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1 # From rivers to marine environments: a constantly evolving microbial
2 # community within the plastisphere

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13 ## Abstract

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

Keywords

Hydrocarbonoclastic bacteria, pathogenic bacteria, 16S rRNA amplicon sequencing, biofilm, plastic debris

1. Introduction

European plastic demand was to 50.7 million tons in 2019, mainly for packaging and the building and construction sector (Plastic 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 (PlasticsEurope, 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 1,000 and 3,000 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 *et al*., 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

51 structure from marine environment evolves gradually with the immersion time of the polymer:
52 *Gamma-* and *Alphaproteobacteria* constitute the primary colonizers, while *Bacteroidetes*
53 represent the secondary colonizers (De Tender *et al*., 2017a,b). Moreover, the position in the
54 water column (floating plastics *vs* plastics on the sediment), geographical location, chemical
55 composition of plastic polymer, or seasons can influence the bacterial community structure
56 (Delacuvellerie *et al*., 2019, 2021; Oberbeckmann *et al.*, 2014,2021, Frère *et al*., 2018, Zettler
57 *et al*., 2013, Debroas *et al.,* 2017). Two studies have shown that the plastics' shape and size do
58 not influence the microorganism structure (Frère *et al*., 2018; Cheng *et al*., 2021), while one
59 recent study has shown the influence of the polymer colors (Wen *et al*., 2020). Amaral-Zettler
60 and colleagues described the "cycle life" of plastic showing that plastics originate from land
61 source and, are transported via rivers to the ocean rivers (Amaral-Zettler *et al*., 2020). It is
62 therefore essential to study the bacterial structure of plastics sampled across a transect that
63 includes rivers, estuary and inshore from the same geographical zone, in our case the Ligurian
64 Sea.

65 Marine PD impact the ecosystem's health because species can use it as dispersion vector by
66 invasive or pathogenic species, changing the structure of natural ecosystems (De Tender *et al*.,
67 2015; Aliani & Molcard, 2003). Harmful algae, *e.g., Coolia*, *Ostreopsis* and *Alexandrium*, have
68 been detected on the surface of marine PD (Garcés & Camp, 2003), and the *Vibrio* genus,
69 containing numerous pathogenic species, is often found in higher concentrations on plastic than
70 in the natural environment (lower than 1% in seawater; Thompson *et al*., 2006; Frère *et al*.,
71 2018). Many bacteria attached to the PD surface are opportunistic microorganisms and can
72 grow on other types of support such as wood, glass or leaves (Lyons *et al.*, 2010). Moreover,
73 bacteria specialized in complex carbon degradation are selected on plastics, such as
74 hydrocarbon-degrading bacteria (*Hyphomonas*, *Oceaniserpentilla*, etc), supporting the fact that
75 these microorganisms can play a role in plastic degradation (Oberbeckmann *et al*., 2016; Zettler

76 *et al*., 2013). Our previous study that compared plastics from the same geographical location
77 showed significant differences in the bacterial community structure found on floating marine
78 PD in respect to PD collected in sediments (Delacuvellerie *et al*., 2019). Moreover, a
79 enrichment culture was used to select candidates for the plastic degradation, highlighting the
80 statistically significative enrichment of a hydrocarbon-degrading bacteria, *Alcanivorax* genus,
81 on PE (Delacuvellerie *et al*., 2019).

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

93 2. Materials and methods

94 2.1. Plastic sample collection

95 PD were collected in the Ligurian Sea from the river Var to the Port of Saint Louis du
96 Rhone, from July 21st, 2019 to August 9th, 2019 as part of Expedition MED 2019 Citizen
97 Science laboratory aboard the sailing boat Free Soul, in four aquatic ecosystems: river, estuary,
98 harbor and inshore (**Fig.S1**). Inshore plastic sampling was performed using a manta net towed
99 by the boat for a period of 30 min at the average speed of 2 knots. From the collected samples

100 with the manta net, the PD items of bigger dimensions and with a more evident biofilm were
101 selected. Additional samples of PD were collected manually in five harbors (Port of Saint
102 Laurent du Var, Vielle Darse de Toulon, Port Saint Louis du Rhône, Ecluse du port de Saint
103 Louis du Rhône, Vieux Port de Marseille) and in one river (Var). Once collected, PD were
104 immersed in water collected from the sampling location in sterile 50 ml falcon tubes and stored
105 at 4°C during transportation. A table summarizes all the PD sampled by Expédition MED
106 (**Table 1**) as well as the physico-chemical parameters of the water (**Table S1**).

107 **2.2. Plastic sample processing**

108 Microbial biofilms were removed from the PD surface and used for the bacterial community
109 structure analysis and the enrichment culture. In this way, PD were rinsed in sterile seawater
110 (35 g/L of Sigma Sea Salt) for marine samples or in sterile freshwater for river samples to
111 remove microorganisms not attached to the biofilm. Biofilms were scrapped with a sterile
112 scalpel blade to recover a maximum of the biomass. Subsequently, the plastics were rinsed with
113 ethanol 70% (V/V) and deionized water to remove organic coatings and dried at 30°C for 1
114 day. After this, the PD were used for analyses of their chemical composition.

115 From the total 107 PD samples, only 92 had enough biofilm to carry out DNA extraction to
116 study microbial communities. For the bacterial community analyses, the biofilm recovered from
117 PD was used for the DNA extraction (**Table S1**; number of samples sequenced by aquatic
118 ecosystems: 4 for estuary, 4 for freshwater river, 19 for harbors, 65 for seawater). From 11 of
119 these 92 PD samples that had a thicker biofilm, allowed us to save a portion of the biofilm for
120 enrichment cultures to study the ability of bacteria to degrade plastic (Delacuvellerie *et al.*,
121 2019) (**Table S2**).

122 **2.3. Polymer chemical composition**

123 The chemical composition of the plastic was analyzed using Attenuated Total Reflectance
124 Fourier Transform Infrared (ATR-FTIR) spectroscopy (Bruker, Tensor 27) with OPUS 6.5
125 software. The spectra were acquired over the wavelength range of 4000 − 600 cm^{-1} with 64
126 spectral scans (Mahoney *et al.*, 2013). The size and the color of each plastic sample was
127 collected in order to classify the plastic samples: macroplastics (25-1000 mm), mesoplastics (5-
128 25 mm) and microplastics (<5 mm) (GESAMP, 2019). The color code used were chosen as
129 suggested in EMODnet (Galgani *et* al. 2017).

