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## Microplastic bacterial communities in the Bay of Brest: Influence of polymer type and size

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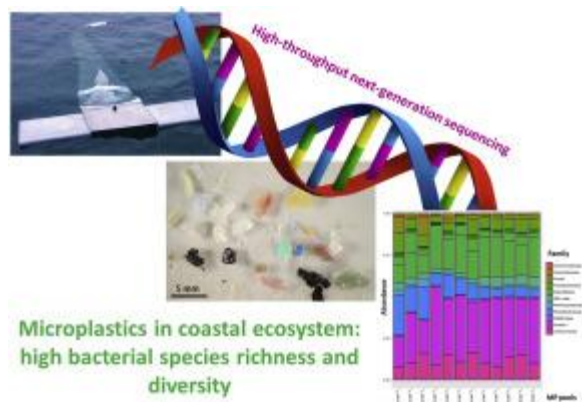
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### Abstract :

Microplastics (<5 mm) exhibit intrinsic features such as density, hydrophobic surface, or high surface/volume ratio, that are known to promote microbial colonization and biofilm formation in marine ecosystems. Yet, a relatively low number of studies have investigated the nature of microplastic associated bacterial communities in coastal ecosystems and the potential factors influencing their composition and structure. Here, we characterized microplastics collected in the Bay of Brest by manual sorting followed by Raman spectroscopy and studied their associated bacterial assemblages using 16S amplicon high-throughput sequencing. Our methodology allowed discriminating polymer type (polyethylene, polypropylene and polystyrene) within small size ranges (0.3–1 vs. 1–2 vs. 2–5 mm) of microplastics collected. Data showed high species richness and diversity on microplastics compared to surrounding seawater samples encompassing both free living and particle attached bacteria. Even though a high proportion of operational taxonomic units (OTU; 94 ± 4%) was shared among all plastic polymers, polystyrene fragments exhibited distinct bacterial assemblages as compared to polyethylene and polypropylene samples. No effect of microplastic size was revealed regardless of polymer type, site and date of collection. The *Vibrio* genus was commonly detected in the microplastic fraction and specific PCR were performed to determine the presence of potentially pathogenic *Vibrio* strains (namely *V. aestuarianus* and the *V. splendidus* polyphyletic group). *V. splendidus* related species harboring

putative oyster pathogens were detected on most microplastic pools (77%) emphasizing the need of further research to understand the role of microplastics on pathogen population transport and ultimate disease emergence.

### Graphical abstract



### Highlights

► Study of marine microplastic bacterial communities using next-generation sequencing. ► High species richness and diversity was observed on microplastics. ► No effect of microplastic size was shown on alpha and beta diversities. ► Polystyrene showed different bacterial communities than polyethylene and propylene. ► *Vibrios* harboring putative oyster pathogens were detected on most microplastics.

**Keywords** : Bacteria, Microplastics, Coastal ecosystem, Metabarcoding, *Vibrios*

## 54 **Introduction**

55 Plastic debris, and notably microplastics (defined as plastic particles < 5mm (Arthur et al.,  
56 2009) contaminate the worldwide marine ecosystems (Eriksen et al., 2014; Lusher, 2015;  
57 Sebille et al., 2015) leading to increased concerns about their ecological impacts (Rochman,  
58 2016). Owing to their global distribution and small size, microplastics are efficiently ingested  
59 by a wide range of marine organisms, from zooplankton (Cole et al., 2011) to mollusks (Van  
60 Cauwenberghe and Janssen, 2014), fishes (Foekema et al., 2013) and even marine mammals  
61 (Fossi et al., 2012) making their way into all marine food chains and posing a critical threat to  
62 marine organisms. Besides direct physical impacts upon microplastics ingestion (Wright et  
63 al., 2013) and indirect toxicity related to the release of chemicals carried by microplastics  
64 (plasticizers, pigments, monomers, adsorbed pollutants) (Koelmans et al., 2014, 2016),  
65 concerns are raising regarding the potential for microplastics to represent new substrates for  
66 microorganisms, especially harmful and pathogenic ones (Kirstein et al., 2016; Lusher, 2015;  
67 Maso et al., 2003).

