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From rivers to marine environments: A constantly evolving microbial community within the plastisphere

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ABSTRACT

Plastics accumulate in the environment and the Mediterranean Sea is one of the most polluted sea in the world. The plastic surface is rapidly colonized by microorganisms, forming the plastisphere. Our unique sampling supplied 107 plastic pieces from 22 geographical sites from four aquatic ecosystems (river, estuary, harbor and inshore) in the south of France in order to better understand the parameters which influence biofilm composition. In parallel, 48 enrichment cultures were performed to investigate the presence of plastic degrading-bacteria in the plastisphere. In this context, we showed that the most important drivers of microbial community structure were the sampling site followed by the polymer chemical composition. The study of pathogenic genus distribution highlighted that only 11% of our plastic samples contained higher proportions of *Vibrio* compared to the natural environment. Finally, results of the enrichment cultures showed a selection of hydrocarbon-degrading microorganisms suggesting their potential role in the plastic degradation.

1. Introduction

European plastic demand was to 50.7 million tons in 2019), mainly for packaging and the building and construction sector (Plastics Europe, 2020). The most widely used polymers in Europe are polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PU), polyethylene terephthalate (PET) and polystyrene (PS). Forty-two percent of plastics across Europe are reported to be recycled (Plastics Europe, 2020), while a major part of discarded polymers ends up in landfills and finally in oceans causing a global environment issue (Geyer et al., 2017). The five subtropical oceanic gyres have been identified as a vast accumulation zones in the ocean, but the Mediterranean Sea has comparable average density of plastic debris (PD), *e.g.*, between 1000 and 3000 tons of floating plastics in 2013 (Cózar et al., 2015). The hydrodynamics of the Mediterranean semi-enclosed basin, added to the high human pressure, can explain the floating plastic accumulation (Cózar et al., 2015; Boucher and Friot, 2017). The combination of mechanical abrasion, hydrolysis, photo- or thermal-oxidation and the biodegradation of PD leads to the formation of three categories of size fragments: macro- (25–1000 mm), meso- (5–25 mm) and microplastics (<5 mm) (Andrady, 2011; GESAMP, 2019). Due to their variation in density, size and surface area, the composition and the quantity observed in the different environmental compartments are different, *e.g.*, PP and the PE are mainly found in the surface water (Debroas et al., 2017), while PET is mainly found on sediment (Andrady, 2011).

Once entered in the aquatic environment, PD are quickly colonized by microorganisms such as bacteria, fungi, algae and tiny invertebrates, forming a distinct ecological niche named the "plastisphere" (Zettler et al., 2013; Delacuvellerie et al., 2022). Plastic bacterial biofilm structure from marine environment evolves gradually with the immersion time of the polymer: *Gamma-* and *Alphaproteobacteria* constitute the primary colonizers, while *Bacteroidetes* represent the secondary colonizers (De Tender et al., 2017a, b). Moreover, the position in the water column (floating plastics *vs* plastics on the sediment), geographical location, chemical composition of plastic polymer, or seasons can influence the bacterial community structure (Delacuvellerie et al., 2019,

Abbreviations: PP, polypropylene; PE, polyethylene; PVC, polyvinyl chloride; PU, polyurethane; PET, polyethylene terephthalate; PS, polystyrene.

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2021; Oberbeckmann et al., 2014, 2021; Frère et al., 2018; Zettler et al., 2013; Debroas et al., 2017). Two studies have shown that the plastics' shape and size do not influence the microorganism structure (Frère et al., 2018; Cheng et al., 2021), while one recent study has shown the influence of the polymer colors (Wen et al., 2020). Amaral-Zettler and colleagues described the "cycle life" of plastic showing that plastics originate from land source and, are transported *via* rivers to the ocean rivers (Amaral-Zettler et al., 2020). It is therefore essential to study the bacterial structure of plastics sampled across a transect that includes rivers, estuary and inshore from the same geographical zone, in our case the Ligurian Sea.

Marine PD impact the ecosystem's health because species can use it as dispersion vector by invasive or pathogenic species, changing the structure of natural ecosystems (De Tender et al., 2015; Aliani and Molcard, 2003). Harmful algae, *e.g.*, *Coolia*, *Ostreopsis* and *Alexandrium*, have been detected on the surface of marine PD (Garcés and Camp, 2003), and the *Vibrio* genus, containing numerous pathogenic species, is often found in higher concentrations on plastic than in the natural environment (lower than 1% in seawater; Thompson and Polz, 2006; Frère et al., 2018). Many bacteria attached to the PD surface are opportunistic microorganisms and can grow on other types of support such as wood, glass or leaves (Lyons et al., 2010). Moreover, bacteria specialized in complex carbon degradation are selected on plastics, such as hydrocarbon-degrading bacteria (*Hyphomonas*, *Oceaniserpentilla*, etc.), supporting the fact that these microorganisms can play a role in plastic degradation (Oberbeckmann et al., 2016; Zettler et al., 2013). Our previous study that compared plastics from the same geographical location showed significant differences in the bacterial community structure found on floating marine PD in respect to PD collected in sediments (Delacuvellerie et al., 2019). Moreover, an enrichment culture was used to select candidates for the plastic degradation, highlighting the statistically significative enrichment of a hydrocarbon-degrading bacteria, *Alcanivorax* genus, on PE (Delacuvellerie et al., 2019).

Most publications reporting on the plastisphere structure focus on one or two parameters influencing the bacterial communities in marine environment. In this present study, our extensive sampling, *i.e.*, 107 pieces of plastics in 22 geographical locations, allowed us to compare and contrast the impact of numerous physico-chemical parameters on the plastisphere: (1) type of polymer (*e.g.*, PP, PE, PS); (2) size (macro-, meso-, microplastic), (3) color; (4) environment (seawater inshore, harbor, freshwater river) and (5) sampling site. In this way, the primary aim of this study was to determine the most important drivers controlling the composition of bacterial communities on PD using 16S rRNA amplicon sequencing. In addition, we characterized the distribution of pathogenic bacteria colonizing the polymer surface and the hydrocarbon-degrading bacteria by enrichment cultures, that could constitute candidates for plastic degradation.

2. Materials and methods

2.1. Plastic sample collection

PD were collected in the Ligurian Sea from the river Var to the Port of Saint Louis du Rhone, from July 21st, 2019 to August 9th, 2019 as part of Expedition MED 2019 Citizen Science laboratory aboard the sailing boat Free Soul, in four aquatic ecosystems: river, estuary, harbor and inshore (Fig. S1). Inshore plastic sampling was performed using a manta net towed by the boat for a period of 30 min at the average speed of 2 knots. From the collected samples with the manta net, the PD items of bigger dimensions and with a more evident biofilm were selected. Additional samples of PD were collected manually in five harbors (Port of Saint Laurent du Var, Vielle Darse de Toulon, Port Saint Louis du Rhône, Ecluse du port de Saint Louis du Rhône, Vieux Port de Marseille) and in one river (Var). Once collected, PD were immersed in water collected from the sampling location in sterile 50 ml falcon tubes and stored at 4 °C during transportation. A table summarizes all the PD sampled by Expédition MED (Table 1) as well as the physico-chemical parameters of the water (Table S1).

2.2. Plastic sample processing

Microbial biofilms were removed from the PD surface and used for the bacterial community structure analysis and the enrichment culture. In this way, PD were rinsed in sterile seawater (35 g/l of Sigma Sea Salt) for marine samples or in sterile freshwater for river samples to remove microorganisms not attached to the biofilm. Biofilms were scrapped with a sterile scalpel blade to recover a maximum of the biomass. Subsequently, the plastics were rinsed with ethanol 70% (V/V) and deionized water to remove organic coatings and dried at 30 °C for 1 day. After this, the PD were used for analyses of their chemical composition.