130 **2.4. Enrichment culture to assess plastic degradation**

131 The thick biofilms recovered from 11 PD (four plastics from river, three from harbors and
132 four from inshore**; Table S2**) were cultured in glass tubes containing 5 ml of low carbon source
133 media (0.05% (W/V) of yeast extract), as described in Delacuvellerie *et al.*, 2019 and with 2
134 cm^2 of clean plastic film (all the plastics were in film except the low molecular weight
135 polyethylene (LMWPE) that which is in the pellet form). Briefly, the marine medium is
136 composed of: 0.05% yeast extract, 0.2% ammonium sulfate, 3.5% salts (W/V, Sigma Sea Salt)
137 and 1% trace elements (0.1% $MgSO_4.7H_2O$, 0.1% $FeSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$ 0.01%
138 $CuSO_4.5H_2O$ and 0.01% $MnSO_4.5H_2O$) in 20mM (N-morpholino) propanesulfonic acid
139 (MOPS) at pH 8, adapted from Yoshida *et al.*, 2016). Regarding the freshwater river samples,
140 the same medium without sea salt was used. Five polymers were tested for each sample: LDPE,
141 LMWPE, PET, PS and PET (**Table S3**). The plastics were sterilized in 70% ethanol overnight
142 and dried in petri dishes under a laminar flow hood. Enrichment cultures were shaken at 140
143 rpm at 30 °C. After 80 days of culture, formation biofilms were visible with the naked eye on
144 48 tubes (**Table S2**). The bacterial communities from these biofilms were analyzed by 16S
145 rRNA amplicon sequencing.

146 **2.5. DNA extraction and 16S rRNA amplicon sequencing**

147 DNA was extracted from the biofilm on both the PD samples collected in the field and from
148 the plastic pieces used in the enrichment cultures. The DNA extraction was performed with the
149 biofilm DNA isolation kit (NORGEN BIOTEK CORP) following the manufacturer's
150 instructions. Only samples with a minimum of 1 ng/µl of DNA concentration were sequenced
151 (**Table 1** and **Table S1**). A total of 92 samples from plastics debris collected in the field were
152 sequenced (4 from estuary, 4 from freshwater river, 19 from harbors and 65 from seawater) and
153 48 samples from the enrichment cultures (**Table S2**).

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

158 Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[341F]

159 Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[806R]

160 The high-throughput sequencing by the GIGA (Liège, Belgium) was used to perform the
161 sequencing of 2x300 bp paired-end with the Illumina® MiSeq ® platform (Illumina, San Diego,
162 CA, USA) according to the manufacturer's instructions. The MG-RAST pipeline (version 4.0.3,
163 https://www.mg-rast.org/) was used for the contingency table on the mate pairs (forward and
164 reverse) at the genus level, at a sequence identification level of 97%, using *Greengenes* database
165 (Keegan *et al*., 2016). The following parameters were chosen on MG-RAST: maximum low
166 quality basepairs of 6 and minimum quality of 25 pb. Adapter sequences were removed by a
167 bit-masked k-difference matching algorithm. Sequences were filtered based on length, quality
168 values and number of ambiguous bases. Finally, contamination by host DNA and PCR artifacts

169 were removed. 16S rRNA amplicon sequences were deposited at the SRA (Sequence Read
170 Archive) in NCBI under the accession number PRJNA724000.

171 2.6. Diversity indexes

172 The bacterial diversity of the plastic samples from the field and from enrichment cultures
173 were studied. Rarefaction curves were performed to verify the sequencing quality using the
174 *PAST* software (**Fig.S2**) (Hammer *et al.*, 2001). The richness and equitability indices,
175 corresponding to the alpha-diversity, were calculated on the rarefied data (14,387 reads counts
176 for *in-situ* sampling and 6770 reads counts for the enrichment cultures, *Limma* RGui package).
177 One sample containing less than 6770 reads was excluded (P4-01-PET; 5,425 reads counts,
178 **Fig.S2**). Using OTU presence/absence, Venn diagrams were created to assess the distribution
179 of these OTUs according the sampling site or chemical composition of polymer, using
180 *VennDiagram* RGui package (Hanbo & Paul, 2011). Multivariate analysis PERMANOVA was
181 used to study the beta-diversity using *vegan* RGui package (Wang *et al.*, 2012). The statical
182 significative variation between the conditions was calculated using the Bray-curtis dissimilarity
183 with 10, 000 permutations. Principal Component Analysis (PCA) using arrows to show the
184 influence of the taxonomy was used (Krause *et al.,* 2020). The presence of human pathogenic
185 bacteria was investigated using the Bode Science Center database (https://www.bode-science-
186 center.com/center/relevant-pathogens-from-a-z.html) and pathogenic species of marine flora
187 and fauna were investigated in literature (Kirstein *et al*., 2016; Viršek *et al.,* 2017; McCormick
188 *et al*., 2014; Amaral-Zettler *et al*., 2020).

189 2.7. Heatmap and validation of response groups (RGs)

190 Heatmap was performed on the bacterial communities from the enrichment cultures. OTUs
191 statistically significantly affected by the condition were identified using a negative binomial
192 distribution and Generalized Linear Model (nbGLM). This deviance analysis revised by 1,000
193 resampling iterations of the residual variance (*mvabund* Rgui package; Dixon, 2003). Eighty-

194 three OTUs affected by salinity (seawater *vs* freshwater) were plotted on a heatmap. The Monte-
195 Carlo simulation, comparing the RG clustering with a null-model containing all the OTUs,
196 validated four response groups (**Fig.S3**).

197 ## 3. Results and discussion

198 ### 3.1. Chemical characterization of plastic polymer sampled across the Ligurian Sea

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

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

211 ### 3.2. Impact of physico-chemical parameters on the plastic bacterial composition

212 3.2.1. From rivers to oceans: an evolving plastisphere

213 The richness and equitability indexes were approximately 150 and 0.50 for each aquatic
214 ecosystem (*i.e.*, inshore, estuary, harbor, river), respectively (**Fig.1A**). The PERMANOVA
215 analyses significatively highlighted the influence of different aquatic ecosystems and more
216 precisely, the influence of the sampling site (**Table 2; Fig.S1**) on the bacterial community
217 structure, both with a p-value of 1e-05. The result can be explained by differences in the

218 location-related properties, such as salinity, pH, and temperature (**Table S1**). In our study,
219 although a consistent number of replicates for aquatic ecosystems would have facilitated the
220 statistical analysis, we observed that the microbial structure was affected by different levels of
221 salinity (seawater, freshwater, brackish water) with a p-value of 1e-05 (**Table 2**). This is
222 accordance with previous study showing that location-related environmental parameters, such
223 as salinity, temperature and oxygen content, appeared to be correlated to the bacterial
224 community diversity (De Tender *et al*., 2015).

