)
Morphologically, the post-incubation MBs, either initially virgin or initially colonized, were similar to each other and many showed geometric shapes resembling the sea urchins madreporite holes (Murano et al., 2020) from which they were possibly egested.

### 6.3.2 16S rRNA sequencing

A total of 81,388 sequences were retrieved, for a total of 2,607 OTUs. As much as 22,902 sequences were retrieved from MB-attached pre-colonized samples taken before the incubation (PRE_BIOF_MB), for a total of 781 OTUs; 22,194 sequences have been retrieved from MB-attached samples taken after the incubation (POST_BIOF_MB), for a total of 858 OTUs; 26,947 sequences have been retrieved from virgin MB taken after the incubation (POST_VIRG_MB), for a total of 886 OTUs. 2,398 sequences have been retrieved from the free prokaryotic samples taken at the end of the experiment from the tank with previously incubated MBs (POST_BIOF_FREE), for a total of 172 OTUs; 6,947 sequences have been retrieved from the free prokaryotic samples taken from the tank with virgin MBs (POST_VIRG_FREE), for a total of 303 OTUs. Total alpha diversity was significantly different between MBs analysed after 1-week incubation in seawater.
(PRE_BIOF_MB), after 48 h in the tanks (POST_BIOF_MB), and the virgin MBs after the incubation with sea urchins (POST_VIRGIN_MB), suggesting that under the experimental conditions, colonization could take place as short as after 48 h, but also that this time was not long enough to induce a convergence towards the same prokaryotic community (Table 6.1).

Table 6.1. Diversity indices (Observed and Shannon) of prokaryotic communities grown on MBs. PRE_BIOF_MB are beads incubated for 1 week in natural seawater under controlled conditions. POST_BIOF_MB are the same beads after 48 h in the tank with the sea urchins. POST_VIRGIN_MB are untreated beads (only diluted in distilled water, vortexed and diluted in the sea urchin tanks), after 48h incubation.

<table>
<thead>
<tr>
<th></th>
<th>Observed</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE_BIOF_MB</td>
<td>411.00</td>
<td>5.21</td>
</tr>
<tr>
<td>POST_BIOF_MB</td>
<td>493.00</td>
<td>5.69</td>
</tr>
<tr>
<td>POST_VIRGIN_MB</td>
<td>509.00</td>
<td>5.50</td>
</tr>
</tbody>
</table>

The alpha diversity values were similar to those reported in other studies, from microplastics retrieved at sea (Zettler et al., 2013) or from incubation experiments with artificial plastic pellets (Oberbeckmann et al., 2018). Non-metric multidimensional scaling using the dissimilarity matrix calculated from Bray-Curtis distance showed three clusters; the pre-incubation beads (PRE_BIOF_MB) and the post incubation communities (POST_BIOF_MB and POST_VIRGIN_MB) (with their corresponding free community, POST_BIOF_FREE and POST_VIRG_FREE) (Figure 6.4). This highlights the similarity of the attached communities of biofilm, virgin and the free communities associated with the virgin beads. It also underlines a difference between the pre-incubation beads and their correspondant free community from the same environment.
Figure 6.4. Non-metric multidimensional analysis of the Bray-Curtis dissimilarity matrix of the samples analysed. PRE_BIOF_MB are beads incubated for 1 week in natural seawater under controlled conditions. POST_BIOF_MB are the same beads after 48 h in the tank with the sea urchins. POST_VIRGIN_MB are untreated beads (only diluted in distilled water, vortexed and diluted in the sea urchin tanks), after 48h incubation. FREE are the free-living counterpart of the attached communities studied.

This similarity among free and attached communities from the post incubation was surprising, also considering that the water used to grow the biofilm on MBs was the same used for the following incubations with the sea urchins. In order to explore this similarity, an analysis of shared and exclusive OTUs was performed. Figure 6.5 shows that only 31 OTUs (1.7%) were in common. While the attached communities shared 3.2% of total OTUs, the free communities (virgin and biofilm) do not share any (0%). Similarly, very few OTUs are shared between each of the conditions (biofilm/covered and virgin) (10 for virgin MBs, and 5 for biofilm/covered MBs). The attached communities showed more exclusive OTUs than their free counterparts (36% vs 6.2% biofilm; 37.4% vs 9.9% virgin), showing a general distinction among free and attached communities.
Despite similar alpha diversity, only <1% of the OTUs were shared by all attached communities, especially belonging to Flavobacteriaceae, Rhodobacteraceae and Saprospiraceae families (Table 6.2), which are common MP colonizers (Zettler et al., 2013; Oberbeckmann et al., 2014; Dussud et al., 2018; Amaral-Zettler et al., 2021). This little amount of OTUs shared highlighted the high difference between the three communities.

