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Parity in bacterial communities and resistomes: Microplastic and natural organic particles in the Tyrrhenian Sea

Andrea Di Cesare [a,1], Maria Belen Sathicq [b,1], Tomasa Sbaffi [a], Raffaella Sabatino [a], Dario Manca [a], Florian Breider [c], Sylvain Coudret [c], Lee J. Pinnell [d], Jeffrey W. Turner [e], Gianluca Corno [a,*]

[a] National Research Council of Italy – Water Research Institute (CNR-IRSA) Molecular Ecology Group (MEG), Verbania, Italy
[b] Instituto de Limnología "Dr. Raúl A. Ringuelet" (ILPLA) CONICET-UNLP, Bv. 120 y 62 n1437, La Plata, Buenos Aires, Argentina
[c] Ecole Polytechnique Fédérale de Lausanne — EPFL, Central Environmental Laboratory, IIE, ENAC, Station 2, CH-1015 Lausanne, Switzerland
[d] Veterinary Education, Research, and Outreach Program, School of Veterinary Medicine & Biomedical Sciences, Texas A&M University, Canyon, TX, USA
[e] Department of Life Sciences, Texas A&M University, Corpus Christi, TX, USA

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ABSTRACT

Petroleum-based microplastic particles (MPs) are carriers of antimicrobial resistance genes (ARGs) in aquatic environments, influencing the selection and spread of antimicrobial resistance. This research characterized MP and natural organic particle (NOP) bacterial communities and resistomes in the Tyrrhenian Sea, a region impacted by plastic pollution and climate change. MP and NOP bacterial communities were similar but different from the free-living planktonic communities. Likewise, MP and NOP ARG abundances were similar but different (higher) from the planktonic communities. MP and NOP metagenome-assembled genomes contained ARGs associated with mobile genetic elements and exhibited co-occurrence with metal resistance genes. Overall, these findings show that MPs and NOPs harbor potential pathogenic and antimicrobial resistant bacteria, which can aid in the spread of antimicrobial resistance. Further, petroleum-based MPs do not represent novel ecological niches for allochthonous bacteria; rather, they synergize with NOPs, collectively facilitating the spread of antimicrobial resistance in marine ecosystems.

1. Introduction

The Mediterranean Sea is strongly impacted by anthropogenic forcing and climate change. In 2018, the Mediterranean Sea was defined as a "plastic trap" given the large amount of plastic waste and the relatively small exchange of water between the Sea and the Atlantic Ocean (Alessi and Di Carlo, 2018). Multiple studies have characterized Mediterranean Sea microplastics (MPs) and the microbial biofilms that develop on MPs (e.g., Cincinelli et al., 2019; Amaral-Zettler et al., 2021; Amaral-Zettler, 2022; Delacuvellerie et al., 2022). Further, the Mediterranean region is a "hotspot" for climate change (Tuel and Eltahir, 2020) and the Mediterranean Sea has experienced dramatic changes in temperature, atmospheric circulation, precipitation, extreme events, and sea-level rise (summarized by MedECC, 2020). Climate change is predicted to aggravate human pathogenic diseases (Mora et al., 2022) and increases in sea surface temperature have been linked to the spread and risk of mesophilic bacterial pathogens (Semenza et al., 2017). Concurrently, the spread of bacterial pathogens is predicted to coincide with a spread in antibiotic resistance and thermal adaptation is predicted to select for bacterial strains more tolerant to antibiotic challenge (reviewed by Rodriguez-Verdugo et al., 2020).

Thanks to the studies where the chemical composition of the MPs has been investigated, we could assess that the specificity of the plastisphere (Amaral-Zettler et al., 2020; Zettler et al., 2013) is not only controlled by biogeographical patterns and external factors (e.g., nutrients availability, physical factors, local pollution sources) but also by the type of polymer, its aging and its physical characteristics (Zhai et al., 2023) and the presence of additives such as plasticizers and UV stabilizers (Pinto et al., 2019; Staples et al., 1997; Tarafdar et al., 2022; Wright et al., 2020). For example, it has been demonstrated the preference by fast-

* Corresponding author at: National Research Council of Italy – Water Research Institute (CNR-IRSA) Molecular Ecology Group (MEG), Largo Tonolli 50, 28921 Verbania, Italy.
E-mail address: gianluca.corno@cnr.it (G. Corno).
[1] Co-first authors.

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growing opportunistic bacteria (e.g. *Pseudomonas*, *Acinetobacter*) for more complex MPs, such as tire wear particles (Sathicq et al., 2022), while other allochthonous antibiotic resistant bacteria seem to prefer more labile MPs or bioplastics (e.g., *Bacillus cereus*; Di Cesare et al., 2021).

The studies assessing the polymers constituting MPs floating in the Mediterranean Sea and, even more specifically in the Tyrrhenian Sea, suggest the strong predominance of PE, PP, PET, and other hard-degradable polymers (e.g., nylon, PS), while other plastics are often absent or limited to very small proportions (Dussud et al., 2018). This can be due to fast degradation (bioplastic particles) or sinking time (tire wear particles), or simply because they are discharged in limited amounts into the environment.

Natural organic particles (NOPs) and MPs are concomitantly present in the water column. NOPs include detritus, fecal pellets, exopolysaccharides, and decomposing protists. NOPs are well-known hotspots for microbial diversity and productivity, since they offer spatial refuge and nutrition both in eutrophic coastal waters and in oligotrophic open oceans (Hutchinson, 1957). Their influence on the dynamics of microbial communities has been studied since the early days of aquatic biology (e.g., Hutchinson, 1957; Patrick, 1949), and the continuous shower of NOPs falling from the photic zone to the deep ocean (i.e., marine snow, sensu Alldredge and Silver, 1988) plays an important role in carbon sequestration (Turley, 2002). However, despite their concurrence, few studies have compared the microbial communities inhabiting NOPs and MPs.

A recent model suggested that marine snow may accumulate and remove MPs from the photic zone (Kvale et al., 2020). Similarly, a laboratory-based microcosm showed that marine snow can indeed facilitate MP sinking, increasing its availability to filter-feeding molluscs (Porter et al., 2018). By contrast, a separate laboratory-based microcosm showed that marine snow containing buoyant MP fibers demonstrated less cohesion and slower sinking, potentially reducing the efficiency of the biological carbon pump (Roberts et al., 2023). Removal by marine snow may constitute a significant biological MP transport pathway, but field-based experimentation is needed for confirmation.

Here, we (i) define the general impact that MPs can have in marine waters, serving as either a selecting factor for new members of the bacterial community or an additional substrate for already present bacteria and, (ii) assess the persistence of allochthonous bacteria derived from coastal inputs within the marine communities. To address the latter, we comprehensively examined the bacterial community composition on MPs, NOPs, and in the surrounding water of the Tyrrhenian Sea, while also exploring their resistome (total content of antimicrobial resistance genes, ARGs). This allowed us to identify ARGs of different origin and, through metagenome assembled genomes (MAGs) reconstruction, to assign them to various bacterial taxa, ranging from typical marine, to potential pathogens and allochthonous taxa.