From the total 107 PD samples, only 92 had enough biofilm to carry out DNA extraction to study microbial communities. For the bacterial community analyses, the biofilm recovered from PD was used for the DNA extraction (Table S1; number of samples sequenced by aquatic ecosystems: 4 for estuary, 4 for freshwater river, 19 for harbors, 65 for seawater). From 11 of these 92 PD samples that had a thicker biofilm, allowed us to save a portion of the biofilm for enrichment cultures to study the ability of bacteria to degrade plastic (Delacuvellerie et al., 2019) (Table S2).

2.3. Polymer chemical composition

The chemical composition of the plastic was analyzed using Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy (Bruker, Tensor 27) with OPUS 6.5 software. The spectra were acquired over the wavelength range of 4000–600 cm^{-1} with 64 spectral scans (Mahoney et al., 2013). The size and the color of each plastic sample was collected in order to classify the plastic samples: macroplastics (25–1000 mm), mesoplastics (5–25 mm) and microplastics

Table 1
Summary of the plastic debris sampling in 22 geographical sites with the number of plastic pieces, the polymer chemical composition (polyethylene (PE), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET), not determined (N.D.)), the size and the colors by habitat types. The size classification and the color code was as based on GESAMP (2019) and Galgani et al. (2017), respectively.

Habitat types	Number of geographical sites	Number of plastic pieces	Polymer chemical composition	Colors	Size
River	1	4	PE: 3 PS: 1	Black: 1 None: 1 White: 2	Microplastic: 4
Estuary	1	4	PE: 4	None: 3 White: 1	Microplastic: 2 Mesoplastic: 2
Harbor	5	19	PE: 8 PS: 8 PP: 3	5 none 13 white 1 white and red	Microplastic: 7 Mesoplastic: 9 Macroplastic: 3
Inshore	13	65	N.D.: 6 PE: 44 PET: 1 PS:6 PP: 8	Black: 2 Blue: 8 Brown: 1 None: 20 Red: 4 White: 30	Microplastic: 27 Mesoplastic: 28 Macroplastic: 10

(<5 mm) (GESAMP, 2019). The color code used were chosen as suggested in EMODnet (Galgani et al., 2017).

2.4. Enrichment culture to assess plastic degradation

The thick biofilms recovered from 11 PD (four plastics from river, three from harbors and four from inshore; Table S2) were cultured in glass tubes containing 5 ml of low carbon source media (0.05% (W/V) of yeast extract), as described in Delacuvellerie et al., 2019 and with 2 cm^2 of clean plastic film (all the plastics were in film except the low molecular weight polyethylene (LMWPE) that which is in the pellet form). Briefly, the marine medium is composed of: 0.05% yeast extract, 0.2% ammonium sulfate, 3.5% salts (W/V, Sigma Sea Salt) and 1% trace elements (0.1% $MgSO_4{\cdot}7H_2O$, 0.1% $FeSO_4{\cdot}7H_2O$, 0.01% $ZnSO_4{\cdot}7H_2O$ 0.01% $CuSO_4{\cdot}5H_2O$ and 0.01% $MnSO_4{\cdot}5H_2O$) in 20 mM (N-morpholino) propanesulfonic acid (MOPS) at pH 8, adapted from Yoshida et al., 2016). Regarding the freshwater river samples, the same medium without sea salt was used. Five polymers were tested for each sample: LDPE, LMWPE, PET, PS and PET (Table S3). The plastics were sterilized in 70% ethanol overnight and dried in petri dishes under a laminar flow hood. Enrichment cultures were shaken at 140 rpm at 30 °C. After 80 days of culture, formation biofilms were visible with the naked eye on 48 tubes (Table S2). The bacterial communities from these biofilms were analyzed by 16S rRNA amplicon sequencing.

2.5. DNA extraction and 16S rRNA amplicon sequencing

DNA was extracted from the biofilm on both the PD samples collected in the field and from the plastic pieces used in the enrichment cultures. The DNA extraction was performed with the biofilm DNA isolation kit (Norgen Biotek Corp.) following the manufacturer's instructions. Only samples with a minimum of 1 ng/μl of DNA concentration were sequenced (Table 1 and Table S1). A total of 92 samples from plastics debris collected in the field were sequenced (4 from estuary, 4 from freshwater river, 19 from harbors and 65 from seawater) and 48 samples from the enrichment cultures (Table S2).

A 460 bp fragment of the hypervariable V3–V4 region of the 16S rRNA gene of bacteria and archaea was amplified by PCR using the following primers: 806R (5′-GGACTACNNGG GTATCTAAT-3′) and 341F (5′-CCTAYGGGRBGCASCAG-3′) (Nunes et al., 2016) supplemented by overhang (adaptator illumina):

Forward overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[341F]

Reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[806R]

The high-throughput sequencing by the GIGA (Liège, Belgium) was used to perform the sequencing of 2 × 300 bp paired-end with the Illumina® MiSeq® platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The MG-RAST pipeline (version 4.0.3, https://www.mg-rast.org/) was used for the contingency table on the mate pairs (forward and reverse) at the genus level, at a sequence identification level of 97%, using *Greengenes* database (Keegan et al., 2016). The following parameters were chosen on MG-RAST: maximum low quality basepairs of 6 and minimum quality of 25 pb. Adapter sequences were removed by a bit-masked k-difference matching algorithm. Sequences were filtered based on length, quality values and number of ambiguous bases. Finally, contamination by host DNA and PCR artifacts were removed. 16S rRNA amplicon sequences were deposited at the SRA (Sequence Read Archive) in NCBI under the accession number PRJNA724000.

2.6. Diversity indexes

The bacterial diversity of the plastic samples from the field and from enrichment cultures were studied. Rarefaction curves were performed to verify the sequencing quality using the *PAST* software (Fig. S2) (Hammer et al., 2001). The richness and equitability indices, corresponding to the alpha-diversity, were calculated on the rarefied data (14,387 reads counts for *in-situ* sampling and 6770 reads counts for the enrichment cultures, *Limma* RGui package). One sample containing less than 6770 reads was excluded (P4-01-PET; 5425 reads counts, Fig. S2). Using OTU presence/absence, Venn diagrams were created to assess the distribution of these OTUs according the sampling site or chemical composition of polymer, using *VennDiagram* RGui package (Hanbo and Paul, 2011). Multivariate analysis PERMANOVA was used to study the beta-diversity using *vegan* RGui package (Wang et al., 2012). The statistically significative variation between the conditions was calculated using the Bray-Curtis dissimilarity with 10,000 permutations. Principal Component Analysis (PCA) using arrows to show the influence of the taxonomy was used (Krause et al., 2020). The presence of human pathogenic bacteria was investigated using the Bode Science Center database (https://www.bode-science-center.com/center/relevant-pathogens-from-a-z.html) and pathogenic species of marine flora and fauna were investigated in literature (Kirstein et al., 2016; Virsek et al., 2017; McCormick et al., 2014; Amaral-Zettler et al., 2020).