225 The plastisphere in river was mainly represented by *Cyanobacteria*, *Bacteroidetes*,
226 *Betaproteobacteria*, *Cyanobacteria* and *Deinococcus-Thermus* with a relative abundance of
227 10.7%, 7.5%, 7.5% and 5.2%, respectively (**Fig. S5 & 2**). Few studies reported the biofilm
228 structure in rivers but, as in the marine environment, plastic is a distinct environmental niche
229 mainly composed of *Beta*-, *Gammaproteobacteria* and *Bacteroidetes* (Hoellein *et al*., 2014;
230 McCormick *et al.,* 2014; Mccormick *et al.,* 2016; Amaral-Zettler *et al*., 2020, Amaral-Zettler
231 *et al*., 2021). *Cyanobacteria* and diatoms were shown to inhabit the surface of PD, thus
232 contributing to the primary production (Amaral-Zettler *et al*., 2020; Delacuvellerie *et al*., 2022).
233 The most represented genera from river samples were *Chamaesiphon*, *Deinococcus* and
234 *Hymenobacter* with 8.3 %, 5.2% and 1.3 %, respectively (**Fig. 2**). Samples from estuary, inshore
235 and harbors displayed a similar bacterial structure at the phylum level (**Fig.S5**). In accordance
236 with the literature (Bhagwat et al., 2021; Zettler *et al*., 2013), the bacterial communities were
237 mainly composed of *Bacteroidetes* (26%), *Gammaproteobacteria* (10%) and
238 *Alphaproteobacteria* (22%). *Gamma*- and *Alphaproteobacteria* were characteristic of the
239 primary colonizers in the plastisphere in the marine environment, while *Bacteroidetes* are
240 known to be secondary colonizers (De Tender *et al*., 2015). Amaral-Zettler and colleagues
241 performed taxonomic analyses of the plastisphere in the Ligurian Sea in 2018 (Amaral-Zettler
242 *et al*., 2021). Although the taxonomy of the Ligurian Sea samples showed *Alphaproteobacteria*,

243 *Gammaproteobacteria* and *Cyanobacteria* as dominating bacterial groups (Amaral-Zettler *et*
244 *al*., 2021), we observed that *Cyanobacteria* were less represented in our marine PD. Exposure
245 time and season are factors influencing biofilm formation and the bacterial composition vary
246 over time (Oberbeckmann *et al*., 2016 and 2014). At the genus level, *Cytophaga*, *Saprospira*,
247 *Tenacibaculum*, unclassified from *Gammaproteobacteria* and from *Alphaproteobacteria* were
248 the most genera represented across the inshore, estuary and harbor samples with small
249 percentage variations (**Fig.2**). The *Tenacibaculum* genus, with most species forming biofilm,
250 contains species pathogens for several fish, *e.g., T. maritimum*, *T. soleae*, *T. discolor* or *T.*
251 *gallaicum* (Fernández-Álvarez *et al*., 2018), and has already been associated with bacteria
252 composing the plastisphere (Oberbeckmann *et al*., 2016). *Saprospira* has already been
253 associated with plastic community from PP (Zettler *et al.*, 2013). Despite the different
254 geographical sample, there was a homogeneity in the most represented genera. However, some
255 genera were mainly represented on one site. For example, *Marinobacter*, bacteria degrading
256 hydrocarbon, was mainly represented on EM19-P1 (7%) (Duran, 2010). *Cyclobacterium* (11%)
257 and Pseudoalteromonas (9%) were most represented on EM19-01 and EM19-P5, respectively.

258 The presence of several genera such as *Cellulophaga*, *Paenibacillus* or *Brevibacillus,*
259 less represented on the PD were interesting. Indeed, *Cellulophaga* genus was represented on
260 average at less than 0.1% on all sampling site, except for river PD, which did not show this
261 genus. *Cellulophaga*, mainly found in marine alga and beach mud, also known as cellulose-
262 degrading bacteria (Abt *et al.,* 2011), synthesizing extracellular hydrolases can metabolize
263 cellulose as carbon source. Plastic oxidized by UV, or other physico-parameters leading to the
264 production of ester-link in the plastic matrix, could be altered by *Cellulophaga*'s enzymes
265 (Krueger *et al*., 2015). Indeed, natural polymers, such as proteins, chitin or cellulose, are
266 depolymerized via the cleavage of the hydrolytic bonds, *e.g*., ester-links. Therefore, plastics
267 containing hydrolysable backbone structures might be degraded by these enzymes (Krueger *et*

268 *al*., 2015). *Paenibacillus* genus, representing 0.1% of the river bacterial communities, contains
269 also cellulose-degrading bacterium (Wang *et al*., 2008), while the *Brevibacillus* genus, found
270 on EM19-F1 and P3 samples at 0.4%, showed the following species, *i.e.*, *Brevibacillus*
271 *borstelensis* - known to degrade PE - as well other pathogenic species of invertebrates, *e.g.*, *B.*
272 *thuringiensis* and *B. laterospora* (Hadad *et al*., 2005; Ruiu, 2013; Bravo *et al*., 2007). Bacteria
273 present in the plastisphere could have a role in the plastic degradation and/or in the transfer of
274 pathogenic species.

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

284 3.2.2 Influence of the polymer chemical composition on the plastisphere

285 The second studied parameter was the chemical composition of PD. The richness and
286 equitability indexes were similar between the polymers from marine environment (PP, PET,
287 PS, PE; **Fig.1B**). However, the bacterial community structure was affected by the polymer
288 composition (PERMANOVA analysis, Table 2, p-value =0.00913). Results from the previous
289 section highlighted that the bacterial communities from plastics were dependent on the
290 environment parameters, *e.g*., sampling site. However, the results of polymer type analysis
291 provide an insight of the plastisphere composition expected in the aquatic environment since

292 these PD were sampled from a large study area composing by 18 sampling sites in marine water
293 (harbor and inshore samples) influenced by different environmental parameters (Table 1). As
294 shown in the Venn diagram, the overlap of bacterial genera according to the pooled plastic
295 chemical composition sampled in the seawater (**Fig.3**). Interestingly, the majority of genera
296 (147) were shared between the four types of plastic (PET, PP, PS and PE). However, several
297 genera were identified as specific for a given plastic, *e.g.*, 24% of genera composing the PE
298 bacterial communities were specific to this polymer. The variation of the taxonomic
299 classification depending on the polymer chemical composition is shown on Figure 4.
300 Interestingly, *Bacteroidetes* was most abundant on PP than the others plastics. At the family
301 level, *Cyclobacteriaceae* and *Cytophagaceae* are most represented on PP while
302 *Oceanospirillaceae* and *Bacillariaceae* are most abundant on PET. *Saprospiraceae,*
303 *Rhodobacteraceae, Alteromonadaceae* and *Flavobacteriaceae* were abundant on the plastics
304 and were already found on previous study in marine environment (Oberbeckmann *et al.*, 2016;
305 Zettler *et al.*, 2013; Bhagwat *et al.*, 2021). Interestingly, these families contained members
306 known for their ability in the complex carbon degradation and for their marine biofilm lifestyles
307 (Oberbeckmann *et al.*, 2016). Finally, some genera were more present on one type of polymer
308 than the others, *e.g.*, *Marinobacter* on PS or *Cyclobacterium* on PP.