The sea urchins (and their released organic compounds) may have supported the proliferation of specific taxa despite the presence of different prokaryotes already established on the MBs as a result of the previous incubation. This supported by the highest number of shared OTUs in this analysis (149, 6.4%) occurred between the MBs of the two tanks at the end of the experiment (POST_BIOF_MB and POST_VIRGIN_MB) (Figure 6.6). The 149 OTUs shared between the two post/incubation treatments belong to 56 taxa, including some related to common colonizers like Flavobacteriaceae, Vibrionaceae and Rhodobacteraceae,
but also some other OTUs belonging to phyla less known as colonizers like Fibrobacteres, Spirochaetes, Tenericutes, Fusobacteria, Lentisphaerae.

![Venn diagram](image)

**Figure 6.6.** Venn diagrams showing the number and percentages (in brackets) of OTUs shared by prokaryotic communities attached to MBs under the three conditions. PRE_BIOF_MB are beads incubated for 1 week in natural seawater under controlled conditions. POST_BIOF_MB are the same beads after 48h in the tank with the sea urchins. POST_VIRGIN_MB are untreated beads only diluted in distilled water, vortexed and diluted in the sea urchin tanks, after 48h incubation.
Table 6.2. Taxa shared among all the three communities.

<table>
<thead>
<tr>
<th>Taxa</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__Saprospira ceae;D_5__Aureispira</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Chitinophagales;D_4__Saprospira ceae;D_5__Lewinella;D_6__uncultured bacterium</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Aquibacter</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Maribacter</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__NS3a marine group</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Polaribacter 4</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Flavobacteriales;D_4__Flavobacteriaceae;D_5__Winogradskyella</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Bacteroidetes;D_2__Rhodothermia;D_3__Balneolales;D_4__Balneolaceae;D_5__Balneola</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Pseudophaeobacter</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodobacterales;D_4__Rhodobacteraceae;D_5__Sulfitobacter</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Colwelliaceae;D_5__Thalassotalea;D_6__uncultured bacterium</td>
</tr>
<tr>
<td>D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Marinobacteraceae;D_5__Marinobacter</td>
</tr>
</tbody>
</table>

The prokaryotic community attached to MBs incubated in natural seawater was dominated by Bacteroidetes (68%) and Proteobacteria (30%), with a minor contribution of Planctomycetes (1%). At the end of the experiment, this community shifted to 46% Proteobacteria, 45% Bacteroidetes and 2% Planctomycetes. The initially non-colonized (“virgin”) MBs showed a prokaryotic community formed by 52% of Bacteroidetes, 30% of
Proteobacteria and 1.8% of Planctomycetes (Figure 6.7). Both post-treatment prokaryotic communities showed over 5 to 7 times more OTUs belonging to Firmicutes than the ones pre-treatment. As a matter of fact, Firmicutes is one of the most abundant phyla belonging to the sea urchin internal microbiome (La Port et al., 2018). As such, this could suggest a contribution from the internal animal microbiome to the attached beads community, possibly through its released faeces attaching to free MBs or also through their passage through the digestive system.

![Prokaryotic community composition at the phylum level. N=2 biological replicates. PRE_BIOF_MB are beads incubated for 1 week in natural seawater under controlled conditions. POST_BIOF_MB are the same beads after 48h in the tank with the sea urchins. POST_VIRGIN_MB are untreated beads only diluted in distilled water, vortexed and diluted in the sea urchin tanks, after 48h incubation.](image)

When analyzed at a finer taxonomic level, the prokaryotic community on MBs incubated in natural seawater showed lower evenness, with Saprospiraceae, Rhodobacteraceae and Flavobacteriaceae dominating, together with Alteromonadaceae and Cryomorphaceae (Figure 6.8) and Haliaeaceae and Sphingomonadaceae as minor contributors (not shown in Figure 6.7 because not reaching the threshold of 2%). Marinilabiliaceae emerged consistently incubated in presence of the sea urchins, also with Saprospiraceae, Rhodobacteraceae, Flavobacteriaceae and the uncultured family of Bacteroidetes VC 2.1_Bac22 (Figure 6.8). This is consistent with previous observations that the prokaryotic plastosphere offers favourable growth conditions to members of the so-called “rare biosphere” which are usually found in very low amount in the seawater. This confirms that MBs represent new niches allowing an adaptive advantage to some prokaryotes with respect
to others. Whether this is due simply by mechanical support or protection or if there is also a chemical interaction with the different polymers, is still under debate.