The design of our study, comprising two coastal sampling sites, one characterized by a very limited human presence (Cinque Terre, CT) and one with a strong coastal impact of anthropogenic origin (Forte dei Marmi, FdM), and a third site in pelagic waters (defined as open ocean: OO, thus free from direct coastal inputs). This enabled us to assess the actual magnitude of coastal impacts in the Tyrrhenian Sea, an environment characterized by strong surface streams rapidly recirculating the waters within the whole sea basin (Fig. 1).

2. Materials and methods

2.1. Sampling procedure and sample processing

The sampling was carried out in September 2019 aboard a sailing boat (Bavaria 46 "Malice"). Surface plastic samples were collected using a manta trawl (200 μm mesh size, 38 × 68 cm mouth opening), towed on the water surface at 3 knots for 30 min for each sampling session, kept at a distance of about 70 m from the boat to avoid the turbulence induced by the wake of the ship (Baini et al., 2018). Three surface tows were carried out for each sampling point, two of them were used for quantitative and qualitative analysis of MPs, and one for the DNA extraction from MPs and NOPs biofilms. For each sampling, the manta net was rinsed thoroughly from the outside of the net with seawater, in the direction from the manta mouth to the cod end in order to concentrate all particles attached to the net. To quantify the water filtered the net was equipped with a flowmeter. To prevent contamination throughout the analyses, all the materials used for sample collection, including the nets, were cleaned and rinsed before any tow. Each sample was passed by

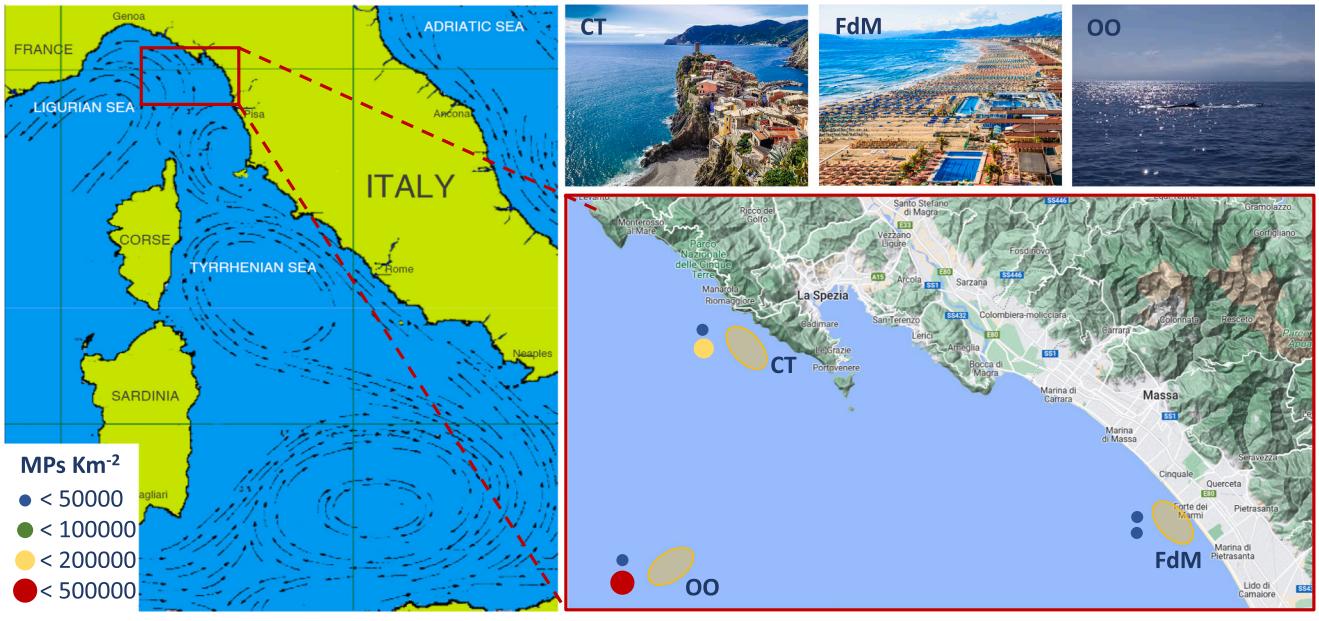

Fig. 1. On the left, the sampling area in the Northern Tyrrhenian Sea. The map on the left depicts the surface streams in September 2019 (from the Atlas of Surface streams in Italian Seas of the Italian Navy, 2020). On the right, a high-resolution picture (from Google Maps) of the sampling area, with the three transect zones (CT: Cinque Terre, 44.0554421 N 9.8225649 E; FdM: Forte dei Marmi, 43.9559771 N 10.1576700 E; OO: open ocean, 43.9655302 N 9.7816679 E), the number of collected MPs for the two tows per site, and, on the top, a particular of the sampling sites.

three consecutive stainless steel sieves (sizes 5'000, 1'000, and 200 μm). The stainless-steel sieves were carefully washed with MPs-free deionized water and the retained particulate was transferred with the help of a glass funnel and stain steel tools to a glass bottle. The samples for MPs identification were fixed with ethanol (final concentration ~35 %). All the samples were kept at 4 °C until further processing. For the assessment of biofilm microbiomes, the retained particulate of each sample was vacuum-filtered onto custom-made 25 μm polyester net filters (with hot-sealed border to avoid particles release) and washed with autoclaved deionized water. MPs and NOPs isolation were performed through visual sorting under a stereomicroscope with the help of stainless-steel tools. MPs and NOPs then separately underwent DNA extraction using a commercial kit (Qiagen Powersoil), according to the manufacturer instructions.

Particles were quantified according to the protocols by Rivers et al. (2019): in detail the number of particles per m^3 was obtained by dividing the total number of particles detected in each tow by the total waterflow (Q) in the manta net, quantified in situ by a flowmeter (in $m^3 sec^{-1}$) multiplied for the duration of the tow (1800 s). These values were further normalized per km^2 by dividing the total number of particles per sample by the sampled area, where the sampled area was calculated by multiplying the sampling distance by the width of the opening of the manta net.

For each sampling site 10 L of water from about 1 m below the surface was collected and kept at 4 °C in the dark, in order to assess the free-living microbial communities. Water samples were then prefiltered on 25 μm pore-size nets, and the ultrafiltrate was then collected on 0.2 μm pore-size polycarbonate filters (Nucleopore). From each filter DNA was extracted using a commercial kit (Qiagen Powersoil), according to the manufacturer instructions. All DNA samples were quantified by Qubit (Invitrogen) and kept at −20 °C until sequencing.