2.7. Heatmap and validation of response groups (RGs)

Heatmap was performed on the bacterial communities from the enrichment cultures. OTUs statistically significantly affected by the condition were identified using a negative binomial distribution and Generalized Linear Model (nbGLM). This deviance analysis revised by 1000 resampling iterations of the residual variance (*mvabund* Rgui package; Dixon, 2003). Eighty-three OTUs affected by salinity (seawater *vs* freshwater) were plotted on a heatmap. The Monte-Carlo simulation, comparing the RG clustering with a null-model containing all the OTUs, validated four response groups (Fig. S3).

3. Results and discussion

3.1. Chemical characterization of plastic polymer sampled across the Ligurian Sea

The present analysis was based on the sampling of 107 pieces of floating PD across the Ligurian Sea (Fig. S1). The chemical identification of polymer composition by ATR-FTIR showed that PE, PS and PP were the most abundant type of surface marine plastics (Fig. S4), with 69%, 18% and 12%, respectively. These polymers have a low density and have specific gravities of approximately 0.94 (PE), 1.05 (PS) and 0.84 (PP), which are lower than the specific gravity of seawater (approximately 1.025). Only one plastic was identified as PET, plastic mainly found on the sediment (1.37 of gravity) (Andrady, 2011).

PE, PS and PP are commodity and mainly single use plastics packaging and are the most common types of PD floating at sea surface in the marine environment, in accordance with their worldwide production (Debroas et al., 2017; Plastics Europe, 2020). They are the most abundant types of polymers in other studies of floating marine PD (Amaral-Zettler et al., 2021; Suaria et al., 2016).

3.2. Impact of physico-chemical parameters on the plastic bacterial composition

3.2.1. From rivers to oceans: an evolving plastisphere

The richness and equitability indexes were approximately 150 and 0.50 for each aquatic ecosystem (*i.e.*, inshore, estuary, harbor, river), respectively (Fig. 1A). The PERMANOVA analyses significatively highlighted the influence of different aquatic ecosystems and more precisely, the influence of the sampling site (Table 2; Fig. S1) on the bacterial community structure, both with a *p*-value of 1e−05. The result can be explained by differences in the location-related properties, such as salinity, pH, and temperature (Table S1). In our study, although a consistent number of replicates for aquatic ecosystems would have

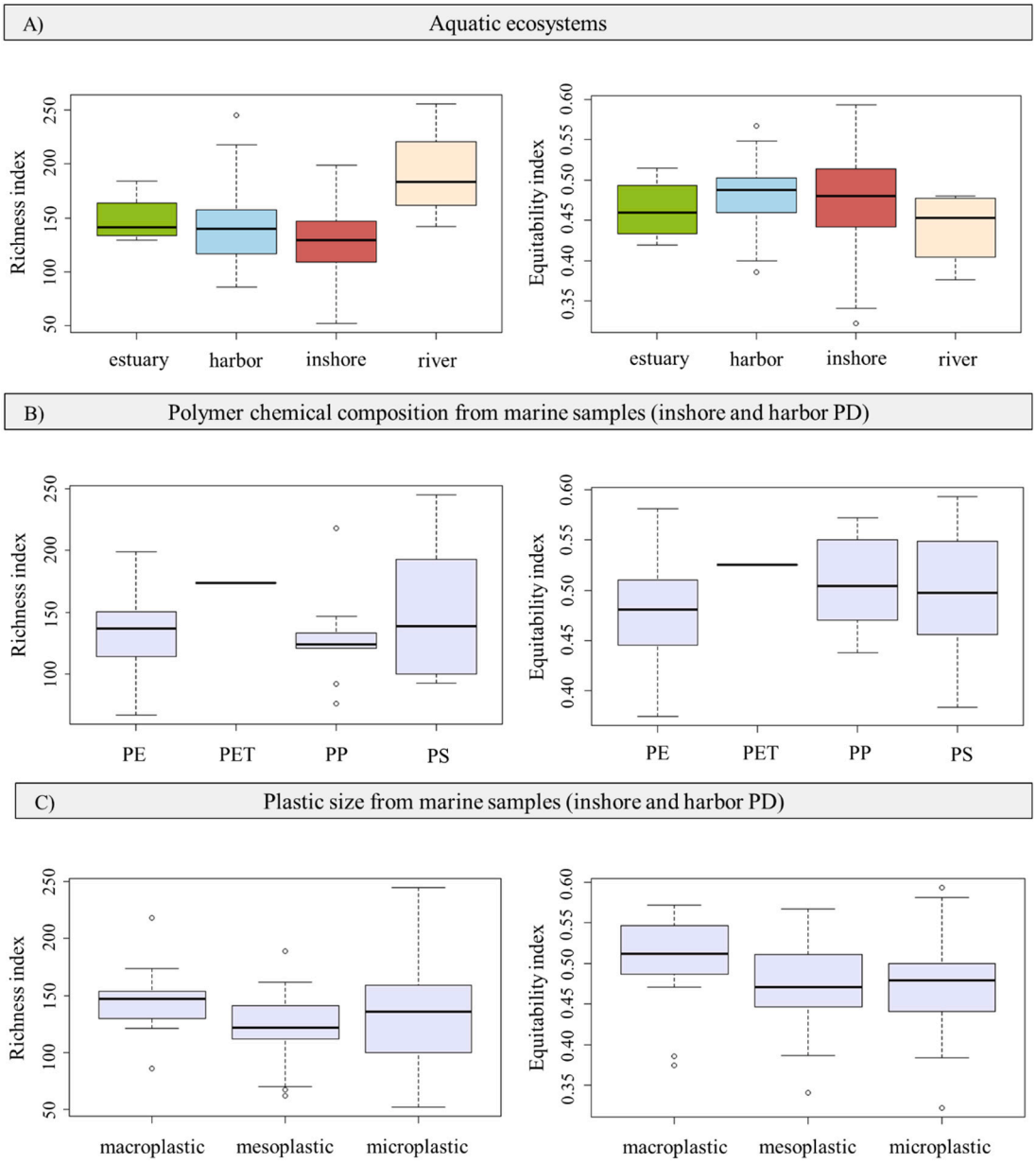

Fig. 1. Richness and equitability indexes of bacterial communities according to (A) the type of aquatic ecosystems: estuary (n = 4), inshore (n = 65), harbor (n = 19), river (n = 4); (B) the polymer chemical composition from marine water samples (inshore and harbor samples): polyethylene (PE; n = 52), polystyrene (PS; n = 14), polypropylene (PP, n = 11), polyethylene terephthalate (PET, n = 1) and (C) the plastic size from marine water samples (inshore and harbor samples): micro- (n = 34), meso- (n = 37), macroplastics (n = 13) obtained from 16S rRNA amplicon sequencing. ANOVA showed no significant difference.

facilitated the statistical analysis, we observed that the microbial structure was affected by different levels of salinity (seawater, freshwater, brackish water) with a *p*-value of 1e−05 (Table 2). This is accordance with previous study showing that location-related environmental parameters, such as salinity, temperature and oxygen content, appeared to be correlated to the bacterial community diversity (De Tender et al., 2015).