Figure 6.8. Prokaryotic community composition at the Family level. Bars are biological replicates. PRE_BIOF_MB are beads incubated for 1 week in natural seawater under controlled conditions. POST_BIOF_MB are the same beads after 48h in the tank with the sea urchins. POST_VIRGIN_MB are untreated beads only diluted in distilled water, vortexed and diluted in the sea urchin tanks, after 48h incubation.

Although they were not dominant, representatives of the genera *Arcobacter*, *Vibrio*, and *Colwellia*, hosting potential pathogenic strains, were observed in the post-experiment attached communities, even though they were not detected among the initially attached prokaryotes (data not shown). All these genera have been found responsible of the “bald sea urchin disease” (Girard et al., 2012; Boudouresque et al., 2013). This suggests that in particular conditions, such as in the presence of high organic matter, also combined with substrate specificity, potential pathogens might find favorable conditions for growth.
6.4 Conclusions

These data showed that colonization on the surface of MPs takes place within 48 hours of introduction in the aquatic environment. Indeed, our SEM microphotographs showed that after 1 week the biofilm formation completely covered the surface of MBs. This 1-week biofilm had the same order of magnitude of OTUs comparing with works retrieving MPs from marine environment by manta-net. We found differences among pre-incubation and post-incubation communities attached to MBs. This of course might also be linked to the sea urchins, their microbiome, and their released organic compounds, which supported the proliferation of specific taxa despite the presence of different prokaryotes already established on the MBs as a result of the previous incubation. The environmental conditions showed also differences in the effect in the sea urchins.

In terms of realistic exposure scenarios, currently projections suggest that in coastal areas MP concentrations could reach higher levels than predicted causing an increase in the toxicity thresholds for marine organisms (Everaert et al., 2018). In this context, the outcome of this work provides the basis for future investigations on MPs ecotoxicity, encouraging the use of colonized rather than virgin plastics, considering that colonization occurs as soon as plastics enter the marine environment and are therefore their most realistic form. *P. lividus* also confirms as a useful model organism also for their relevance in economic aspects linked its human consumption.
Chapter 7. General conclusions
7.1 MP distribution patterns

In order to reach a Good Environmental Status (GES) in European waters, descriptor number 10 within the Marine Strategy Framework Directive (MSFD), is the assessment of marine litter (Jahnke et al., 2013). In this PhD thesis, the focus was towards indicator 10.1.2: floating litter. Although this indicator includes large (macro) as well as small (micro) litter, and not only plastics, MP is included in this indicator, since MP pollution is increasingly recognized as a potential threat to any environment, and the marine one in particular.

MP pollution is widespread, from the surface of the water column to the bottom of the oceans, including very deep trenches and remote areas (Peng et al., 2018). In this study, the focus was on floating microplastics, as the fraction included between 330 µm and 5 mm (Thompson et al., 2009), that is named “microplastics”. Several factors determine MP distribution in space and time: natural variability, local hydrodynamism and circulation, together with human impacts, in particular in areas close to urban concentrations, where productive activities like agriculture, industries, transportation, fishing, tourism and related discharges are concentrated. All these activities use plastics for their functioning and MPs continue to increase due to changing demographics favoring immigration to coastal regions, together with extensive fishing, recreational and maritime uses of the ocean (Ribic et al., 2010). It is estimated that 80% of litter found at sea is delivered by terrestrial sources such as public littering, improper waste disposal, waste dump run-offs, tourism, industrial activities, together with combined sewer systems (Andrady et al., 2011). In this study, it was observed that seasonal differences were reported at different sites, with higher MP concentrations in the summer, probably related to the higher demographic concentration of people and activities closer to the coasts, substantially affecting production and disposal of new plastics. This increase was noted as reported in sections 4.1 (Gulf of Napoli and Bay of Pozzuoli). Another important aspect highlighted in the present study is the spatial variability in terms of MP pollution. Especially that urban and discharge zones are the most impacted from this kind of pollution. This was found in subchapter 4.3, where MP concentrations were much higher in coastal areas such as the GoN (subchapter 4.3.1) and the BoP (subchapter 4.3.2), rather than more offshore sampled areas like Cilento (subchapter 4.3.3) and the northern Adriatic Sea (subchapter 4.3.5). In more detail, within the coastal areas, as in the case of the GoN, stations closer to the coast and in proximity of high-density population areas, fishery, maritime and discharge activities like st.1 in the GoN (Portici), station 2 in the BoP, and Paloma, N5 and S1 in the Adriatic Sea, showed higher MP concentrations than...
stations farther offshore. In addition to these factors, MP distribution can be further influenced by wind mixing, distributing MPs in the water column, also drawing them from the very surface (Kukulka et al., 2012).