2.2. Metagenome shotgun sequencing and bioinformatic analyses

2.2.1. Metagenomic shotgun sequencing and reads pre-processing

Samples were sent to an external service (IGA Technology Services, Italy) for metagenomic shotgun sequencing, negative controls were previously checked for DNA presence and, due to the absence of detectable DNA, they were not sent for sequencing. Celero™ DNA-Seq Library Preparation Kit (Tecan, Switzerland) was used for library preparation following the manufacturer's instructions. Sequencing was performed using a NovaSeq 6000 instrument (Illumina, USA) and 2 × 150 bp paired-end chemistry under the company's protocol/standard settings. The reads were assessed using FastQC (version 0.11.9; Andrews, 2010) and MultiQC (version 1.12) (Ewels et al., 2016). The assessment considered the overall quality of the sequenced reads, their average length, the duplicates, and an overview of the GC content present in each sample. The quality scores and the other metrics were used to guide the required parameterization of the metagenomic profiling procedure. Trimmomatic (version 0.39) (Bolger et al., 2014) was used to quality filter reads and remove Illumina adapters (using the following parameters: LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36). Merging of paired reads was done with vsearch version 2.17.1 (Rognes et al., 2016) with default parameters. Samples contained on average 29.6 M ± 12.5 M reads. On average, >97.4 % of raw reads passed the quality filtering step and of those, an average of 40.9 % successfully merged. Raw reads were publicly available in NCBI under the accession number PRJNA1021400 https://dataview.ncbi.nlm.nih.gov/object/PRJNA1021400?reviewer=nrdnv8j1p5kk3flldkbli4qo36. Raw reads and high-quality reads are summarized in Supplementary Table 1.

2.2.2. Taxonomic and resistome profiling

Metagenomic taxonomical profiling was done with the Metaxa pipeline. Read pairs were scanned with Metaxa 2.0 (Bengtsson et al., 2011; Bengtsson-Palme et al., 2015) to extract bacterial 16S rRNA (SSU) sequences (default options). Read pairs identified as bacterial SSUs by Metaxa were assigned to and grafted on their closest matching bacterial rRNA sequences in Silva (latest release, 138.1) and clustered using Mothur (version 1.42.1) (Schloss, 2020; Schloss et al., 2009), with the function classify.seqs (cutoff: 90). The counts were normalized as a percentage and exported for further analysis. ARG-like reads were obtained through the annotation to the DeepARG database (deepARG-DB-v1.1.1) (Arango-Argoty et al., 2018) with the pipeline function *deeparg short_reads_pipeline*. Alignment thresholds on sequence identity and minimum alignment length were set, as described in Li et al. (2015a, 2015b) (E-value < 1e-10, identity > 90 %, and minimum alignment length of 25aa). Copies of 16S rRNA genes were inferred by mapping against the 2013 release of the GREENGENES database (DeSantis et al., 2006) as a default in the *deeparg* pipeline. The number of 16S rRNA gene-like hits per sample was used for the normalization of ARG-like fragments abundances, as described in Li et al. (2015a, 2015b). Pivot tables of relative abundance were created for the ARG-like sequences and used for statistical analyses.

2.2.3. Assembly of MAGs

To retrieve MAGs, raw sequence reads were trimmed of low-quality bases and adapter sequences with TrimGalore! Version 0.4.4 (https://github.com/FelixKrueger/TrimGalore), which is a wrapper for Cutadapt (Martin, 2011) and FastQC (Andrews, 2010). Overlapping reads were merged with FLASH version 1.2.11 (Magoč and Salzberg, 2011) using a maximum allowed overlap of 150 bp. Processed reads were co-assembled using MEGAHIT (Li et al., 2015a) version 1.1.1 to produce four co-assemblies that were composed of three replicates each (seawater n = 3, ceramic n = 3, PET n = 3, PHA n = 3). Co-assembly was performed using the 'meta-large' preset for large and complex communities and a minimum contig length of 1000 bp. MAGs were recovered using a previously described method (Parks et al., 2017). Briefly, the processed reads were mapped to the co-assembled metagenomic contigs using BWA-MEM algorithm version 0.7.15-r1142 with default parameters (Li and Durbin, 2010). Genomes were recovered with MetaBAT version 2.12.1 using default MetaBat2 settings (Kang et al., 2019). The resulting bins were merged, filtered, and refined using CheckM version 1.0.11 (Parks et al., 2015) and RefineM version 0.0.23 (Parks et al., 2017) with default parameters. Only MAGs with a quality score >50, defined by Parks et al. (2017) as the estimated completeness of a genome minus five times its estimated contamination, were retained for further analysis. High-quality MAGs were assigned taxonomically with classifications based on the Genome Taxonomy Database (GTDB) (Parks et al., 2018, 2020; Rinke et al., 2021) using GTDB-Tk version 2.1.0 (Chaumeil et al., 2020) with release 07-RS207 of the GTDB. The 'classify_wf' command was employed with default settings. Predicted coding sequences (pCDS) for each MAG were created using prodigal version 2.6.3 (Hyatt et al., 2010).

2.2.4. Detection of resistome in MAGs

To investigate the MAG antibiotic and metals resistance gene content, the proteomes of each MAG was annotated against the DeepARG database (deepARG-DB-v1.1.1) (Arango-Argoty et al., 2018) and the BacMet database of experimentally confirmed resistance genes (BacMet v2.0) (Pal et al., 2014) using DIAMOND blastp (flags used: –sensitive –id 60 –evalue 1e-10). Further analysis was performed on MAGs that contained at least one ARG. Mobile genetic elements (MGE) genes presence was inferred by annotation of MAGs proteome against the mobileOG database, a comprehensive database based on 10 million hallmark proteins sequences of all major classes of MGEs (includes ICEBerg, ACLAME, NCBI Plasmid RefSeq, COMPASS, immedb, and Isfinder and pVOG) using the mobileOGs-pl-kyanite tool (using the following settings: -e 1e-10 -p 70 -q 70) (Brown et al., 2022). All genomic maps were built with Proksee (https://proksee.ca). The tools Alien Hunter (Vernikos and Parkhill, 2006) and CRISPR/Cas Finder (Couvin et al., 2018) available in Proksee were used to highlight putative horizontal gene

transfer (HGT) regions of the contigs identified as "alien" therefore considered originated from HGT and find CRISPR arrays and eventual associated Cas proteins, respectively.