68 The first mention of plastic debris being colonised by microorganisms (for instance diatoms  
69 and bacteria) was done by Carpenter et al. (1972). For the past decade several field studies  
70 have demonstrated that plastic debris and microplastics represent a novel substrate for habitat  
71 and transport of a wide range of marine organisms. Microplastic-associated rafting  
72 communities were observed to be composed of macrobenthic organisms such as arthropods,  
73 mollusks, bryozoans and cnidarians (Bryant et al., 2016; Goldstein et al., 2014) and  
74 eukaryotic microorganisms such as dinoflagellates, diatoms, invertebrate eggs and fungus  
75 (Maso et al., 2003; Oberbeckmann et al., 2014; Reisser et al., 2014); thus raising question  
76 about the transfer of potentially invasive rafting taxa to pristine ecosystems (Galgani et al.,  
77 2013). Dispersal of non-indigenous species through attachment to natural substrate (wood,  
78 vegetal, pumice) has been widely described (Jokiel, 1990), however the buoyant, persistent  
79 and ubiquitous nature of microplastics may significantly exacerbate the survival and long-  
80 distance transport of various hitchhikers. A recent example of this enhanced dispersal of  
81 organisms by plastic debris is the identification of nearly 300 Japanese species (mainly  
82 invertebrate) that reached the U.S. Pacific Northwest shores as a consequence of the 2011  
83 East Japan earthquake and tsunami. Interestingly, most species were attached to the remains  
84 of manmade debris primarily composed by plastics (Carlton et al., 2017). Colonization of  
85 plastic debris and microplastics by prokaryotes has also been shown in various environments  
86 from freshwater to seawater, marine sediments and beaches (reviewed in Oberbeckmann et

87 al., 2015). All these studies demonstrated a high diversity and richness of microorganisms  
88 colonizing microplastics, constituting a unique marine environment called the “Plastisphere”  
89 (Zettler et al., 2013). In addition, bacterial families harboring well-known human, fish and  
90 shellfish pathogenic strains (Vibrionaceae, Campylobacteraceae, Flavobacteriaceae and  
91 Aeromonadaceae) have been regularly detected on microplastics (Dussud et al., 2018;  
92 Kirstein et al., 2016; Schmidt et al., 2014; Viršek et al., 2017; Zettler et al., 2013). As a  
93 consequence, more research to understand the spatiotemporal patterns of plastic colonizing  
94 microorganisms and the ecological risks for marine ecosystems, food safety and public health  
95 is needed (GESAMP, 2016; Harrison et al., 2011; Keswani et al., 2016).

96 The aim of the present study was to investigate microplastic-associated bacterial communities  
97 collected in the coastal ecosystem of the bay of Brest (Brittany, France). The bay of Brest was  
98 recently studied for microplastic contamination; the mean concentration was estimated around  
99 0.24 floating microplastic.m<sup>-3</sup> dominated by polyethylene (PE), polypropylene (PP) and  
100 polystyrene (PS) fragments (Frère et al., 2017). In this study, floating microplastics collected  
101 during two sampling surveys and at two stations in the bay were characterized by manual  
102 sorting followed by Raman spectroscopy and associated bacterial communities were analyzed  
103 using high-throughput 16S rRNA gene amplicon sequencing to investigate: (a) taxa associated  
104 to microplastics and to the surrounding seawater encompassing both free-living and particle  
105 attached communities, and (b) the influence of the polymeric nature and size ranges of  
106 microplastics (5-2 mm, 2-1 mm and 1-0.3 mm) on the composition and structure of the  
107 bacterial communities. Because the genus *Vibrio* was commonly detected on microplastics,  
108 specific PCR were also performed to determine the presence / absence of potentially  
109 pathogenic *Vibrio* strains (namely *V. aestuarianus* and the *V. splendidus* polyphyletic group).