The plastisphere in river was mainly represented by *Cyanobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Cyanobacteria* and *Deinococcus-Thermus* with a relative abundance of 10.7%, 7.5%, 7.5% and 5.2%, respectively (Figs. S5 & 2). Few studies reported the biofilm structure in rivers but, as in the marine environment, plastic is a distinct environmental niche mainly composed of *Beta-*, *Gammaproteobacteria* and *Bacteroidetes* (Hoellein et al., 2014; McCormick et al., 2014; McCormick et al., 2016; Amaral-Zettler et al., 2020, Amaral-Zettler et al., 2021). *Cyanobacteria* and diatoms were shown to inhabit the surface of PD, thus contributing to the primary production (Amaral-Zettler et al., 2020; Delacuvellerie et al., 2022). The most represented genera from river samples were *Chamaesiphon*, *Deinococcus* and *Hymenobacter* with 8.3%, 5.2% and 1.3%, respectively (Fig. 2). Samples from estuary, inshore and harbors displayed a similar bacterial structure at the phylum level (Fig. S5). In accordance with the literature (Bhagwat et al., 2021; Zettler et al., 2013), the bacterial communities were mainly composed of *Bacteroidetes* (26%), *Gammaproteobacteria* (10%) and *Alphaproteobacteria* (22%). *Gamma-* and *Alphaproteobacteria* were characteristic of the primary colonizers in the plastisphere in the marine environment, while *Bacteroidetes* are known to be secondary colonizers (De Tender et al., 2015). Amaral-Zettler and colleagues performed taxonomic analyses of the plastisphere in the Ligurian Sea in 2018 (Amaral-Zettler et al., 2021). Although the taxonomy of the Ligurian Sea samples showed *Alphaproteobacteria*, *Gammaproteobacteria* and *Cyanobacteria* as dominating bacterial groups (Amaral-Zettler et al., 2021), we observed that

Table 2
PERMANOVA analyses using Bray-Curtis dissimilarity with 10,000 permutations on the different conditions: sampling site, type of aquatic ecosystems, plastic size, plastic color and chemical composition. The salinity having an impact on the bacterial community composition, the followed factors, *i.e.*, plastic debris size, plastic debris color and plastic debris chemical composition (polyethylene (PE), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET)), were calculated from seawater samples (Table S1). Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Factors tested	*p*-Value	r^2	Signif.
One-way PERMANOVAs (Bray-Curtis dissimilarity, 10,000 permutations)			
1. Sampling site	1.00E−05	0.23474	***
2. Type of aquatic ecosystems (river (n = 4), harbor (n = 19), estuary (n = 4), inshore (n = 65))	1.00E−05	0.10138	***
3. Salinity (brackish water (n = 4), seawater (n = 83), freshwater (n = 4))	1.00E−05	0.0767	***
4. Plastic size (micro- (n = 34)/meso- (n = 37)/macroplastic (n = 13))			
From seawater (harbor and inshore):	0.09694	0.03207	/
5. Plastic color (black (n = 2), blue (n = 8), brown (n = 1), none (25), red (n = 4), white (n = 43))			
From seawater (harbor and inshore)	0.4349	0.06141	/
6. Plastic chemical composition (PE (n = 52), PS (n = 14), PP (n = 11))			
From seawater (harbor and inshore)	0.00913	0.08334	**

Cyanobacteria were less represented in our marine PD. Exposure time and season are factors influencing biofilm formation and the bacterial composition vary over time (Oberbeckmann et al., 2014, 2016). At the genus level, *Cytophaga*, *Saprospira*, *Tenacibaculum*, unclassified from *Gammaproteobacteria* and from *Alphaproteobacteria* were the most genera represented across the inshore, estuary and harbor samples with small percentage variations (Fig. 2). The *Tenacibaculum* genus, with most species forming biofilm, contains species pathogens for several fish, *e.g.*, *T. maritimum*, *T. soleae*, *T. discolor* or *T. gallaicum* (Fernández-Álvarez and Santos, 2018), and has already been associated with bacteria composing the plastisphere (Oberbeckmann et al., 2016). *Saprospira* has already been associated with plastic community from PP (Zettler et al., 2013). Despite the different geographical sample, there was a homogeneity in the most represented genera. However, some genera were mainly represented on one site. For example, *Marinobacter*, bacteria degrading hydrocarbon, was mainly represented on EM19-P1 (7%) (Duran, 2010). *Cyclobacterium* (11%) and Pseudoalteromonas (9%) were most represented on EM19-01 and EM19-P5, respectively.

The presence of several genera such as *Cellulophaga*, *Paenibacillus* or *Brevibacillus*, less represented on the PD were interesting. Indeed, *Cellulophaga* genus was represented on average at less than 0.1% on all sampling site, except for river PD, which did not show this genus. *Cellulophaga*, mainly found in marine alga and beach mud, also known as cellulose-degrading bacteria (Abt et al., 2011), synthesizing extracellular hydrolases can metabolize cellulose as carbon source. Plastic oxidized by UV, or other physico-parameters leading to the production of ester-link in the plastic matrix, could be altered by *Cellulophaga*'s enzymes (Krueger et al., 2015). Indeed, natural polymers, such as proteins, chitin or cellulose, are depolymerized *via* the cleavage of the hydrolytic bonds, *e.g.*, ester-links. Therefore, plastics containing hydrolysable backbone structures might be degraded by these enzymes (Krueger et al., 2015). *Paenibacillus* genus, representing 0.1% of the river bacterial communities, contains also cellulose-degrading bacterium (Wang et al., 2008), while the *Brevibacillus* genus, found on EM19-F1 and P3 samples at 0.4%, showed the following species, *i.e.*, *Brevibacillus borstelensis* – known to degrade PE – as well other pathogenic species of invertebrates, *e.g.*, *B. thuringiensis* and *B. laterospora* (Hadad et al., 2005; Ruiu, 2013; Bravo et al., 2007). Bacteria present in the