2.3. Data analyses

Differences among the sampling sites in the total counts of MPs were analyzed by the non parametric Kruskal-Wallis rank sum test. Then, we calculated the richness (as the number of different OTUs in each sample) of the bacterial community and the total ARG abundances per samples. In both cases, differences among samples were evaluated by ANOVA, with a Tukey post-hoc test, applying as explanatory variables both the sampling site and the matrix. For both OTU and ARG datasets, a hierarchical clustering of samples was done using "hellinger" method on the distance matrices ("bray" method), to investigate sample composition. Compositional correlation of the two distance matrices was further tested by Mantel test (method = "spearman", permutations = 9999); the same matrices were used to test the composition of microbiome and resistome against the variables site and matrix by PERMANOVA. ARG abundances were arcsine transformed prior to being analyzed (Warton and Hui, 2011). All the tests were performed in the R environment v4.2.1 (R Core Development Team, 2021). The pathobiome (potential pathogenic bacterial genera) was obtained by subsetting the OTU dataset selecting the genera present in the "relevant pathogens A-Z" list of the Hartmann Science Center (https://www.hartmann-science-center.com/en/hygiene-knowledge/pathogens-a-z). Similarly, starting from the ARG dataset, we defined a set of ARGs considered to be high-risk for human health, according to what was reported by Zhang et al. (2021).

3. Results

3.1. Microplastic particle abundance and chemical composition

The number of MPs in the three sampling sites was highly variable within replicates, still all measured abundances were in the range of 15–250 × 10^3 MPs km^{-2} (or 0.08–0.87 MPs m^{-3}) (Fig. 1). No significant variation between sites could be determined ($p = 0.56$, Supplementary Table 2). The size distribution of the MPs particles was rather consistent between sites, with medium sized MPs being always the most abundant (up to 70 % in OO); small particles were less abundant in OO (<10 %) than in the coastal samples (about 40 %) (Fig. 2). The polymers forming MPs were very similar in each sampling site: PE was the most represented MP in the three sampling sites and in each transect, reaching between 67.8 and 90.3 % of the total plastics; PP was the second most abundant MP, accounting from 3.3 to 19.3 % of the particles. PS was rather abundant only in CT, where it accounted for 15.0 % of the collected MPs. Other types of MPs were always limited to very small proportions. In general, in every sample, >80 % of the identified MPs were PE and PP (Fig. 2).

3.2. Bacterial community composition

Richness of the bacterial community varied based on the sampling site ($p = 0.040$, Supplementary Table 3), with CT displaying higher OTU richness compared to OO ($p = 0.049$, Supplementary Table 3). No differences were found between the other sites (Supplementary Table 3). There were no significant differences in richness among water, NOPs, and MPs ($p = 0.124$, Supplementary Table 3).

In terms of composition the situation was different: the microbial communities within the biofilm of both MPs and NOPs were composed (and dominated) by the same OTUs, limited in number when compared to the surrounding water, where other bacteria were predominant (Supplementary Fig. 1). This distribution was common to all the sampled sites (Fig. 3). In detail, in the plastisphere and in the biofilm on NOPs, *Vibrio* and *Alivibrio* spp. as well as *Shewanella* and *Buchnera* spp. accounted for at least 50 % of the detected OTUs (about 90 % on NOPs in OO); while, the same bacteria were hardly detectable in water. *Cetobacterium* resulted to be abundant on MPs (7 %) and NOPs (19 %) only in CT. Water samples were largely dominated by the SAR11 and SAR116 groups (up to 40 %) and by *Alteromonas* and *Pseudoalteromonas* (up to 45 %). At the same time SAR groups and *Alteromonas* were nearly absent in the biofilms; while, *Pseudoalteromonas* was present in limited proportions. Other Burkholderiales and Cyanobacteria were present in every sample, independent of site and substrate (5–25 %) (Fig. 3).

Independently by the sampling site, the pathobiome was almost absent in water (always largely below 0.1 % of the reads) but it became a larger proportion of the microbiome in the biofilms, where it accounted for up to 26 % of the overall community (NOPs in OO) (Fig. 4). Within biofilms, the pathobiome was largely dominated by *Vibrio* (Fig. 4), and no differences in terms of colonization between NOPs and MPs could be observed. Excluding the Vibrionaceae, the other potential pathogens were present in extremely low numbers, and no clear trends in their

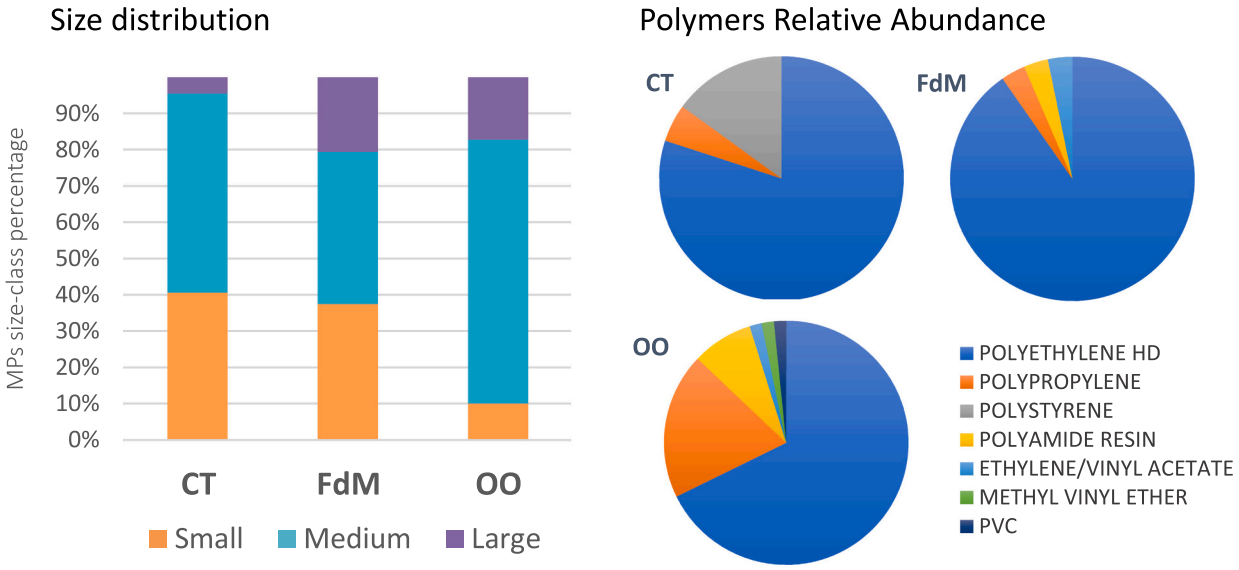

Fig. 2. Size distribution of the MPs and the relative abundance of polymers in the three sampling sites. Small particles size: 0.2–0.99 mm; Medium particle size: 1.0–4.99 mm; Large particle size: >5.0 mm.