309 The chemical composition of plastics can significantly influence the bacterial
310 communities: (i) the chemical structure of the polymer, (ii) the particle shape (ropes and sheets,
311 De Tender *et al.*, 2017a), and (iii) chemical additives (plasticizers) (De Tender *et al.*, 2015).
312 The chemical function of the surface, the roughness, the hardness, the electric charge and the
313 hydrophobicity all play a role and influence the biofilm formation due to the physico-chemical
314 properties of the bacterial cell surface (Zhang *et al.*, 2015; Renner & Weibel, 2011; Ganesan *et*
315 *al.*, 2022). For example, one recent study had shown that the same bacterial species (*Bacillus*

316 *subtilis* and *Bacillus pumilus*) adhered better to PE and PVC surfaces than to PP and PET
317 surfaces due to the intrinsic surface properties of the plastic's surface (Cai *et al*., 2019).

318 3.2.3. Plastic debris size and color parameters not impacting the bacterial communities

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

329 **3.3. Dispersion of pathogenic bacteria**

330 Floating plastics are free support, known to assemble the ideal conditions for the
331 microbial development and spreading out and represent a dispersion way for microorganisms,
332 among which there might be also harmful and/or invasive microorganisms in new habitats. For
333 example, a study showed that when corals are in contact with plastic debris, the likelihood of
334 disease significantly increases, from 4% to 89% (Lamb *et al*., 2018). To better understand the
335 related risks for human health, aquaculture or fisheries, the distribution of genera containing
336 pathogenic species was investigated. In **Figure 2**, *Vibrio* and *Tenacibaculum* were genera
337 represented in marine water, which includes numerous pathogenic species, *e.g,*. *Vibrio*
338 *parahaemolyticus*, *T. maritimum*, *T. soleae*, *T. discolor* or *T. gallaicum* (Kirstein *et al*., 2016;
339 Fernández-Álvarez *et al*., 2018). Previous studies have shown that the *Vibrio* genus can

340 represent up to 24% of the biofilm communities (Zettler *et al.,* 2013) and the *Vibrionaceae*
341 family up to 20% of the bacterial population on floating plastics (Delacuvellerie *et al.,* 2019).
342 Microbial pathogens optimize the exploitation of their host specializing in a surface-associated
343 lifestyle, such as aquatic aggregates (Colwell *et al*., 2003; Danovaro *et al.,* 2009) and marine
344 PD (Oberbeckmann *et al.,* 2016). **Figure S8** represents the percentage of several genera
345 containing pathogenic species for humans and fishes (Bode Science Center database; Kirstein
346 *et al*., 2016; Viršek *et al.,* 2017; McCormick *et al*., 2014; Amaral-Zettler *et al*., 2020), thus
347 allowing the pathogen dispersion in all the sampling sites. Eleven percent of the total plastic
348 samples had a percentage of *Vibrio* higher than 1% and only two PD, EM19-33-02 and EM19-
349 32-05 from seawater inshore, had a percentage higher than 10% with 17% and 14%,
350 respectively. The *Vibrio* genus was a little more represented at three locations: EM19-33 and
351 EM19-03 (inshore); EM19-P5 (harbor) with a mean of 3.7%, 2% and 2%, respectively. EM19-
352 P5 was in the harbor at Marseille and EM19-33 station was close to the harbor. The Marseille
353 Port is a commercial harbor with high boat traffic and passenger numbers, increasing the waste
354 which could explain the higher number of *Vibrio*. The third location, EM19-03, was in the
355 Ligurian Sea west Cannes, a well-known touristic region with numerous marinas. EM19-27-04
356 and EM19-14-02 possessed more than 40% of the populations represented by genera which
357 include pathogenic species (*Tenacibaculum, Pseudomonas*, *Arcobacter*, *Aeromonas* and
358 *Vibrio*). In summary, pathogenic bacteria were scattered across our sampling locations with
359 slight variations, excepted for EM19-27 and EM19-14 that contained a higher percentage of
360 pathogenic genera. Moreover, only 11% of our PD samples possessed a percentage of *Vibrio*
361 higher than the seawater (< 1%, Thompson & Ploz, 2006) and the proportion of potential
362 pathogen in the plastisphere remained constant across all sampling sites. In concordance with
363 recent studies, our taxonomic analysis did not indicate that the enrichment of the *Vibrio* genus
364 in the plastisphere of PD from the Ligurian Sea would pose an alarming risk to human health

365 and/or fisheries (Oberbeckmann *et al*., 2021; Delacuvellerie *et al*., 2022) regarding the *Vibrio*
366 genus in the Ligurian Sea.

367 **3.4. Hydrocarbonoclastic bacteria**

368 3.4.1. Hydrocarbon-degrading bacteria present in the natural environment

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

381 3.4.2. Selection of putative plastic degrading candidates by enrichment culture

382 Previous studies showed that culture enrichment containing plastic as the main carbon
383 source allow to select putative degrading-bacteria*, e.g.*, *Ideonella sakaiensis* and *Alcanivorax*
384 *borkumensis* (Delacuvellerie *et al*., 2019; Yoshida *et al*., 2016). The phylum comparison of
385 enrichment culture clearly showed a significative distinctness between the marine and
386 freshwater samples, with a higher proportion of *Betaproteobacteria* in freshwater (**Fig.S10**).
387 The PERMANOVA analysis confirms these results (Table 3). Moreover, the bacterial
388 communities from the enrichment culture in freshwater contained a higher proportion of

389 *Gammaproteobacteria*, a primary colonizer (De Tender *et al*., 2017a,b). Finally, *Bacteroidetes*
390 was mainly found on marine environment compared to freshwater samples. A PCA also
391 confirmed the distinction of the bacterial communities from rivers at t_0 and t_{80}, with the selection
392 of genera such as *Novispirillum*, *Ensifer*, *Clostridium* or *Acinetobacter* at t_{80} **(Fig.S11)**. The
393 enriched bacterial communities in marine medium was distinct from enrichment in freshwater
394 with the selection of, *e.g*., *Vibrio*, *Sagittula*, *Cytophaga* or *Alcanivorax*. Regarding the diversity
395 indexes for freshwater samples, the richness was statistically significantly higher at t_0 than t_{80}
396 on the five plastic chemical compositions (**Fig.S12**), explained by the fact that a selection of
397 genera took place after the culture. Moreover, some bacteria did not survive due to the growing
398 conditions (medium containing 0.05% of yeast extract, 2% ammonium sulfate, 3.5% salts and
399 1% trace elements at pH 8 in 200 mM MOPS) being too distinct from the natural environment.