In the coastal areas sampled MP pollution appears to derive from weathering and fragmentation of larger secondary MPs, based on the observed higher abundance of MPs ranging between 1-5 mm in size. This is also due to photo oxidation, the main responsible for weathering and plastic break down in pieces, which increases with increasing temperatures (Andrady et al., 2011). When mixing is low, as in the summer and closer to the coast, plastic items reside longer at surface and therefore undergo greater photo oxidation. The dominant input of plastic into oceans is from land-based sources, whereas only a small fraction is produced directly at sea from vessels, fisheries, and mariculture (UNEP report, 2016). In this, rivers are key players, as they transport plastic into lakes, seas, and the ocean. Rivers and lakes are also active secondary MP producers via mechanical fragmentation of the plastic litter abandoned along their paths (Kataoka et al., 2019).

7.2 A dynamic community: the Plastisphere

Soon after plastic enters the aquatic realm, it is colonized by microorganisms. On MPs a complex and dynamic community is soon established, mainly composed of prokaryotes and diatoms, named the plastisphere (Zettler et al. 2013). The composition of the microbial plastisphere has been the object of many studies (e. g. Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015; Bryant et al., 2016; Dudek et al., 2020; Amaral-Zettler et al., 2021), but many questions remain open about its composition, dynamics and succession. Considering the results from this PhD thesis, subchapter 4.2 showed how temporal variability in terms of community structure plays a relevant role in the plastisphere. Seemingly each and every sampling from the GoN and BoP mirrors a different MP-attached community, this highlighting the randomness and the high level of stochasticity of community structure in plastisphere in coastal areas. On the other hand, spatial variability was also found to be another relevant discriminant in terms of parameters shaping the plastisphere community. The latter mostly found at molecular level rather than morphological. Indeed, from coastal to offshore areas, the plastisphere seemed to have quite an unchanged community, where similar genera of diatoms were found (e. g. Navicula, Amphora, Nitzschia). Inter-spatial difference was more relative to “rare” taxa, indeed were found only at very deep levels of taxonomy, highlighted from the Venn diagrams. In terms of community succession, the
results showed that MP colonization appears to take place almost as soon as its introduction in the aquatic environment. This was found along the Sarno river and its estuary (subchapter 4.4.3), where MP pieces even at the source station (SU) were already colonized. Other evidences were found from the incubation experiments (Chapter 6). It was evident that as short as one week after incubation of commercial microbeads in seawater under controlled conditions, a thick biofilm had formed. This 1-week biofilm contained the same order of magnitude of OTUs when compared with the one grown on MPs retrieved from the marine environment.

Also environmental factors, such as salinity and silicates (section 4.4.6), appeared to be strong determinants shaping the biofilm in quantity and quality, even though their relative importance changes with the season and the region, also confirming previous observations (Oberbeckmann et al., 2019; Amaral-Zettler et al., 2020). After all these discriminants shaping the attached communities, evidences of a general marine biofilm “core community” of a few bacterial taxa present in low quantity were found (Kirstein et al., 2019). This is shared among different MPs, suggesting that plastic “specific” microorganisms might be represented by rather rare species, as also found by Scales et al. (2021). When considering all data produced in the present research work, a “core community” could also be identified within the total OTUs, formed by known plastic colonizers such as Flavobacteriaceae, Rhodobacteraceae or genera like Dokdonia and Oleiphilus (Table 6.1). Part of this community also harbors putative pathogens and plastic degraders like Vibrio, Pseudomonas, Acinetobacter (Table 7.2), as also discussed in chapter 5.
Table 7.1. Families and Genera of MP colonizers detected in different areas in this study. GoN is Gulf of Napoli; BoP Bay of Pozzuoli, PL is Paracentrotus lividus (sea urchin) from incubation experiments, as described in Chapter 6.