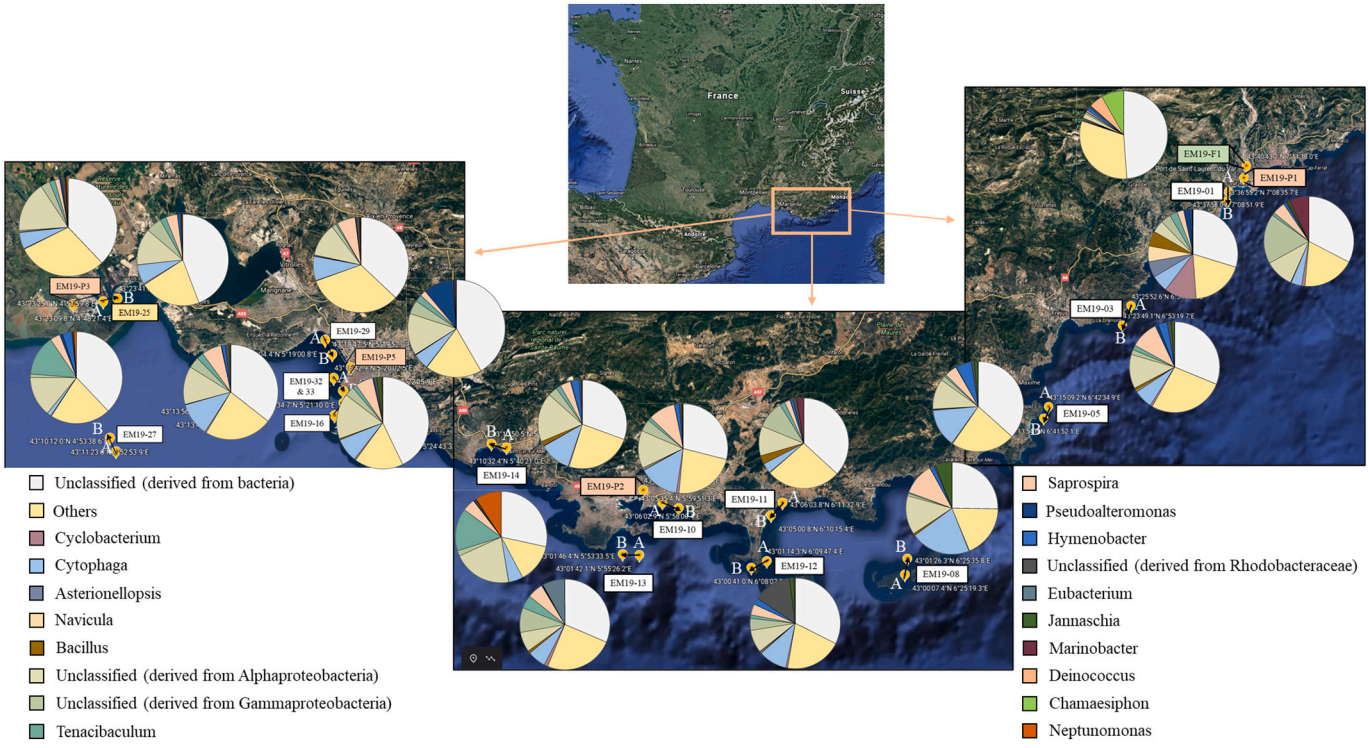

Fig. 2. Map of all the 2019 sampling from the Mediterranean Sea. Samplings by a manta net are represented as followed: first point (A) shows the beginning of the sampling and the second (B), the end; the arrow show the direction of the boat. The plastic samples in a harbor are represented in orange rectangle; the freshwater river in green; marine water inshore in white and estuary in yellow. The pie chart represented the percentage of genera based on 16S rRNA amplicon sequencing for all sampling sites. Taxa displaying a proportions <5% were gathered into "Others" category. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

plastisphere could have a role in the plastic degradation and/or in the transfer of pathogenic species.

Finally, the Venn diagram (Fig. S6) showed the genus dispersion according to the aquatic ecosystems showing that 178 genera (corresponding to 48% and 24% of river and inshore genera, respectively) were shared between the four ecosystems, confirming previous findings, demonstrating that a "core" of bacteria was shared among all polymers (Kirstein et al., 2018). Interestingly, 80 genera were shared between marine and freshwater samples and could be explained by the fact that a fraction of PD was exported by rivers into the marine environment, biofilm development starting in freshwater and continuing in seawater (Schmidt et al., 2017; Amaral-Zettler et al., 2020). Or there might be bacteria that are generalist and can live both in seawater and freshwater.

3.2.2. Influence of the polymer chemical composition on the plastisphere

The second studied parameter was the chemical composition of PD. The richness and equitability indexes were similar between the polymers from marine environment (PP, PET, PS, PE; Fig.1B). However, the bacterial community structure was affected by the polymer composition (PERMANOVA analysis, Table 2, p-value = 0.00913). Results from the previous section highlighted that the bacterial communities from plastics were dependent on the environment parameters, *e.g.*, sampling site. However, the results of polymer type analysis provide an insight of the plastisphere composition expected in the aquatic environment since these PD were sampled from a large study area composing by 18 sampling sites in marine water (harbor and inshore samples) influenced by different environmental parameters (Table 1). As shown in the Venn diagram, the overlap of bacterial genera according to the pooled plastic chemical composition sampled in the seawater (Fig.3). Interestingly, the majority of genera (147) were shared between the four types of plastic (PET, PP, PS and PE). However, several genera were identified as specific for a given plastic, *e.g.*, 24% of genera composing the PE bacterial communities were specific to this polymer. The variation of the taxonomic classification depending on the polymer chemical composition is shown on Fig. 4. Interestingly, *Bacteroidetes* was most abundant on PP than the others plastics. At the family level, *Cyclobacteriaceae* and *Cytophagaceae* are most represented on PP while *Oceanospirillaceae* and *Bacillariaceae* are most abundant on PET. *Saprospiraceae*, *Rhodobacteraceae*, *Alteromonadaceae* and *Flavobacteriaceae* were abundant on the plastics and were already found on previous study in marine environment (Oberbeckmann et al., 2016; Zettler et al., 2013; Bhagwat et al., 2021). Interestingly, these families contained members known for their ability in the complex carbon degradation and for their marine biofilm lifestyles (Oberbeckmann et al., 2016). Finally, some genera were more present on one type of polymer than the others, *e.g.*, *Marinobacter* on PS or *Cyclobacterium* on PP.

The chemical composition of plastics can significantly influence the bacterial communities: (i) the chemical structure of the polymer, (ii) the particle shape (ropes and sheets, De Tender et al., 2017a), and (iii) chemical additives (plasticizers) (De Tender et al., 2015). The chemical function of the surface, the roughness, the hardness, the electric charge and the hydrophobicity all play a role and influence the biofilm formation due to the physico-chemical properties of the bacterial cell surface (Zhang et al., 2015; Renner and Weibel, 2011; Ganesan et al., 2022). For example, one recent study had shown that the same bacterial species (*Bacillus subtilis* and *Bacillus pumilus*) adhered better to PE and PVC surfaces than to PP and PET surfaces due to the intrinsic surface properties of the plastic's surface (Cai et al., 2019).

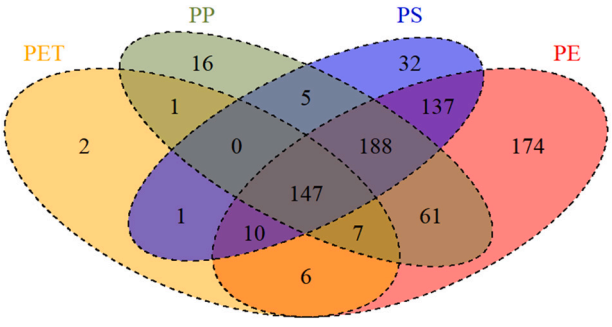

Fig. 3. Venn diagram showing overlap of bacterial OTUs according to the pooled plastic chemical composition (polyethylene terephthalate (PET, n = 1), polypropylene (PP, n = 10), polystyrene (PS, n = 14) and polyethylene (PE, n = 56) from all the samples excepted freshwater plastic. Shared or unique OTUs are represented by numbers inside the circles for a given sample type.

3.2.3. Plastic debris size and color parameters not impacting the bacterial communities

There were no difference in alpha- and the beta-diversity of the bacterial communities in relation to the size or the color of the PD (Fig. 1C; Table 2). Our results were in contradiction with a recent study showing that the microbial richness was higher on PE mesoplastic than microplastic (Debroas et al., 2017). Other research showed that the apparent size effect can be due the difference in the surface to volume ratio (named specific ratio) and not from the size itself. For example, for a similar mass of polymers, a material containing an irregular surface has a larger available surface than a regular one. In accordance with previous results, the materials size had no effect on the bacterial diversity and composition (Cheng et al., 2021; Frère et al., 2018). In agreement with the literature, the plastic's color did not influence the diversity index (Wen et al., 2020).