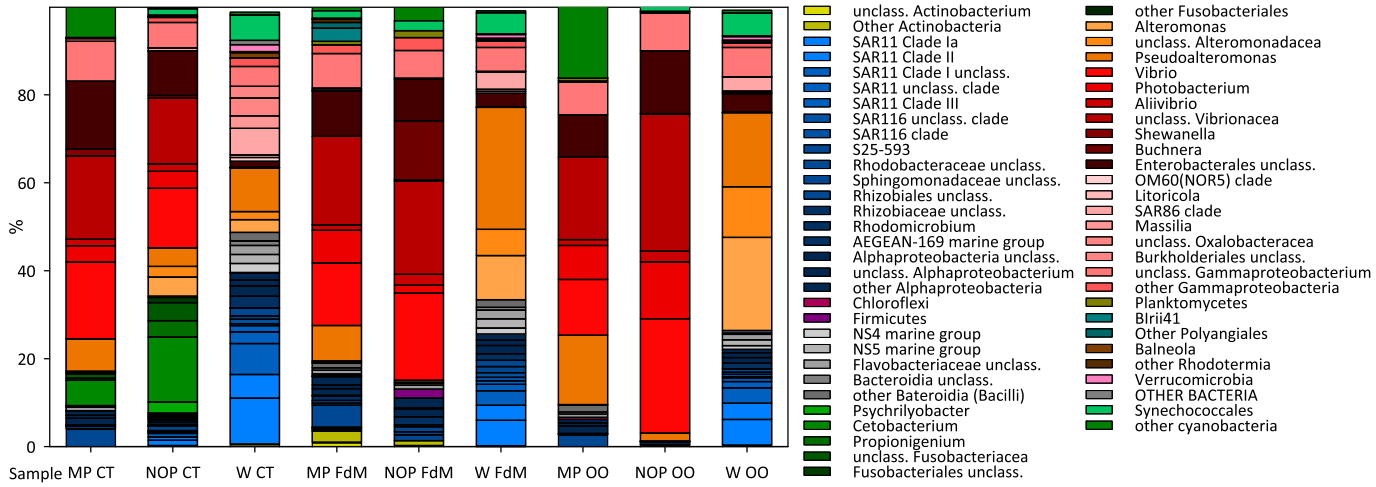

Fig. 3. Microbial community composition, depicted as relative abundance. Sampling sites: CT: Cinque terre; FdM: Forte dei Marmi; OO: Open Ocean.

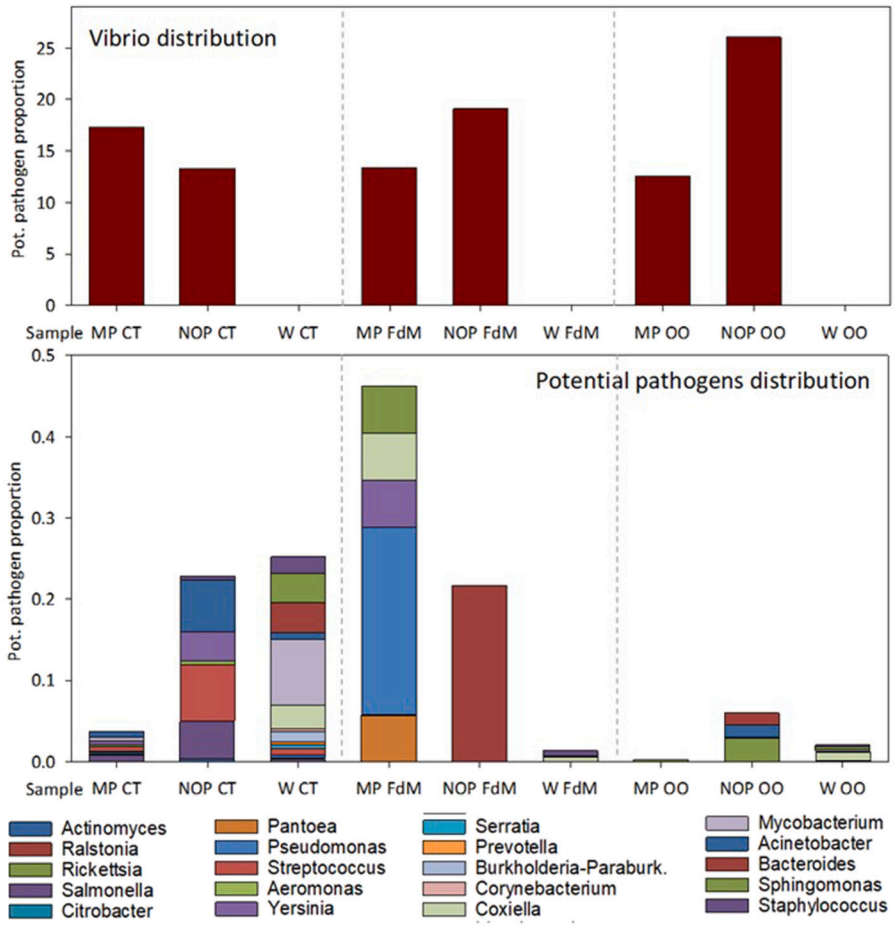

Fig. 4. Composition of the different pathobiomes; in the upper graph the relative distribution of the genus Vibrio, in the lower graph the relative distribution of all the other potential pathogens (Y axis: Percentage of potential pathogens on the overall number of bacterial reads).

distribution could be detected (Fig. 4).

3.3. Resistome abundance and composition

The ARG abundance normalized on the 16S rRNA gene was higher on NOPs than in water ($p = 0.0075$, Fig. 5, Supplementary Table 4). No significant variations were observed for the other substrates or among the sampling sites (Supplementary Table 4). The most abundant resistance classes were related to multidrug, unclassified, and tetracycline resistances while other resistances, generally related to anthropogenic pollution, were limited to smaller numbers. This result was confirmed at the more detailed level of ARG (Fig. 6). In detail, it was possible to identify two different resistomes, one characterizing the biofilms (independently by the substrate) and another characterizing the water, independently by the location (Supplementary Fig. 1). The presence of ARGs considered a high-risk for human health (according to