<table>
<thead>
<tr>
<th>Family</th>
<th>Sampled areas</th>
<th>Genus</th>
<th>Sampled areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavobacteriaceae</td>
<td>Adriatic/GoN/BoP/Sarno/PL</td>
<td>Dodkonia</td>
<td>Adriatic/GoN</td>
</tr>
<tr>
<td>Rhodobacteriaceae</td>
<td>Adriatic/GoN/BoP/Sarno/PL</td>
<td>Winogradskyella</td>
<td>Adriatic/GoN</td>
</tr>
<tr>
<td>Vibrionaceae</td>
<td>GoN/BoP/PL</td>
<td>Winogradskyella</td>
<td>Adriatic/GoN</td>
</tr>
<tr>
<td>Cyanobacteriaceae</td>
<td>Adriatic / GoN</td>
<td>Synechococcus</td>
<td>Adriatic/GoN</td>
</tr>
<tr>
<td>Pirellulaceae</td>
<td>GoN / Sarno</td>
<td>Oleiphilus</td>
<td>GoN / Sarno</td>
</tr>
<tr>
<td>Caulobacteriaceae</td>
<td>GoN/BoP</td>
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</tr>
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<td>Alteromonadaceae</td>
<td>GoN/BoP</td>
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<td>Pseudoalteromonadaceae</td>
<td>GoN/BoP</td>
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<td></td>
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<tr>
<td>Phycisphaeraceae</td>
<td>GoN/BoP/Sarno</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomonadaceae</td>
<td>GoN/BoP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderiaceae</td>
<td>GoN/Sarno</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleiphilaceae</td>
<td>GoN/Sarno</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.2. Genera of putative pathogens and plastic degraders detected in this study. GoN is Gulf of Napoli; BoP Bay of Pozzuoli.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Sampled areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio</td>
<td>GoN/BoP/Cilento</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>GoN /Sarno</td>
</tr>
<tr>
<td>Tenacibaculum (Oberbeckmann et al., 2014)</td>
<td>BoP/Adriatic</td>
</tr>
<tr>
<td>Acinetobacter (Debroas et al., 2017)</td>
<td>GoN/BoP/Cilento/Adriatic</td>
</tr>
<tr>
<td>Marinobacter (Delacuvellerie et al., 2019)</td>
<td>GoN/Sarno/BoP</td>
</tr>
<tr>
<td>Oleiphilus (Erni-Cassola et al., 2019)</td>
<td>GoN</td>
</tr>
<tr>
<td>Pseudoalteromonas (Laganà et al., 2018)</td>
<td>BoP</td>
</tr>
</tbody>
</table>
7.3 Future perspectives

While data on community composition and single members of the plastisphere are now widespread, little is known about activities of microbes living attached to MPs. This would help in understanding the role of the plastisphere in the whole ecosystem functioning, especially in colonization by invasive species or contribution to local productivity. Rate estimates of primary and/or secondary production of attached communities could help understanding the contribution of the microbial plastisphere to local and global ecosystem functioning (Reisser et al., 2014; Bryant et al., 2016) for instance, in terms of DMS or other secondary metabolites, produced by diatoms, which are known biogeochemically important molecules. In this, metatranscriptomics could have a role, at least to identify putative functions. Although methodological limitations are foreseen, such as the amount of RNA needed, this could be a suitable direction. KEGG pathways on MPs have been reported already (Xu et al., 2019), but more information is needed, also considering the extreme space and time variability of these communities.

These dynamic measurements could also be relevant in better understanding the potential role of MPs in spreading of harmful organisms (Masò et al., 2003) and/or pathogens, like *Vibrio* and *Aeromonas* genera, *Campylobacteraceae* family and *Aeromonas salmonicida* species (Zettler et al., 2013; Oberbeckmann et al., 2019; Amaral-Zettler et al., 2020). Another aspect which deserves more attention is the less abundant, yet potentially very important organisms often found on MPs, such as fungi, choanoflagellates, ciliates, and metazoan which have been reported at times to be relevant in the community also in terms of degradation of polymers (Pramila et al., 2011; Paço et al., 2017). In this study, they have been often observed, although rarely at high concentrations. They need to be better investigated, as they might perform important degradation and predation processes, highly contributing to food web dynamics on MP and ecosystems in general.
References


De Falco, F., Cocca, M., Avella, M., & Thompson, R. C. (2020). Microfiber release to water, via laundering, and to air, via everyday use: a comparison between polyester clothing with differing textile parameters. Environmental science & technology, 54(6), 3288-3296.


Delacuvellerie, A., Cyriaque, V., Gobert, S., Benali, S., Wattiez, R., 2019. The plastisphere in marine ecosystem hosts potential specific microbial degraders including Alcanivorax borkumensis as a key player for the low-density polyethylene degradation.


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River mouth, Gulf of Naples (Italy) produces a biofilm biosorbing Pb (II). Science of the Total Environment 562, 588-595.


https://omnexus.specialchem.com/polymer-properties/properties/density#values

https://www.istat.it/it/

https://www.protezioneecivile.gov.it/it/
APPENDIX
Impact of Microbial Colonization of Polystyrene Microbeads on the Toxicological Responses in the Sea Urchin *Paracentrotus lividus*

Carola Murano, Vincenzo Donnarumma, Ilaria Corsi, Raffaella Casotti, and Anna Palumbo