3.3. Dispersion of pathogenic bacteria

Floating plastics are free support, known to assemble the ideal conditions for the microbial development and spreading out and represent a dispersion way for microorganisms, among which there might be also harmful and/or invasive microorganisms in new habitats. For example, a study showed that when corals are in contact with plastic debris, the likelihood of disease significantly increases, from 4% to 89% (Lamb et al., 2018). To better understand the related risks for human health, aquaculture or fisheries, the distribution of genera containing pathogenic species was investigated. In Fig. 2, *Vibrio* and *Tenacibaculum* were genera represented in marine water, which includes numerous pathogenic species, *e.g.*, *Vibrio parahaemolyticus*, *T. maritimum*, *T. soleae*, *T. discolor* or *T. gallaicum* (Kirstein et al., 2016; Fernández-Álvarez and Santos, 2018). Previous studies have shown that the *Vibrio* genus can represent up to 24% of the biofilm communities (Zettler et al., 2013) and the *Vibrionaceae* family up to 20% of the bacterial population on floating plastics (Delacuvellerie et al., 2019). Microbial pathogens optimize the exploitation of their host specializing in a surface-associated lifestyle, such as aquatic aggregates (Colwell et al., 2003; Danovaro et al., 2009) and marine PD (Oberbeckmann et al., 2016). Fig. S8 represents the percentage of several genera containing pathogenic species for humans and fishes (Bode Science Center database; Kirstein et al., 2016; Virsek et al., 2017; McCormick et al., 2014; Amaral-Zettler et al., 2020), thus allowing the pathogen dispersion in all the sampling sites. Eleven percent of the total plastic samples had a percentage of *Vibrio* higher than 1% and only two PD, EM19-33-02 and EM19-32-05 from seawater inshore, had a percentage higher than 10% with 17% and 14%, respectively. The *Vibrio* genus was a little more represented at three locations: EM19-33 and EM19-03 (inshore); EM19-P5 (harbor) with a mean of 3.7%, 2% and 2%, respectively. EM19-P5 was in the harbor at Marseille and EM19–33 station was close to the harbor. The Marseille Port is a commercial harbor with high boat traffic and passenger numbers, increasing the waste which could explain the higher number of *Vibrio*. The third location, EM19-03, was in the Ligurian Sea west Cannes, a well-known touristic region with numerous marinas. EM19-27-04 and EM19-14-02 possessed more than 40% of the populations represented by genera which include pathogenic species (*Tenacibaculum*, *Pseudomonas*, *Arcobacter*, *Aeromonas* and *Vibrio*). In summary,

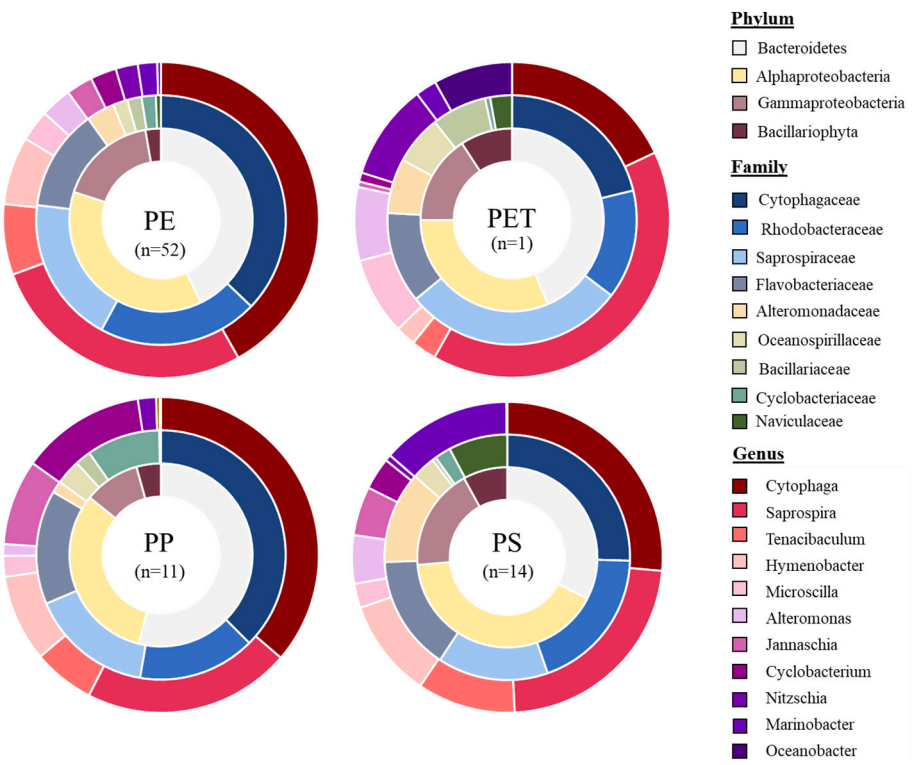

Fig. 4. Average of the most abundant taxonomic groups on different marine plastic polymers namely: polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS) (plastics from inshore and harbor). Inner circles represent phylum classification (excepted for *Alpha-* and *Gammaproteobacteria* being a class), the middle circles are the family level and the outer circles show the genus classification. The group of unclassified bacteria was not presented in this figure for clarity purposes.

pathogenic bacteria were scattered across our sampling locations with slight variations, excepted for EM19-27 and EM19-14 that contained a higher percentage of pathogenic genera. Moreover, only 11% of our PD samples possessed a percentage of *Vibrio* higher than the seawater (<1%, Thompson and Polz, 2006) and the proportion of potential pathogen in the plastisphere remained constant across all sampling sites. In concordance with recent studies, our taxonomic analysis did not indicate that the enrichment of the *Vibrio* genus in the plastisphere of PD from the Ligurian Sea would pose an alarming risk to human health and/or fisheries (Oberbeckmann et al., 2021; Delacuvellerie et al., 2022) regarding the *Vibrio* genus in the Ligurian Sea.

3.4. Hydrocarbonoclastic bacteria

3.4.1. Hydrocarbon-degrading bacteria present in the natural environment

Hydrocarbonoclastic bacteria are commonly found in the plastisphere (Delacuvellerie et al., 2019; Zettler et al., 2013). In the marine environment, hydrocarbon-degrading bacteria are usually found in very low abundance. Their growth is stimulated by contamination of hydrocarbons. These bacteria can degrade carbon-carbon structures, similar to the chemical structure of plastic, and could have a role in plastic degradation (Delacuvellerie et al., 2019). Fig. S9 shows an overview of the putative hydrocarbonoclastic bacteria dispersion according to the sampling location. The hydrocarbon-degrading bacterial percentage was a little higher when the sampling location was closed to the coast and the harbor. Many chemical compounds, such as hydrocarbons, bind and accumulate on plastics (Rochman, 2015), explaining the presence of hydrocarbon degraders on plastics, especially in the harbor. Even when hydrocarbonoclastic bacteria were characterized, they were poorly represented, and would not tend to degrade plastics (Delacuvellerie et al., 2021; Oberbeckmann et al., 2021).

3.4.2. Selection of putative plastic degrading candidates by enrichment culture

Previous studies showed that culture enrichment containing plastic as the main carbon source allow to select putative degrading-bacteria, *e.g.*, *Ideonella sakaiensis* and *Alcanivorax borkumensis* (Delacuvellerie et al., 2019; Yoshida et al., 2016). The phylum comparison of enrichment culture clearly showed a significative distinctness between the marine and freshwater samples, with a higher proportion of *Betaproteobacteria* in freshwater (Fig. S10). The PERMANOVA analysis confirms these results (Table 3). Moreover, the bacterial communities from the enrichment culture in freshwater contained a higher proportion of *Gammaproteobacteria*, a primary colonizer (De Tender et al., 2017a, b). Finally, *Bacteroidetes* was mainly found on marine environment compared to freshwater samples. A PCA also confirmed the distinction of the bacterial communities from rivers at t_0 and t_{80}, with the selection of genera such as *Novispirillum*, *Ensifer*, *Clostridium* or *Acinetobacter* at t_{80} (Fig. S11). The enriched bacterial communities in marine medium was distinct from enrichment in freshwater with the selection of, *e.g.*, *Vibrio*, *Sagittula*, *Cytophaga* or *Alcanivorax*. Regarding the diversity indexes for freshwater samples, the richness was statistically significantly higher at t_0 than t_{80} on the five plastic chemical compositions (Fig. S12), explained by the fact that a selection of genera took place after the culture. Moreover, some bacteria did not survive due to the growing