## 110 **Material and methods**

### 111 **1. Samples collection**

112 Sample collection was conducted in the bay of Brest (Brittany, France) during two sampling  
113 surveys conducted on October 21<sup>th</sup>, 2015 and December 9<sup>th</sup>, 2015. Two sites were sampled:  
114 site A1 was located close to a recreational marina in an area subjected to intense  
115 anthropogenic activities (48°22'41.06"N, 4°29'22.60"W), and site M1 was located in the  
116 center of the bay (48°20'34.59"N, 4°30'6.29"W) in an area characterized by the occurrence of  
117 a transitional vortex created by surface current at flood tide, concentrating floating debris  
118 coming from the north and the south of the bay (Frère et al., 2017). Samples were collected at

119 surface water using a Manta trawl (335  $\mu\text{m}$  mesh, rectangular net opening of 0.6 x 0.16 m)  
120 and stored in sterilized glass jar on board. Three liters of surface seawater were also collected  
121 at each sampling station and filtered through 0.22  $\mu\text{m}$  Sterivex filters in sterile conditions.  
122 Filters were stored at -20 °C and used for subsequent DNA extraction in order to assess both  
123 free-living (FL) and particle-attached (PA) communities present in seawater. Surface water  
124 quality parameters were monitored within the scope of the SOMLIT (Service d'Observation  
125 en Milieu LITtoral), the French Coastal Monitoring Network ([http://somlit.epoc.u-  
126 bordeaux1.fr/fr/](http://somlit.epoc.u-bordeaux1.fr/fr/)) and are presented in supplementary table 1. The suspended particulate matter  
127 (SPM) in the Bay of Brest is mainly composed by phytoplankton (82%), river POM (10%)  
128 and macro-algae (8%) (Liénart et al., 2017).

## 129 **2. Samples processing**

130 All collected microparticles were processed within 24 hours upon sample collection in  
131 rigorous sterile conditions throughout their manipulation with minimal freezing steps in order  
132 to avoid DNA alteration, loss or contamination. Morphological and chemical (Raman)  
133 features were recorded prior to DNA extraction in order to allow the clustering of  
134 microplastics as a function of their polymer nature within each size class.

135 Manual microparticles extraction was performed immediately upon return to the lab using  
136 forceps and a dissecting microscope under sterile conditions. All material (petri dish, filter,  
137 forceps) was sterilized, and forceps were systematically rinsed in 10 % chlorine solution and  
138 milliQ water between manipulations of each particle. Visually identified microplastic-like  
139 particles were individually rinsed with sterile seawater before being dried and shortly stored  
140 in sterile WillCo-dish glass dishes at -20 °C prior to spectroscopy analysis. Microplastic  
141 molecular composition was identified by Raman micro-spectroscopy using the method  
142 developed by (Frère et al., 2016), adapted here due to the need to maintain sterile conditions:  
143 extracted particles were kept in closed sterile WillCo-dish glass dishes exhibiting a top thin  
144 glass slide (0.17 mm width) and spectroscopy analyses were realized through this glass slide.  
145 Preliminary manipulations have ensured that the Raman signal was not affected by the glass  
146 slide (data not shown).

147 Microplastics were exclusively made of fragments and they were isolated based on their  
148 collection date (October and December 2015), their sampling site (A1 and M1) and their  
149 polymer family (polyethylene (PE), polypropylene (PP) and polystyrene (PS)) before being  
150 pooled according to size range: 5-2 mm / 2-1 mm / 1-0.3 mm. The pooling rate was adapted

151 for each size class to ensure sufficient DNA quantity for subsequent 16S amplicon sequencing  
152 (especially for the lowest size class 0.3-1 mm). Accordingly, microplastic pools contained  
153  $n=20$  particles in the 1 - 0.3 mm range, 8 for the 2 – 1mm range and 4 for the 5 – 2 mm range.  
154 A total of one to five pools per polymer and size class were processed according to the  
155 available number of particles collected and identified by Raman micro-spectrometry in each  
156 category (Table 1). Pools were stored in 2 ml tube at  $-20^{\circ}\text{C}$  prior to DNA extraction. Overall,  
157 the bacterial communities were investigated on a total of 47 pools of microplastics (MP) and  
158 12 samples of seawater (0.22 $\mu\text{m}$  Sterivex filters containing FL+PA bacteria).