**ABSTRACT:** The sea urchin *Paracentrotus lividus* (P. lividus) was exposed to either virgin or biofilm-covered polystyrene microbeads (micro-PS, 45 μm) in order to test the effect of microbial colonization on the uptake, biodistribution, and immune response. The biofilm was dominated by bacteria, as detected by scanning electron microscopy and 16S rRNA sequencing. A higher internalization rate of colonized micro-PS inside sea urchins compared to virgin ones was detected, suggesting a role of the plastiophere in the interaction. Colonized and virgin micro-PS showed the same biodistribution pattern by accumulating mainly in the digestive system with higher levels and faster egestion rates for the colonized. However, a significant increase of catalase and total antioxidant activity was observed only in the digestive system of colonized micro-PS-exposed individuals. Colonized micro-PS also induced a significant decrease in the number of coelomocytes with a significant increase in viable cells, compared to control and virgin micro-PS-exposed animals. Moreover, a general time-dependent increase in the red/white amoebocytes ratio and reactive oxygen species and a decrease in nitrogen ones were observed upon exposure to both colonized and virgin micro-PS. Overall, micro-PS colonization clearly affected the innate and toxicological responses of the Mediterranean sea urchin *P. lividus* in comparison to virgin micro-PS.

**KEYWORDS:** biofilm, coelomocyte, microplastics, plastiophere, sea urchin

1. INTRODUCTION

The widespread occurrence of microplastics (<5 mm, MPs) in the marine environment from the coast to offshore, from the surface to the deep sea, and from most urbanized to remote regions is the result of their continuous production, dispersal, and long persistence. With its confined borders, tourist attractions, and extremely populated coasts, the Mediterranean Sea is recognized as the sixth great accumulation zone for marine litter worldwide and consequently a hotspot for MP pollution. In particular, the Mediterranean seafloor is considered a long-term sink for MPs and coastal areas are the most affected by plastic pollution. The interactions between the hydrophobic surface of MPs and the seawater significantly affect their properties, density, size, and their position along the water column and enhance their bioavailability to benthic organisms. These interactions lead to the formation of the so-called "corona," a dynamic layer covering the particle surface, which for nanoparticles is formed by proteins or metabolites and for larger particles is represented by a microbial biofilm. As a matter of fact, as soon as MPs enter the sea, their surface is immediately colonized by microbes (heterotrophs, autotrophs, predators, and symbionts) which together form the microbial members of the so-called "plastiophere." The plastiophere harbors distinct communities from the surrounding free-living representatives, thus representing a new marine niche. One of the key species of the benthic Mediterranean habitats is the sea urchin *Paracentrotus lividus* in terms of its trophic peculiarities and ecological contribution to ecosystem functioning. Sea urchins possess an extraordinary immune system formed by an heterogeneous cell population, the coelomocytes, which mediate the effects of a variety of environmental stressors. Moreover, due to their urchin diet selection and intense grazing activity, sea urchins control the organization, structure, and composition of shallow macroalgal assemblages. This feeding behavior contributes to plastic fragmentation, also inducing the release of secondary MPs into the marine environment as observed for other benthic species. Up to now, there is only a study showing MPs occurrence in the wild specimens of some sea urchin species, in particular in the gut, coelomic fluid, and gonads. In our previous work, we showed for the first time a size-
Diversity and predicted inter- and intra-domain interactions in the Mediterranean Plastisphere

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d Thalasso – Marine Research and Science Communication, 60 Rue Francis Duque, 13002, Marseille, France
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Bacterioplankton
Phytoplankton
Gene network analysis
Plastisphere

ABSTRACT

This study investigated the biogeochemistry, the presence and diversity of potentially harmful taxa harbored, and potential interactions between and within bacterial and eukaryotic domains of life on plastic debris in the Mediterranean. Using a combination of high-throughput DNA sequencing (HTS), Causal Network Analysis, and Scanning Electron Microscopy (SEM), we show regional differences and gradients in the Mediterranean microbial communities associated with marine litter, positive causal effects between microbes including between and within domains of life, and how these might impact the marine ecosystems surrounding them. Adjacent seas within the Mediterranean region showed a gradient in the microbial communities on plastic with non-overlapping endpoints (Adriatic and Ligurian Seas). The largest predicted inter-domain effects included positive effects of a novel red algal Plastisphere member on its potential microbiome community. Freshwater and marine samples housed a diversity of fungi including some related to disease-causing microorganisms. Algal species related to those responsible for Harmful Blooms (HABs) were also observed on plastic pieces including members of genera not previously reported on Plastic Marine Debris (PMD).