Table 3
PERMANOVA analyses using Bray-Curtis dissimilarity with 10,000 permutations of enrichment cultures according the type of plastic and the salinity of enrichment cultures (low-density polyethylene (LDPE), low molecular weight polyethylene (LMWPE), polyethylene terephthalate (PET), polystyrene (PS), polyvinyl chloride (PVC)). Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Factors tested	*p*-Value	r^2	Signif.
One-way PERMANOVAs (Bray-Curtis dissimilarity, 10,000 permutations)			
1. Enrichment culture salinity (seawater *vs* freshwater)	1e−05	0.24627	***
2. Plastic chemical composition (LMWPE, LDPE, PET, PS and PVC)	0.01585	0.09502	*
Two-way PERMANIVAs (Bray-Curtis dissimilarity, 10,000 permutations)			
3. Enrichment culture salinity * plastic chemical composition	0.01149	0.09666	*

conditions (medium containing 0.05% of yeast extract, 2% ammonium sulfate, 3.5% salts and 1% trace elements at pH 8 in 200 mM MOPS) being too distinct from the natural environment.

Eighty-three OTUs discriminated (nbGLM, p-value < 0.05) the bacterial communities at t_0 and t_{80}, in marine and freshwater medium and were represented on a heatmap (Fig. 5). Four response groups (RGs) were defined with hierarchical clustering based on center-scaling abundance. The first RG contained genera selected at t_{80} in the marine medium, and these genera were little represented in the communities at t_0, while the second RG highlighted bacterial genera that were common to the bacterial communities at t_0 and t_{80}. Finally, RG 3 represented bacteria mainly in the communities at t_0 in freshwater and RG4, genera mainly selected after the culture (t_{80}). In accordance with the result of the PCA analysis, the *Alcanivorax*, *Vibrio*, *Sagittula*, *Cytophaga* genera were statistically significantly selected in marine medium at t_{80}. The 10 most abundant genera significantly selected at t_{80} are represented in Fig. 6 according to their distribution and the plastic chemical composition (LDPE, LMWPE, PVC, PET and PS). Regarding the marine samples, several genera such as *Ruegeria*, *Cytophaga*, *Vibrio* and *Marinomonas*, were represented homogeneously on the different plastic chemical compositions (*i.e.*, LDPE, LMWPE, PET, PS and PVC; Fig.6), while other genera were mainly selected on one plastic composition, *e.g.*, *Alcanivorax*, *Sagittula* and *Marinobacter*. Interestingly, *Alcanivorax* was selected on the LDPE (75%). *Alcanivorax* is known for its capacity to degrade hydrocarbons and several polymers (Yakimov et al., 1998; Zadjelovic et al., 2020). This genus had a big affinity with the LDPE after enrichment culture and represented more than 60% of the bacterial communities on the LDPE and seemed implicated in the LDPE degradation with a weight loss of 3% after 80 days of culture (Delacuvellerie et al., 2019). LDPE has a solid structure similar to the chemical structure of alkane, both containing carbon-carbon link. In our study, *Alcanivorax* represented up to 15% of the population on the EM19-P4-01 sample (Fig. S13A). These genera of bacteria would be an excellent candidate for petroleum based plastic degradation, such as for LDPE. The *Sagittula* genus, mainly found on polyethylene (LDPE and LMWPE), contains species able to degrade lignin (Gonzalez et al., 1997). Enzymes capable of degrading lignin can also degrade certain plastics including polyethylene due the structural similarity of synthetic polymers with lignin (Krueger et al., 2015). The *Sagittula* genus could also have a role in PE degradation. Finally, *Marinobacter*, mainly found on the PVC and representing up to 55% of the bacterial population (Fig. 6 and Fig. S13A), is also a hydrocarbonoclastic genus (Duran, 2010). The relative abundance of these three genera on plastics indicates that they could be potential degraders of plastic in the marine environment due to their selection on the plastics.

Fig. 6 showing the 10 most abundant genera selected in the freshwater medium highlighted the presence of *Comamonas*, *Acinetobacter* and *Novispirillum* genera, also known in the literature for their capacity to use hydrocarbons (Guo et al., 2020; Bruckberger et al., 2018). Like in the marine medium, bacteria able to degrade hydrocarbons were enriched on plastics after 80 days of enrichment culture. *Comamonas* and *Acinetobacter* were homogenously selected on several plastic compositions while *Novispirillum* was more abundant on PVC. *Comamonas* represented more than 55% of the bacterial community (EM19-F1-04-PS), and *Acinetobacter* and *Novispirillum* more than 40% (Fig. S13B). Finally, the *Ensifer* genus was strongly selected on the PET film, on one sample:

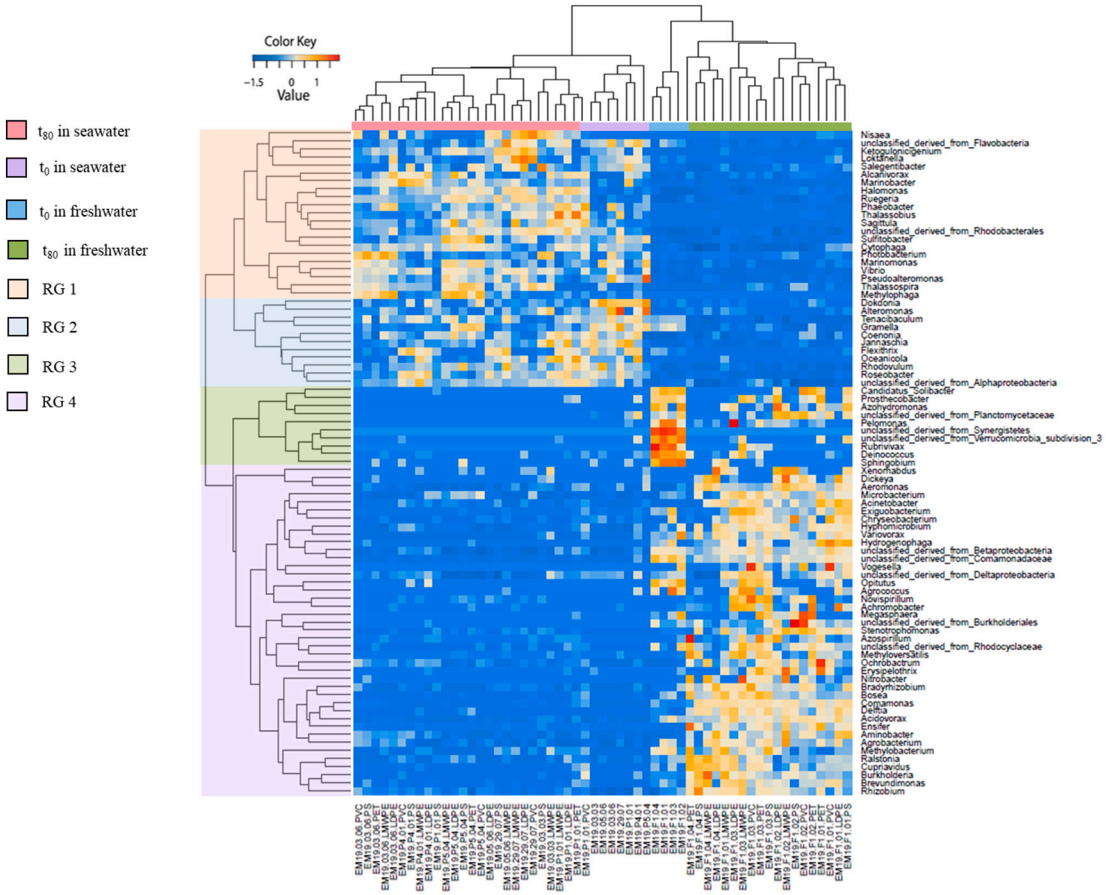

Fig. 5. Heatmap of the 83 genera significantly affected by the culture medium: seawater *vs* freshwater and by the comparison of the initial bacterial community (t_0) and after the enrichment culture (t_{80}) on the different plastic types: low-density polyethylene (LDPE), low molecular weight polyethylene (LMWPE), polyethylene terephthalate (PET), polystyrene (PS), polyvinyl chloride (PVC)). Four response groups (RGs) were defined with hierarchical clustering based on center-scaling abundance.