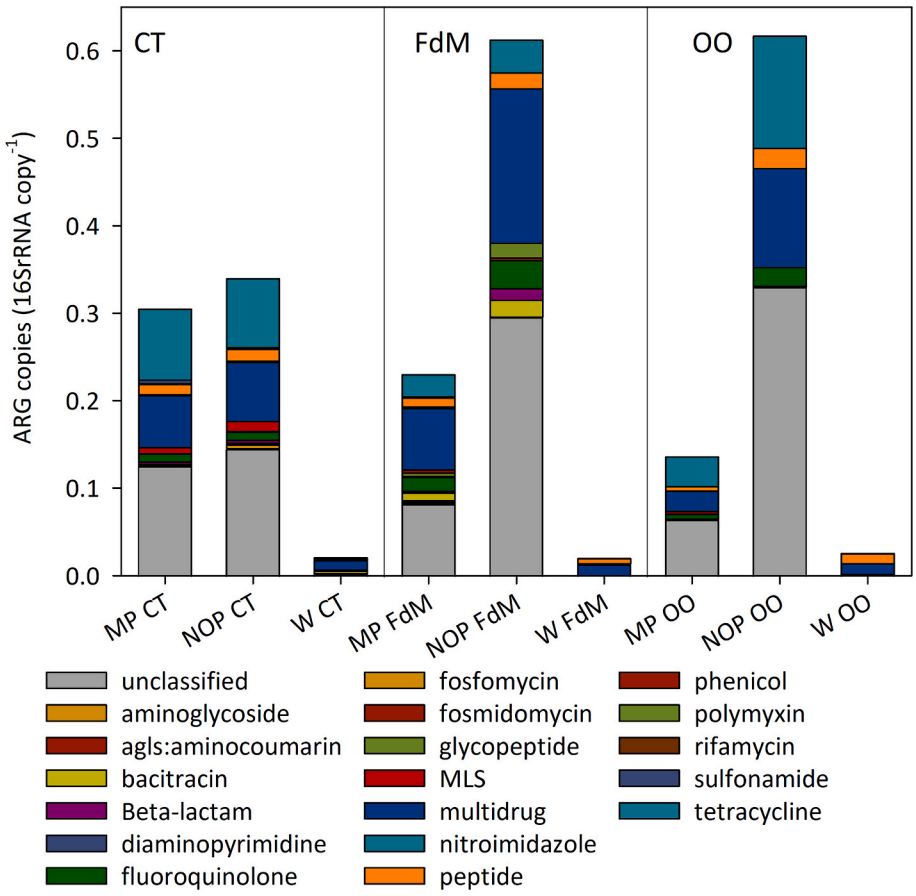

Fig. 5. Antibiotic resistomes presented at the resistance class level in the different samples. The relative measurement is expressed in terms of number of ARG copies per 16SrRNA gene copy.

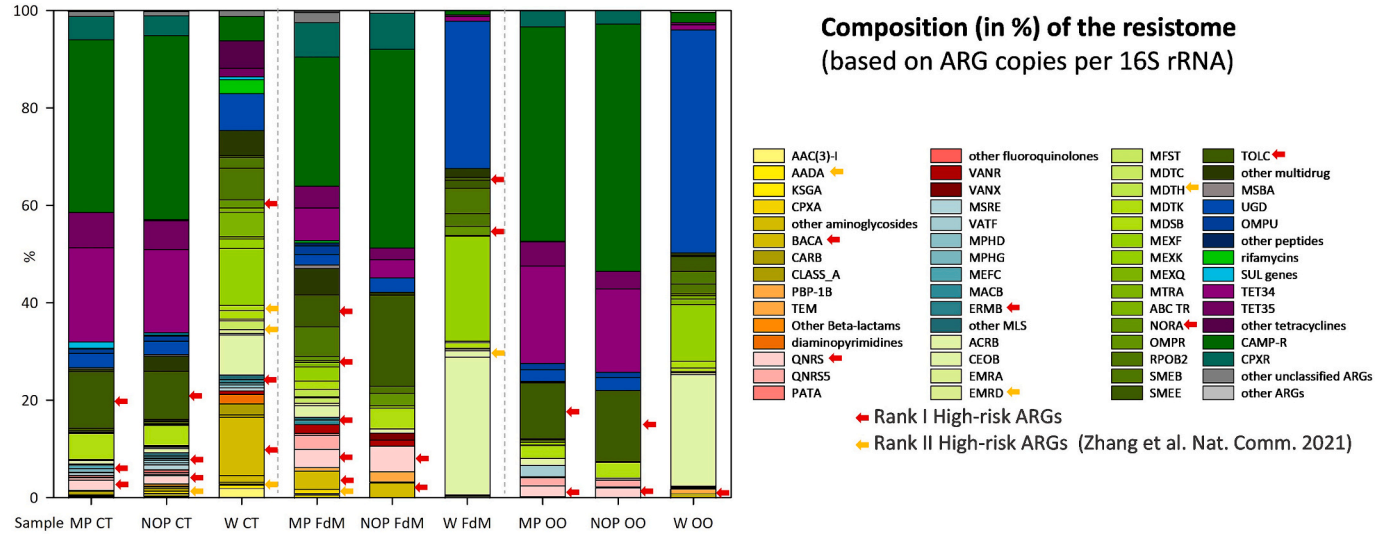

Fig. 6. Composition of the different resistomes, at the level of single ARG (as presented in the table). The different shade of the same colors refer to the different resistance classes. High-risk ARGs are highlighted with arrows on the basis of their rank.

Zhang et al., 2021) did not follow specific trends, and was usually limited to a few genes that represented only a small proportion of the resistome (Fig. 6). The genes providing resistance to fluoroquinolones were present in all the biofilms and were largely represented by the high-risk gene *qnr*S. This gene class was absent in all water samples, linking its potential spread to the presence of particles.

3.4. *Metagenome assembled genomes*

In total, 78 high quality MAGs were recovered (Supplementary Table 5). Of the 78, only 14 did not contain any genes conferring antimicrobial nor metal resistance, which were mostly water-related MAGs (Fig. 7). Almost all MAGs containing ARGs also contained MRGs, and