1. Introduction

The presence of plastic marine debris (PMD) in the world ocean is receiving increasing attention (Rochman, 2020), and the impacts on marine organisms from marine mammals to fish due to ingestion and entanglement are well documented (Feiss et al., 2015; Elkins & van Franeker, 2005; Laist, 1999). PMD is also known to transport non-indigenous and potentially harmful species (Aliani & Mattoni, 2003; Desper, 2002; Massi et al., 2003). In contrast to the visible impact of large pieces of PMD, the role of smaller plastic pieces on marine ecosystems and the transport of microbial taxa between biomes is still relatively unknown (Amaral-Zettler et al., 2021). Microplastics (defined as pieces < 5 mm in maximum dimension) may be too small to impact larger animals directly, but more and more organisms are being discovered that ingest them. Microplastics are the most abundant form of PMD, and also serve as an attachment surface for a wide diversity of

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Available online 2 June 2021
© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)
### Table A1. Temperature, (T °C), Salinity, Chl a (µg l⁻¹) and nutrient concentrations (µM) (Total Inorganic Nitrogen, TIN, SiO₂ and PO₄) relative to the Gulf of Napoli data. NA = data not available.

<table>
<thead>
<tr>
<th>Station</th>
<th>Date</th>
<th>T (°C)</th>
<th>Sal</th>
<th>CHL a (µg l⁻¹)</th>
<th>TIN (µM)</th>
<th>SiO₂ (µM)</th>
<th>PO₄ (µM)</th>
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<tbody>
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<td>31/01/2018</td>
<td>14.504</td>
<td>37.520</td>
<td>3.227</td>
<td>6.749</td>
<td>1.791</td>
<td>0.164</td>
</tr>
<tr>
<td>2</td>
<td>31/01/2018</td>
<td>14.767</td>
<td>37.765</td>
<td>3.576</td>
<td>7.292</td>
<td>1.118</td>
<td>0.202</td>
</tr>
<tr>
<td>3</td>
<td>31/01/2018</td>
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<td>3.721</td>
<td>0.670</td>
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<td>3.227</td>
<td>1.132</td>
<td>0.922</td>
<td>0.060</td>
</tr>
<tr>
<td>2</td>
<td>24/07/2018</td>
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<td>36.988</td>
<td>0.140</td>
<td>7.119</td>
<td>2.900</td>
<td>0.291</td>
</tr>
<tr>
<td>1</td>
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<td>27.485</td>
<td>37.479</td>
<td>3.236</td>
<td>2.927</td>
<td>0.749</td>
<td>0.119</td>
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<td>01/08/2018</td>
<td>26.720</td>
<td>37.680</td>
<td>3.531</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>01/08/2018</td>
<td>26.750</td>
<td>37.890</td>
<td>0.360</td>
<td>1.061</td>
<td>0.289</td>
<td>0.045</td>
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<td>1</td>
<td>27/09/2019</td>
<td>24.336</td>
<td>37.484</td>
<td>3.614</td>
<td>3.946</td>
<td>2.405</td>
<td>0.126</td>
</tr>
<tr>
<td>2</td>
<td>27/09/2019</td>
<td>24.315</td>
<td>37.347</td>
<td>1.816</td>
<td>1.203</td>
<td>1.649</td>
<td>0.047</td>
</tr>
<tr>
<td>3</td>
<td>27/09/2019</td>
<td>24.623</td>
<td>37.116</td>
<td>3.399</td>
<td>6.828</td>
<td>1.687</td>
<td>0.115</td>
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<tr>
<td>1</td>
<td>14/01/2020</td>
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<td>0.682</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>14/01/2020</td>
<td>15.346</td>
<td>37.774</td>
<td>0.449</td>
<td>2.593</td>
<td>2.609</td>
<td>0.085</td>
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<tr>
<td>3</td>
<td>14/01/2020</td>
<td>15.640</td>
<td>37.950</td>
<td>0.460</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</table>

### Table A2. Temperature, salinity and chlorophyll a at the different stations of the Cilento Bay.

<table>
<thead>
<tr>
<th></th>
<th>st. 1</th>
<th>st. 2</th>
<th>st. 3</th>
<th>st. 4</th>
<th>st. 5</th>
<th>st. 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T(°C)</td>
<td>20.713</td>
<td>21.526</td>
<td>20.913</td>
<td>22.179</td>
<td>22.514</td>
<td>22.597</td>
</tr>
<tr>
<td>Sal</td>
<td>37.556</td>
<td>37.534</td>
<td>37.46</td>
<td>37.224</td>
<td>37.194</td>
<td>37.269</td>
</tr>
<tr>
<td>Chla (mg m⁻³)</td>
<td>0.161</td>
<td>0.171</td>
<td>0.089</td>
<td>0.203</td>
<td>0.288</td>
<td>0.193</td>
</tr>
</tbody>
</table>

### Table A3. Single MPs and their characteristics from Station 1 from AMP Cilento.

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<th>CILENTO – STATION 1</th>
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</thead>
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<td>#</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
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</table>