400 Eighty-three OTUs discriminated (nbGLM, p-value<0.05) the bacterial communities at
401 t_0 and t_{80}, in marine and freshwater medium and were represented on a heatmap (**Fig.5**). Four
402 response groups (RGs) were defined with hierarchical clustering based on center-scaling
403 abundance. The first RG contained genera selected at t_{80} in the marine medium, and these
404 genera were little represented in the communities at t_0, while the second RG highlighted
405 bacterial genera that were common to the bacterial communities at t_0 and t_{80}. Finally, RG 3
406 represented bacteria mainly in the communities at t_0 in freshwater and RG4, genera mainly
407 selected after the culture (t_{80}). In accordance with the result of the PCA analysis, the
408 *Alcanivorax, Vibrio*, *Sagittula*, *Cytophaga* genera were statistically significantly selected in
409 marine medium at t_{80}. The 10 most abundant genera significantly selected at t_{80} are represented
410 in **Figure 6** according to their distribution and the plastic chemical composition (LDPE,
411 LMWPE, PVC, PET and PS). Regarding the marine samples, several genera such as *Ruegeria,*
412 *Cytophaga*, *Vibrio* and *Marinomonas*, were represented homogeneously on the different plastic
413 chemical compositions (*i.e.*, LDPE, LMWPE, PET, PS and PVC; **Fig.6**), while other genera

414 were mainly selected on one plastic composition, *e.g.*, *Alcanivorax*, *Sagittula* and
415 *Marinobacter*. Interestingly, *Alcanivorax* was selected on the LDPE (75%). *Alcanivorax* is
416 known for its capacity to degrade hydrocarbons and several polymers (Yakimov *et al.,* 1998;
417 Zadjelavic *et al.,* 2020). This genus had a big affinity with the LDPE after enrichment culture
418 and represented more than 60% of the bacterial communities on the LDPE and seemed
419 implicated in the LDPE degradation with a weight loss of 3% after 80 days of culture
420 (Delacuvellerie *et al.,* 2019). LDPE has a solid structure similar to the chemical structure of
421 alkane, both containing carbon-carbon link. In our study, *Alcanivorax* represented up to 15%
422 of the population on the EM19-P4-01 sample (**Fig.S13A**). These genera of bacteria would be
423 an excellent candidate for petroleum based plastic degradation, such as for LDPE. The *Sagittula*
424 genus, mainly found on polyethylene (LDPE and LMWPE), contains species able to degrade
425 lignin (Gonzalez *et al.,* 1997). Enzymes capable of degrading lignin can also degrade certain
426 plastics including polyethylene due the structural similarity of synthetic polymers with lignin
427 (Krueger *et al.,* 2015). The *Sagittula* genus could also have a role in PE degradation. Finally,
428 *Marinobacter,* mainly found on the PVC and representing up to 55% of the bacterial population
429 (**Fig.6** and **Fig.S13A**), is also a hydrocarbonoclastic genus (Duran, 2010). The relative
430 abundance of these three genera on plastics indicates that they could be potential degraders of
431 plastic in the marine environment due to their selection on the plastics.

432 **Figure 6** showing the 10 most abundant genera selected in the freshwater medium
433 highlighted the presence of *Comamonas*, *Acinetobacter* and *Novispirillum* genera, also known
434 in the literature for their capacity to use hydrocarbons (Guo *et al*., 2020; Bruckberger *et al.,*
435 2018). Like in the marine medium, bacteria able to degrade hydrocarbons were enriched on
436 plastics after 80 days of enrichment culture. *Comamonas* and *Acinetobacter* were
437 homogenously selected on several plastic compositions while *Novispirillum* was more abundant
438 on PVC. *Comamonas* represented more than 55% of the bacterial community (EM19-F1-04-

439 PS), and *Acinetobacter* and *Novispirillum* more than 40% (**Fig.S13B**). Finally, the *Ensifer*
440 genus was strongly selected on the PET film, on one sample: EM19-F1-04-PET (**Fig.6**). Once
441 again, this genus contains species able to degrade polycyclic aromatic hydrocarbons (Muratova
442 *et al*., 2014). After 80 days of enrichment culture in freshwater or marine medium containing
443 the plastic as the main carbon source, hydrocarbonoclastic bacteria were enriched. Our results
444 showed a selection of hydrocarbon degrading microorganisms on PD that suggested their
445 potential ability to hydrolyze plastic. The utilization of plastic-degraders bacteria and their
446 involved enzymes must be investigated to optimize and open new perspectives into the
447 utilization of this knowledge in plastic recycling.

448 **3.5. The "forgotten" bacteria of the plastisphere**

449 In addition to hydrocarbon-degrading and pathogenic bacteria, there were other
450 interesting bacteria. Recent metagenomic and proteomic analyses showed that *Cyanobacteria*
451 were not the most abundant microorganisms into the plastisphere but were the most active while
452 the pathogenic bacteria (*i.e.*, *Vibrio*) were in dormancy, *i.e.*, *Vibrio* were very abundant in the
453 communities but few proteins were detected (Oberbeckmann *et al*., 2021; Delacuvellerie *et al*.,
454 2022). Taking an interest in these bacteria is therefore essential to better understand their role(s)
455 in the plastisphere. Bacterial structure of the freshwater samples contained 11% of
456 *Cyanobacteria*, the percentage decreased until 1%, 0.17% and 0.16% for inshore, harbor and
457 estuary samples, respectively. *Chamaesiphon* and *Leptolyngbya* were most abundant
458 *Cyanobacteria* genera in freshwater samples (**Fig.S14**). Leiser and colleagues (2021),
459 investigated the role of phototrophic sessile *Cyanobacteria* (*Chamaesiphon* spp. and
460 *Leptolyngbya* spp.), in their aggregation on microplastics in freshwater (Leiser *et al.,* 2021).
461 These phototrophic bacteria, forming biofilm on microplastics in eutrophic water, precipitated
462 calcite, increasing the density of the biofilm-associated at microplastic and leading to sinking

463 of plastic particles in the water column. *Cyanobacteria* have a role in the sedimentation of
464 plastic particles (Leiser *et al.,* 2021).

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

470 ## 4. Conclusion

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

486 should be carried out to further decipher the impact of microbial communities developing on
487 PD on the environment.