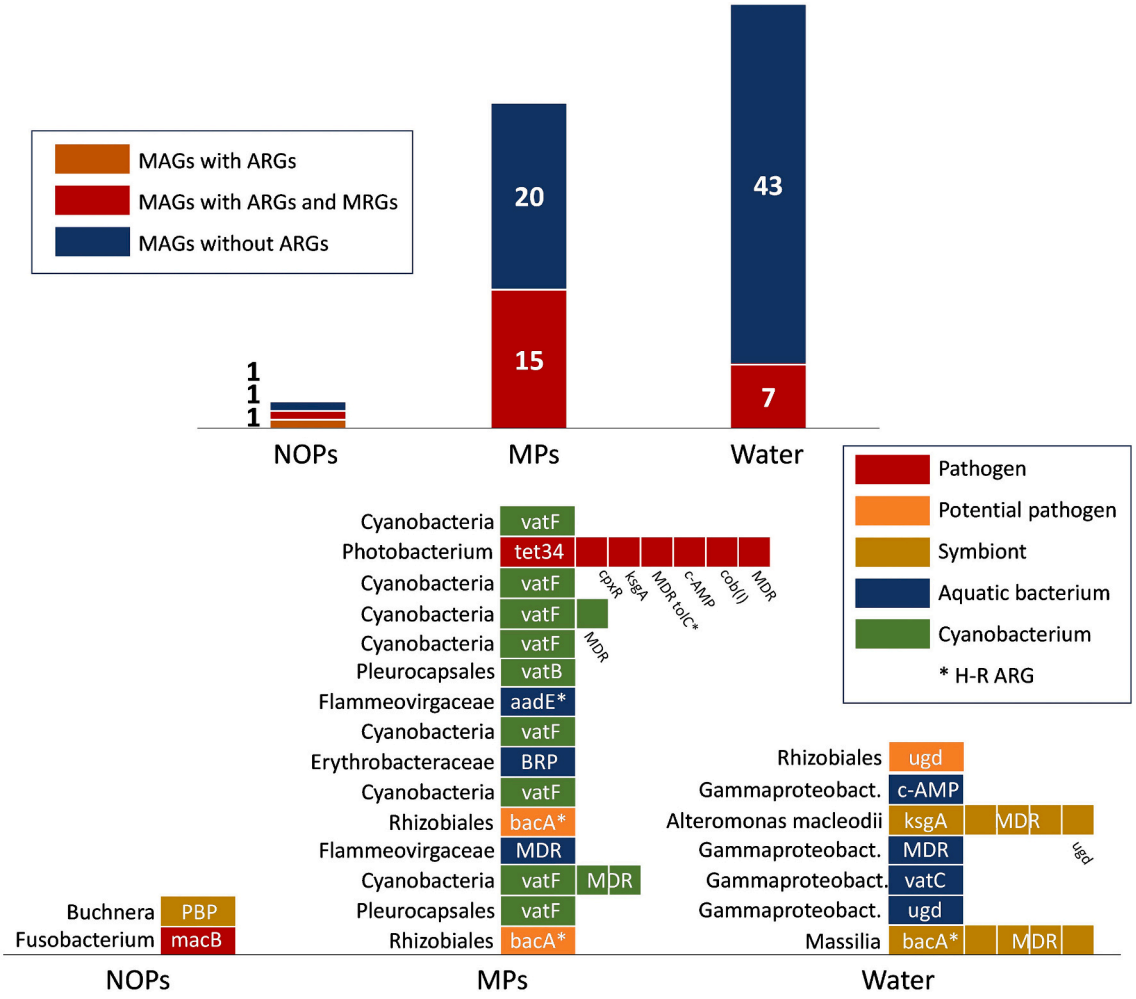

Fig. 7. Distribution of the Metagenome assembled genomes (MAGs). In the upper graph MAGs are presented by substrate and with or without ARGs. In the lower graph MAGs are associated to the bacterium having the MAG in its genome, with additional detail on the identified ARGs and on the bacterium. H-R ARG means high-risk ARG (for human health).

MPs showed the higher proportion of ARGs-containing MAGs than the planktonic communities. In detail, MPs harbored 15 MAGs containing ARGs and MRGs. From the water, 7 MAGs containing ARGs and MRGs were found. From the NOPs only three MAGs could be found, two of which presented ARGs (Fig. 7A). Focusing on the ARG-containing MAGs and related taxonomy, one MAG from NOPs was assigned as a potential pathogenic genus: *Fusobacterium*. Water MAGs were mainly aquatic taxa and symbionts (*Alteromonas macleodii*, genus *Massilia*) as shown in Fig. 7B. MP recovered MAGs were mostly cyanobacterial (nine out of fifteen), three MAGs were potential pathogenic bacteria: the order *Rhizobiales* containing the high risk ARG *bac*A and the genus *Photobacterium*, showing multi-resistance including the high risk ARG *tol*C in two copies and the tetracycline resistance gene *tet*34 (Fig. 7B, Supplementary Table 4). The contigs harboring ARGs underwent further examination to assess the presence of mobile genetic elements (MGEs), resulting in the identification of 8 MAGs and a total of 10 contigs where ARGs and MGEs co-occurred (Supplementary Fig. 2). Cyanobacteria MAGs retrieved from MPs presented a recurring pattern characterized by the presence of the two "phage, structural" and "phage chaperone" genes *gro*S and *gro*L associated with the MLS ARG *vat*F. From the MPs, the *Pleurocapsa* sp. MAG displayed stability/transfer/defense genes in proximity to the MLS *vat*F, while *Photobacterium malacitanum* MAG contained two contigs with multidrug ARGs: *acr*B and the high-risk *tol*C, both associated with replication/recombination/repair genes (*rep* and the topoisomerases *par*C and *par*E, found on plasmids). Both potential symbiont MAGs in water presented ARGs associated with MGEs. The *Massilia* sp. MAG showed the co-presence in the same contig of the high-risk ARG *bac*A and transfer and phage infection/regulation related genes, while another contig contained a multidrug resistance gene *emr*E in proximity to mobility genes related to transfer, replication/recombination/repair, and close to a CRISPR site, suggesting the potential mobility of the genomic site. The contig also presented prophage genes and demonstrated the signature of eight sites of prior HGT (Alien Hunter, green sites, Supplementary Fig. 2). *Alteromonas macleodii* MAG presented the peptide ARG *ugd* in proximity of transposases. From the NOP MAGs, the genus *Buchnera aphidicola* MAG showed the co-presence of two ARGs: the bata-lactam PBP-1B and a mutation conferring resistance to Pulvomycin and phage related mobility genes together with replication/recombination/repair multiple genes; the mobility potential of the genomic area is also underlined by prior HGT events (Alien Hunter green highlighted area, Supplementary Fig. 2).

4. Discussion

We focused our research on surface waters in the Northern Tyrrhenian Sea, an environment we selected for three reasons: it is among the most studied seas in the world (and specifically when it comes to MPs); it is considered one of the seas in the world most exposed to fast and extreme changes due to global warming (MedECC, 2020); and it is affected by long-term plastic pollution, started during the infancy of

plastic production and discharge into the environment.

MP pollution in the Tyrrhenian Sea is largely dominated by PE and PP particles, hard substrates with similar behavior in water, somehow offering very comparable ecological niches to bacteria. The strong prevalence of these substrates is further accentuated by their high buoyancy, potentially introducing a bias in commonly used MP sampling procedures (Sathicq et al., 2022; Wu, 2022). This simplifies our analyses, allowing the evaluation of MPs as a whole, without accounting for the distinct ecological impacts that MPs of varying chemical compositions may have on bacterial communities (e.g., different bacteria forming the biofilms on tire wear particles, hard plastics, bioplastics; Pinnell and Turner, 2019; Sathicq et al., 2022). It also enhances the comparability of our results with the largest majority of the studies on MPs that employ the same methodology.

We observed that the impact of anthropogenic coastal activities in the Tyrrhenian Sea is not the main driver regulating the abundance and diversity of MPs. The strong surface currents that mix the waters of this sea undoubtedly prevail over the former, both when anthropogenic impacts are limited (CT) and when they are more intense (FdM). In fact, it appears that the Tyrrhenian Sea, which suffers from historical MP pollution, is under conditions that do not allow us a straightforward identification of the hotspots for the release of MPs.

We demonstrated that the bacterial communities forming biofilms on the different particles (MPs or NOPs) were very different from the pelagic microbial communities, but very similar to each other. Planktonic (free-living) communities were predominantly composed of well-known marine bacteria (e.g., SAR11, SAR116, *Alteromonas* spp., *Pseudoalteromonas* spp.), as observed in other studies (Logares et al., 2014; Sunagawa et al., 2020). Yet, these taxa were either scarce or entirely absent within the biofilms. This is not surprising as these bacteria are perfectly adapted as free-living forms in open waters and gain competitive advantages in being in environments where the main selective force is the competition for nutrients and micronutrients (Tilman et al., 1982).