### 159 **3. DNA extraction**

160 DNA extraction was performed (i) on the Raman identified microplastics and (ii) directly on  
161 the 0.22 $\mu\text{m}$  filters used for seawater filtration (see section 1. Samples collection). Therefore  
162 the communities revealed in the seawater fraction encompassed both free-living (FL) and  
163 particle associated (PA) bacteria. DNA extraction was done using phenol chloroform: after  
164 adding 800  $\mu\text{l}$  of TNE buffer, 50  $\mu\text{l}$  of SDS 10%, 50  $\mu\text{l}$  of lauryl sarkosyl 10% and 50  $\mu\text{l}$  of  
165 proteinase K (20 mg.g $^{-1}$ ) were added to each tube. Tubes were incubated at  $55^{\circ}\text{C}$  for 2 hours  
166 before being homogenized and transferred in tubes containing silica beads (Lysing Matrix B,  
167 2 mL MP Biomedicals tube). Samples were then centrifuged at 11000 g for 3 min for  
168 mechanical lysis. Aqueous phases were transferred to clean 2 ml tubes, 700  $\mu\text{l}$  of  
169 phenol:chloroform: isoamyl alcohol (25:24:1) were added and tubes were centrifuged at 8000  
170 g for 10 min at room temperature. 700  $\mu\text{l}$  of chloroform was added and tubes were centrifuged  
171 at 8000 g for 10 min at room temperature. Aqueous phases were transferred in clean 2 ml  
172 tubes and DNA precipitation was realized with 1500  $\mu\text{l}$  of absolute ethanol by inversion (this  
173 step was repeated 10 times) after which tubes were centrifuged at 14000 g for 15 min at  $4^{\circ}\text{C}$ .  
174 Aqueous phases were eliminated, 500  $\mu\text{l}$  of ethanol 70% were added and tubes were  
175 centrifuged at 14000 g for 10 min at  $4^{\circ}\text{C}$ . Aqueous phases were eliminated and pellets in  
176 tube's bottom were dried with a SpeedVac for 5 min at  $30^{\circ}\text{C}$ . 30  $\mu\text{l}$  of ultrapure water were  
177 added and tubes with DNA were stored at  $4^{\circ}\text{C}$  before amplicon sequencing analysis. Samples  
178 were further stored at  $-20^{\circ}\text{C}$  prior to PCR analysis.

### 179 **4. 16S amplicons sequencing**

180 Bacterial community assemblages were determined using amplicons sequencing of the 16S  
181 rRNA gene V4-V5 region according to (Huse et al., 2014). We amplified the V4 - V5  
182 hypervariable region of the bacterial 16S rRNA gene using a combination of the barcoded



183 forward primer 518F (5'-CCAGCAGCYGCGGTAAN-3') and a mix of three indexed reverse  
184 primer 926R (5'-TGARTTTNCTTAACTGCC-3'; 5'-TGAGTTTCTTTAACTGCC-3'; 5'-  
185 TNAGTTTCCTTA TCTGCC-3' in 8:1:1 ratio respectively). The following PCR conditions  
186 were used: initial denaturation of thirty cycles at 94°C for 3 min, 94 °C for 30 sec, 58 °C for  
187 45 sec, 72 °C for 1 min following by 72 °C for 2 min and 4 °C at infinite. PCR products were  
188 purified with the Agencourt AMPure XP kit. Due to the presence of ca. 750 bp long  
189 unspecific PCR products, we quantified PCR product of the expected size (ca. 410 bp) on  
190 bioanalyzer high-sensitivity chips (Agilent), to then pool libraries in equimolecular quantities  
191 based on these DNA concentrations. We finally removed unwanted PCR products by size  
192 selecting the library pool on a BluePippin 300-500 bp selection cassette (Sage biosciences).  
193 Amplicon libraries were sequenced in a 2x250 bp paired-end format using the Illumina MiSeq  
194 platform at the Josephine Bay Paul Center Keck facility (Marine Biological Laboratory,  
195 Woods Hole MA, U.S.A). Raw data were deposited on the Ifremer Sextant website  
196 (<http://dx.doi.org/10.12770/c210bf1e-a55c-440f-810f-8f68b1ef9a9d>) and reads with metadata  
197 are publicly available on the VAMPS portal ([www.vamps.mbl.edu](http://www.vamps.mbl.edu)) under the project name  
198 LQM\_MPLA\_Bv4v5.