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499 ## 6. Supplementary Information

500 The supplementary information associated with this article contains additional figures and
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502 ## 7. Conflict of Interests

503 The authors declare no competing financial interest.

504 ## 8. References

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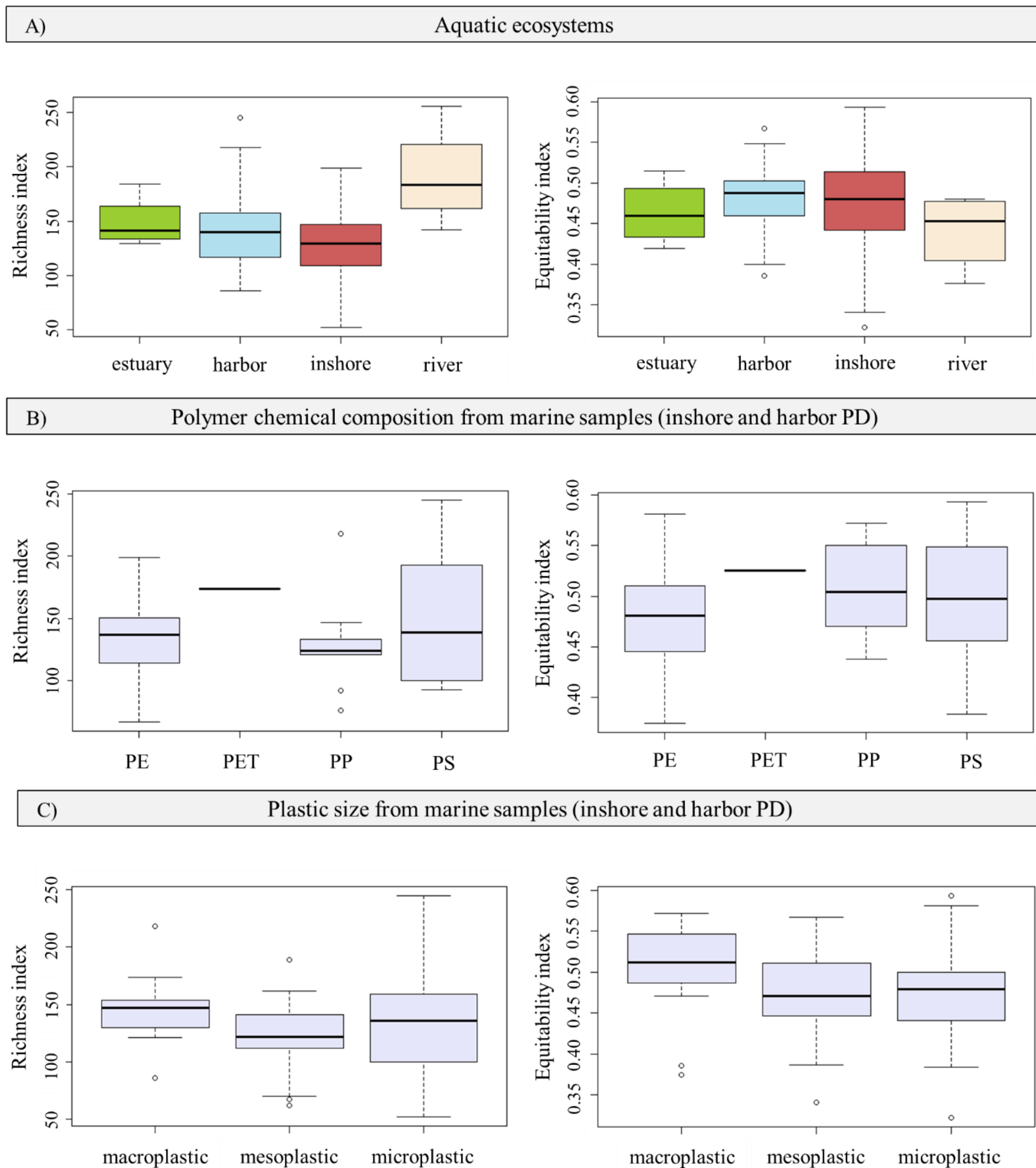
706 **Graphics**



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

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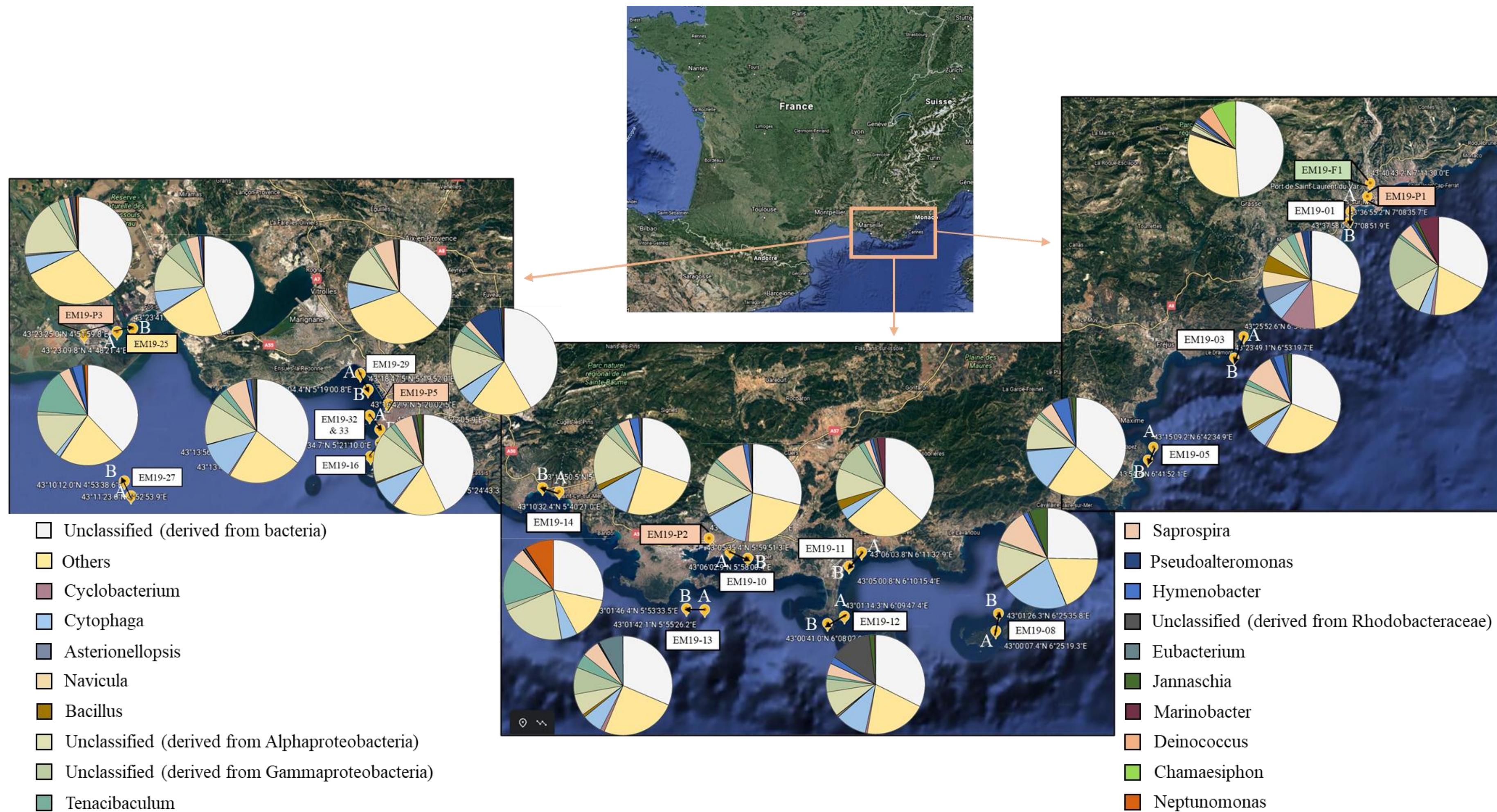