The plastisphere and biofilms on NOPs were dominated by the *Vibrionaceae* family (*Vibrio*, *Aliivibrio*, *Photobacterium*), along with other classical biofilm-forming bacteria (*Buchnera*, *Pseudoalteromonas*). A few bacteria, such as cyanobacteria, *Massilia*, and *Shewanella*, were consistently found in similar proportions across all samples (NOPs, MPs, water) and can thus be considered ubiquitous throughout our sampling campaign.

The overwhelming predominance of *Vibrionaceae* on particles had a negative impact on the establishment of other potential pathogenic bacteria on these substrates. In fact, the pathobiome exhibited exceptionally low diversity within biofilms. On NOPs and on MPs, *Vibrionaceae* accounted for 40 to 70 % of the reads, demonstrating the already well-studied ability of *Vibrio* and other members of the *Vibrionaceae* family to colonize substrates including MPs (Mincer et al., 2023; Pedrotti et al., 2022). The highly limited presence on MPs of other potential pathogens suggests that MPs cannot be considered as a new substrate for allochthonous bacteria of anthropogenic origin in marine waters but, at its best, be considered as an additional substrate to NOPs for *Vibrionaceae* to proliferate.

An easy way to evaluate the possible anthropogenic impact on an environment is to analyze the presence and abundance of ARGs defined as high-risk for human health (Zhang et al., 2021). In our samples the number of high-risk ARGs was limited and randomly distributed, without any specific trend between sites or substrates. However, the fluoroquinolone resistance gene, *qnr*S, was detected within all biofilms but not detected in water. Fluoroquinolones are not only a very important class of human antibiotics of synthetic origin, but they are also, together with tetracyclines, the most used antibiotics in fish farming, where they are administered to prevent or to treat several animal diseases (Amable et al., 2022). The massive use of fluoroquinolones in aquaculture is promoted by the large spectrum of bacteria that can be targeted by this antibiotic class and by its long-term stability in open waters (differently from the very labile beta-lactams; Hektoen et al., 1995). Their use as a growth factor increases the productivity of fish farms, but it is also implicated in the spread of resistances not only in the produced fish but also in marine microbial communities (Buschmann et al., 2012; Tomova et al., 2015). Although the negative effects of the misuse of this antibiotic class are well known (Amable et al., 2022) and their use is regulated in many countries, their impact on marine environments is still strong. The observed enrichment of fluoroquinolone resistance genes on particles suggests a role of NOPs and MPs in the spread and transport of putative fluoroquinolone resistant bacteria across marine waters. Similar patterns are evident for tetracycline resistance genes, but caution is needed when associating their presence to anthropogenic activities, given that tetracyclines are of natural origin unlike fluoroquinolones.

The dominant role as carriers of antibiotic resistances played by particles was also proved when MAGs were reconstructed: about half of the MAGs from particles included ARGs, while in water their proportion was much lower. Partly surprising, none of the MAGs including ARGs were associated with the *Vibrio* genus, and the only *Vibrionaceae* including ARGs was assigned to a potential multidrug resistant *Photobacterium*, reconstructed from a MP sample. In fact, most of the MAGs including ARGs from MP samples belonged to cyanobacteria. While it is true that cyanobacteria have previously been suggested as reservoirs of ARGs and as promoters for their spread (Wang et al., 2020), the gene most frequently detected within cyanobacterial MAGs here was *vat*F, which encodes for a streptogramin A acetyl transferase (Seoane and García Lobo, 2000), which is not considered to be specifically related to anthropogenic pressures. Considering the propension for cyanobacteria to develop biofilms and, thus, to promote cell proximity, our observation calls for more attention to the role of cyanobacteria in the cycle of antimicrobial resistance in marine environments. In MAGs reconstructed (mainly) from biofilms, ARGs (including high-risk ones) were identified in association with genes attributed to bacteriophages, which are recognized as ARG carriers (Calero-Cáceres et al., 2017; Sabatino et al., 2023), as well as with other MGEs like transposons and plasmids. Furthermore, mainly within MAGs reconstructed from the plastispheres, the co-occurrence of ARGs and MRGs suggests a potential co-selection between these two genetic elements, which is consistent with previous findings (Di Cesare et al., 2021). Overall, these results demonstrate the potential of biofilms from MPs and NOPs to act as reservoirs for the selection and dissemination of ARGs within marine ecosystems.

The high similarity between the microbiomes and the resistomes on MPs and NOPs in our study is in partial contrast with the observations by Dussud et al. (2018) from the Tara-Mediterranean expedition (Ghiglione et al., 2023). In their campaign, they could detect a number of differences between the microbial communities on MPs and particulate organic carbon (defined as phycospheres and detritospheres, Ghiglione et al., 2009). In their study, both the composition of MPs and the differences among the bacterial communities within biofilms compared to those in the water were extremely similar to what was observed in our work. However, at the same time, certain distinctions, especially within the rare biosphere (Pedrós-Alió, 2012) and in the distribution of cyanobacteria (not observed in our study) led to the conclusion that MPs could provide a novel exclusive niche for some bacteria. A similar conclusion has also been reached by other researchers based on an experimental study comparing plastics of different chemical compositions (Kirstein et al., 2019). Although we acknowledge our colleagues' conclusion, in our dataset the subtle differences observed between the communities on MPs and NOPs, do not allow us to define MPs as an additional ecological niche for bacteria. Intriguingly, it is possible to speculate that in ecosystems exposed to historical MPs pollution, biofilm-forming bacteria may have adapted to colonize them, erasing once apparent differences.

Instead, our research has highlighted an incremental ecological role of PE- and PP-like MPs in coastal waters, rather than an additive one. This does not imply that MPs are not an environmental threat, because

in a system like the Mediterranean Sea, which will experience significant fluctuations due to climate change, the communities living in biofilms could assume an increasingly important role, and the number of pathogens and antibiotic resistant bacteria selected within them could consequently increase.

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CRediT authorship contribution statement

Andrea Di Cesare: Writing – review & editing, Writing – original draft, Validation, Supervision, Data curation, Conceptualization. **Maria Belen Sathicq:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Tomasa Sbaffi:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Raffaella Sabatino:** Writing – review & editing, Methodology, Investigation. **Dario Manca:** Writing – review & editing, Methodology, Investigation. **Florian Breider:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Sylvain Coudret:** Methodology, Investigation, Data curation. **Lee J. Pinnell:** Writing – review & editing, Software, Methodology, Formal analysis. **Jeffrey W. Turner:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Gianluca Corno:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw reads are publicly available in NCBI under the accession number PRJNA1021400.

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