241
<table>
<thead>
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<th></th>
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<th>FRAGMENT</th>
<th>COLOR</th>
<th>CONDITION</th>
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<tbody>
<tr>
<td>3</td>
<td>0.4</td>
<td>BLACK IN PIECES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>ORANGE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>TRANSPARENT</td>
<td>RICH WITH BIOFILM</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>TRANSPARENT</td>
<td>RICH WITH BIOFILM</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.7</td>
<td>TRANSPARENT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.6</td>
<td>BLACK IN PIECES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.9</td>
<td>GREEN COVERED BY GELATINOUS SUBSTRATE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.4</td>
<td>GREEN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>TRANSPARENT</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>0.7</td>
<td>TRANSPARENT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>TRANSPARENT</td>
<td>IN PIECES</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>BROWN/RED</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>BLACK RIGID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>GREEN THIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>BLUE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.8</td>
<td>WHITE LIKE RUBBER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>GREY IRREGULAR SHAPE, RIGID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>MP Size (mm)</td>
<td>Material</td>
<td>Color</td>
<td>Characteristics</td>
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<tr>
<td>-----</td>
<td>--------------</td>
<td>----------</td>
<td>---------------</td>
<td>--------------------------</td>
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<tr>
<td>2.0</td>
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<td>GREY</td>
<td>IRREGULAR SHAPE, RIGID</td>
</tr>
<tr>
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<td>0.6</td>
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<td></td>
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<td>0.9</td>
<td>FILAMENT</td>
<td>LIGHT BLUE</td>
<td>THIN</td>
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<td>2.3</td>
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<td>2.4</td>
<td>1.2</td>
<td>FILM</td>
<td>TRANSPARENT</td>
<td>ORANGE-Y</td>
</tr>
<tr>
<td>2.5</td>
<td>0.5</td>
<td>FRAGMENT</td>
<td>GREY</td>
<td>RIGID AND IN PIECES</td>
</tr>
<tr>
<td>2.6</td>
<td>0.9</td>
<td>FRAGMENT</td>
<td>LIGHT BLUE</td>
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</tr>
<tr>
<td>2.7</td>
<td>2</td>
<td>FILM</td>
<td>TRANSPARENT</td>
<td>ORANGE-Y</td>
</tr>
<tr>
<td>2.8</td>
<td>1.2</td>
<td>FRAGMENT</td>
<td>GREY</td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>0.8</td>
<td>FRAGMENT</td>
<td>RED</td>
<td>RIGID</td>
</tr>
<tr>
<td>3.0</td>
<td>0.8</td>
<td>FRAGMENT</td>
<td>WHITE</td>
<td>ORANGE-Y</td>
</tr>
<tr>
<td>3.1</td>
<td>1.2</td>
<td>FRAGMENT</td>
<td>BLACK</td>
<td>IRREGULAR SHAPE</td>
</tr>
<tr>
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<td>0.5</td>
<td>FILM</td>
<td>TRANSPARENT</td>
<td>SMALL</td>
</tr>
<tr>
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<td>0.6</td>
<td>FRAGMENT</td>
<td>TRANSPARENT</td>
<td>ORANGE-Y</td>
</tr>
<tr>
<td>3.4</td>
<td>0.7</td>
<td>FRAGMENT</td>
<td>GREY</td>
<td>RIGID</td>
</tr>
<tr>
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<td>0.5</td>
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<td>GREEN</td>
<td></td>
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Table A2. Single MPs and their characteristics from Station 2 from AMP Cilento (Subchapter 4.3.3)
<table>
<thead>
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<th>#</th>
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<td>GLASS-Y</td>
</tr>
<tr>
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<td>0.3</td>
<td>FRAGMENT</td>
<td>BROWN/ORANGE</td>
<td>GLASS-Y</td>
</tr>
<tr>
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<td>BLACK</td>
<td>BICOLOR</td>
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<td>4</td>
<td>0.5</td>
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<td>BLACK</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>FRAGMENT</td>
<td>BLACK</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>FRAGMENT</td>
<td>GREY</td>
<td>IRREGULAR SHAPE</td>
</tr>
<tr>
<td>7</td>
<td>0.7</td>
<td>FRAGMENT</td>
<td>BLACK</td>
<td>IN PIECES</td>
</tr>
<tr>
<td>8</td>
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Table A7. Single MPs and their characteristics from Station 6 from AMP Cilento.
Table A8. Single MPs and their characteristics from Sarno Sea.

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Table A9. Single MPs and their characteristics from Sarno Downstream.

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| lottery ticket |            |

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Table A10. Single MPs and their characteristics from Sarno Upstream.
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