Figure 2: Map of all the 2019 sampling from the Mediterranean Sea. Samplings by a manta net are represented as followed: first point (A) shows the beginning of the sampling and the second (B), the end; the arrow show the direction of the boat. The plastic samples in a harbor are represented in orange 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.

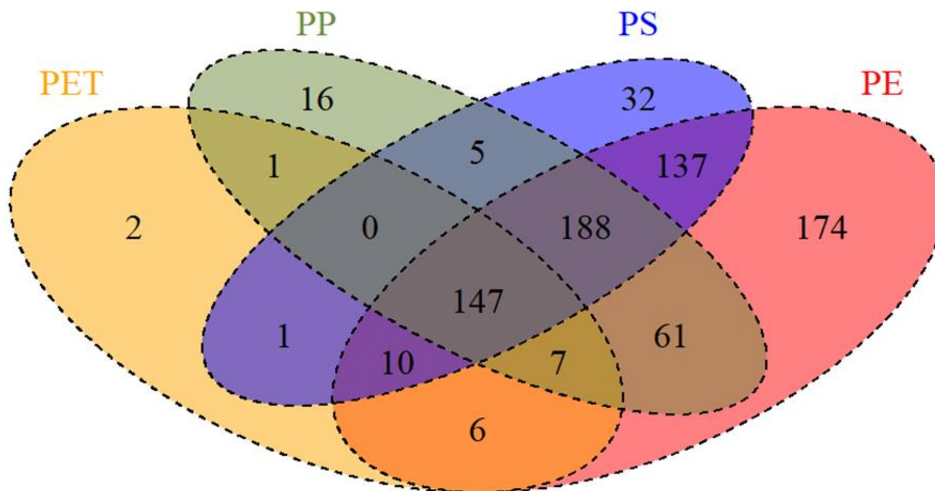

Figure 3: Venn diagram showing overlap of bacterial OTUs according to the pooled plastic chemical composition (polyethylene terephthalate (PET, n=1), polypropylene (PP, n=10), polystyrene (PS, n=14) and polyethylene (PE, n=56) from all the samples excepted freshwater plastic. Shared or unique OTUs are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

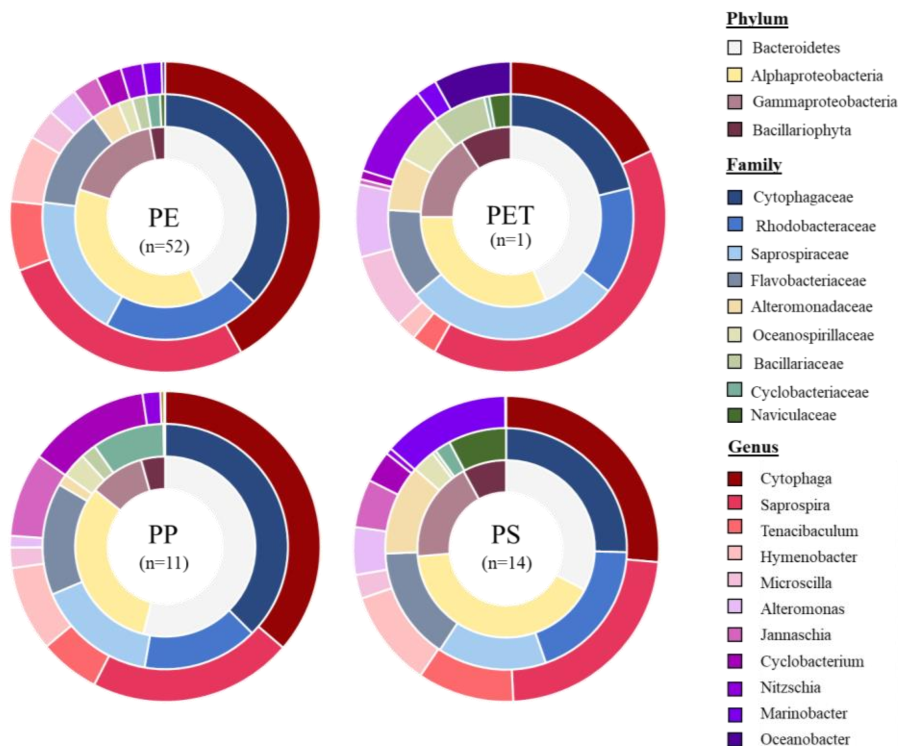

Figure 4: Most abundant taxonomic groups on different marine plastic polymers namely: Polyethylene (PE), Polyethylene terephthalate (PET), Polypropylene (PP), polystyrene (PS) (plastics from inshore and harbor). Inner circles represent phylum classification (excepted for *Alpha*- and

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.