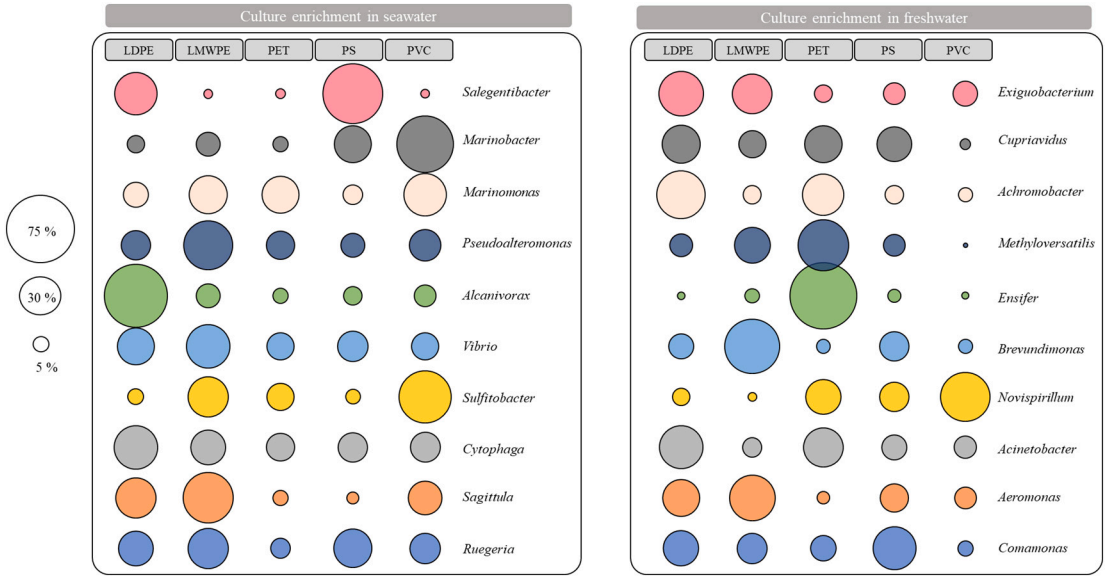

Fig. 6. Distribution of the 10 more abundant genera significantly affected by the culture salinity after the 60 days of enrichment culture (marine or freshwater medium) that have been highlighted on the heatmap according to the 5 polymers used in the enrichment cultures (low-density polyethylene (LDPE), low molecular weight polyethylene (LMWPE), polyethylene terephthalate (PET), polystyrene (PS), and polyvinyl chloride (PVC)).

EM19-F1-04-PET (Fig.6). Once again, this genus contains species able to degrade polycyclic aromatic hydrocarbons (Muratova et al., 2014). After 80 days of enrichment culture in freshwater or marine medium containing the plastic as the main carbon source, hydrocarbonoclastic bacteria were enriched. Our results showed a selection of hydrocarbon degrading microorganisms on PD that suggested their potential ability to hydrolyze plastic. The utilization of plastic-degraders bacteria and their involved enzymes must be investigated to optimize and open new perspectives into the utilization of this knowledge in plastic recycling.

3.5. The "forgotten" bacteria of the plastisphere

In addition to hydrocarbon-degrading and pathogenic bacteria, there were other interesting bacteria. Recent metagenomic and proteomic analyses showed that *Cyanobacteria* were not the most abundant microorganisms into the plastisphere but were the most active while the pathogenic bacteria (*i.e.*, *Vibrio*) were in dormancy, *i.e.*, *Vibrio* were very abundant in the communities but few proteins were detected (Oberbeckmann et al., 2021; Delacuvellerie et al., 2022). Taking an interest in these bacteria is therefore essential to better understand their role(s) in the plastisphere. Bacterial structure of the freshwater samples contained 11% of *Cyanobacteria*, the percentage decreased until 1%, 0.17% and 0.16% for inshore, harbor and estuary samples, respectively. *Chamaesiphon* and *Leptolyngbya* were most abundant *Cyanobacteria* genera in freshwater samples (Fig. S14). Leiser et al. (2021), investigated the role of phototrophic sessile *Cyanobacteria* (*Chamaesiphon* spp. and *Leptolyngbya* spp.), in their aggregation on microplastics in freshwater (Leiser et al., 2021). These phototrophic bacteria, forming biofilm on microplastics in eutrophic water, precipitated calcite, increasing the density of the biofilm-associated at microplastic and leading to sinking of plastic particles in the water column. *Cyanobacteria* have a role in the sedimentation of plastic particles (Leiser et al., 2021).

In addition to *Cyanobacteria*, Fig. 2 showing the percentage of taxonomic profiles of bacterial communities highlighted the fact that a high percentage of the bacterial communities was unclassified. Indeed, around 30% of the bacterial communities from marine water is unclassified while up to 50% of the freshwater samples were unclassified revealing an important gap of knowledge considering plastic-associated bacteria.

4. Conclusion

Our study demonstrated that sampling site is the most important driver of the bacterial structure, followed by the chemical composition of plastic polymer, while the colors and size of plastics did not influence the bacterial biofilm structure. Spatial and seasonal factors seem to be the most important driven of the plastisphere. Some genera were specific of a geographical location, *e.g.*, *Saccharopolyspora* exclusively characterized in PD collected from the Rhone estuary. The geographical location did not influence the proportion of genera containing pathogenic species and only 11% of PD showed higher proportions of *Vibrio* in comparison to the natural environment (<1%). *Cyanobacteria*, *i.e.*, *Chamaesiphon* and *Leptolyngbya* genera, present in the freshwater communities can have a role in the sedimentation of plastic particles. After 80 days in enrichment culture, hydrocarbon-degrading bacteria, potential candidate for plastic degradation, were statistically significantly selected on the different chemical composition of plastic from both seawater and freshwater samples. The utilization of potential plastic-degraders bacteria and their enzymes involved in the polymer degradation must be investigated to open new perspectives in plastic recycling. Moreover, supplementary studies focusing on the functioning of the plastispheres by metagenomic and metaproteomic analyses should be carried out to further decipher the impact of microbial communities developing on PD on the environment.

CRediT authorship contribution statement

Alice Delacuvellerie: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Tosca Ballerini:** Resources, Conceptualization, Writing – review & editing. **Laura Frère:** Resources, Writing – review & editing. **Sabine Matallana-Surget:** Data curation, Writing – review & editing. **Bruno Dumontet:** Resources, Conceptualization. **Ruddy Wattiez:** Supervision, Conceptualization, Methodology, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2022.113660.

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