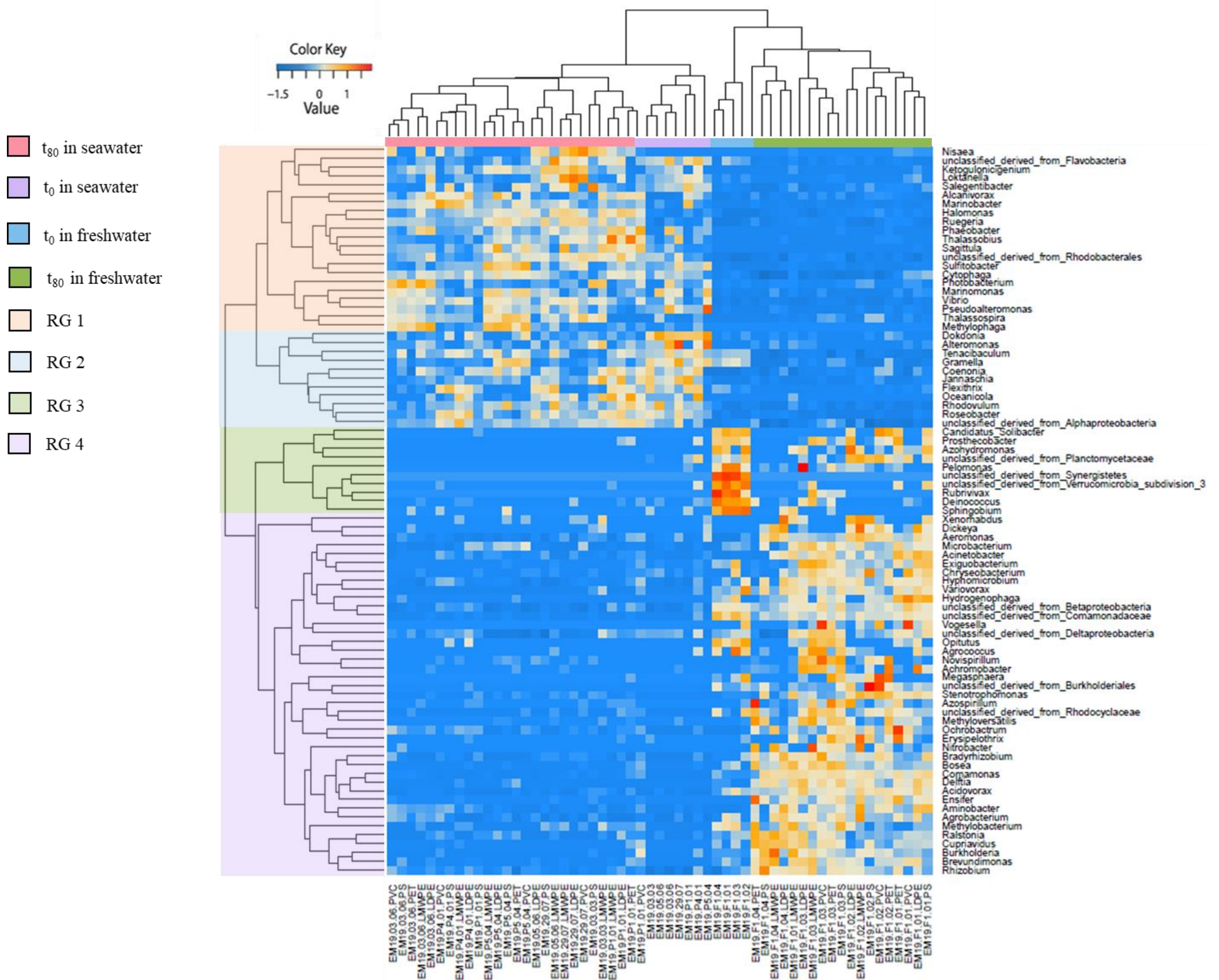

Figure 5: Heatmap of the 83 genera significantly affected by the culture medium: seawater *vs* freshwater and by the comparison of the initial bacterial community (t_0) and after the enrichment culture (t_{80}) on the different plastic types: low-density polyethylene (LDPE), low molecular weight polyethylene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

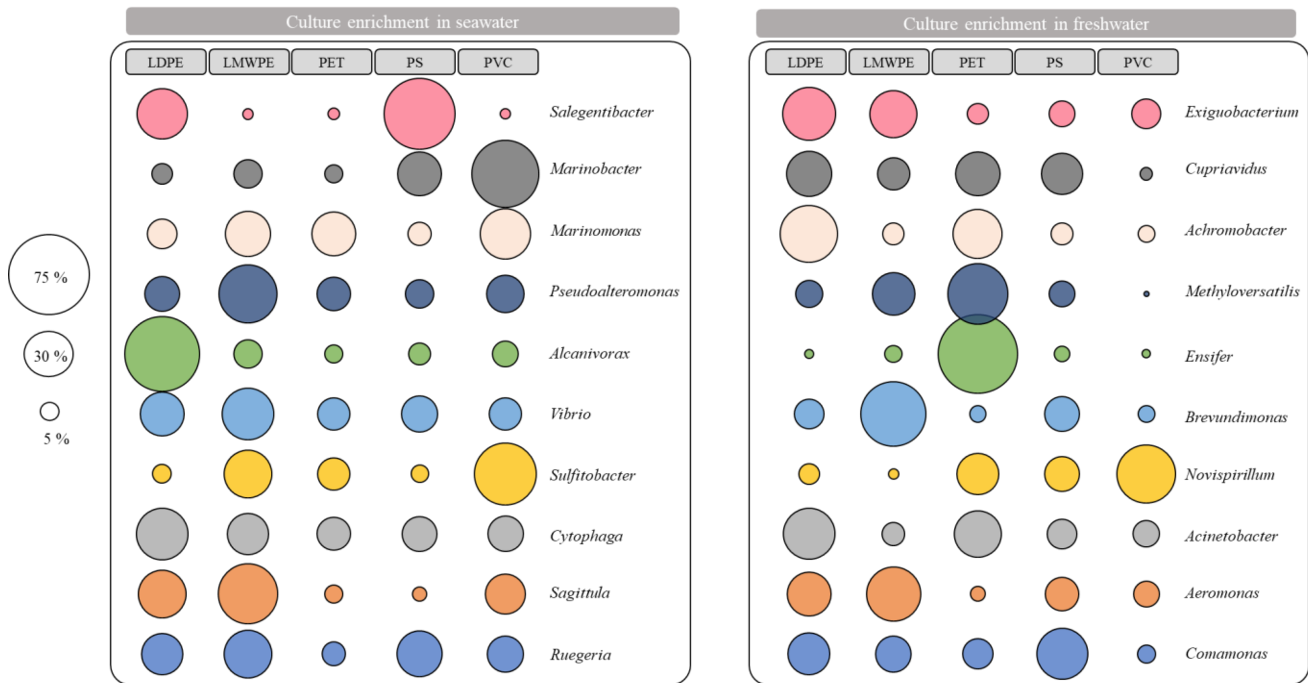

Figure 6: Distribution of the 10 more abundant genera significantly affected by the culture salinity after the 60 days of enrichment culture (marine or freshwater medium) that have been highlighted on the heatmap according to the 5 polymers used in the enrichment cultures (low-density polyethylene (LDPE),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

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	macropastic: 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

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	**

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	*