## 199 **5. Processing sequences**

200 Data were demultiplexed and barcodes were trimmed off the reads by the sequencing  
201 provider. Sequences were filtered, clustered and assigned with the FROGS pipeline (Find  
202 Rapidly OTU with Galaxy Solution) using the Galaxy platform (Escudié et al., 2017). Briefly,  
203 paired-end reads were merged using Flash (1.2.11) with an overlap length of 90 pb and a  
204 minimum length of 340 pb. Next, sequences were filtered using Cutadapt (1.7.1) to remove  
205 primers and using UCHIME (v7) of USEARCH package (1.1.3) to remove chimeras (Edgar,  
206 2010). Dereplication was used to group strictly identical sequences using a homemade script.  
207 SWARM (1.2.2) was used for clustering reads into operational taxonomic units (OTU) with a  
208 first run including an aggregation distance equal to 1 (i.e. high OTU definition linear  
209 complexity) and a second run with an aggregation distance equal to 3 on the seeds of the first  
210 SWARM quadratic complexity (Mahé et al., 2014). Representative sequences were aligned  
211 using NCBI Blast+ (2.2.29) with the database SILVA 123 (Camacho et al., 2009). Singletons  
212 (that is, sequences found once in one sample only) were excluded after quality filtering and  
213 global trimming for downstream analyses.

## 214 **6. Polymerase chain reaction (PCR) analysis**



215 The detection of *Vibrio splendidus* and *V. aestuarianus* by real-time PCR was adapted from  
216 previously published protocols (Saulnier et al., 2009, 2017) allowing the specific detection of  
217 all bacteria from *V. splendidus* polyphyletic group (*V. lentus*, *V. cyclitrophicus*, *V. pomeroyi*,  
218 *V. tasmaniensis*, *V. splendidus*, *V. kanaloae*, *V. gigantis* and *V. crassostreae*), and the specific  
219 detection of *V. aestuarianus* strain. Threshold cycles (Ct), defined as the cycle at which a  
220 statistically significant increase in fluorescence output above background is detected, were  
221 calculated automatically by the thermocycler software. A valid run was defined as a run  
222 exhibiting no amplification of the negative control and amplification of the positive control  
223 fulfilling the following requirements: difference between duplicated values must not exceed  
224 0.5 Ct, and Ct value must be below 37. A sample was defined as positive when it exhibited an  
225 exponential accumulation of fluorescence and a valid cycle threshold.

## 226 **7. Data analysis and statistics**

227 Venn diagrams were generated using the R packages Vegan and Venn, respectively (R Core  
228 team, 2015). For subsequent analyses of alpha- and beta-diversity, read counts were divided  
229 by the total number of reads in each sample to compensate for differential sequencing depth  
230 per sample. Alpha diversity based on observed number of OTU, species richness, Shannon  
231 and Simpson diversity indices were calculated for each sample type (microplastic and  
232 seawater). Whenever one-way analysis of variance (ANOVA) assumptions were met  
233 (normality, heterogeneity of variances, outliers), the latter was used to assess the effect of  
234 sample types on microbial diversity (Chambers et al., 1992), and Tukey HSD (honest  
235 significant difference) test was used for pairwise comparisons. Beta-diversity analyses were  
236 done using the R packages ggplot2 and phyloseq (McMurdie and Holmes, 2013). Bacterial  
237 assemblages of microplastics (PE, PP and PS) and of seawater samples were represented by  
238 mean relative percentage ( $\pm$  standard deviation) and compared using the Jaccard and Bray-  
239 Curtis diversity indices. Results of distance matrix were visualized using nonmetric  
240 multidimensional scaling (nMDS). Statistical comparison of bacterial communities between  
241 sample types, stations and surveys was done by permutational multivariate analysis of  
242 variance (PERMANOVA) and the homogeneity of group dispersions (variances) was  
243 subsequently tested (PERMDISP). Both analyses were performed using the R package Vegan  
244 (Anderson, 2001). Finally, the potential presence of taxonomic groups (i.e., biomarkers) that  
245 may explain the difference between bacterial communities in different sample was explored  
246 with LEfSe (Segata et al., 2011) in the Galaxy framework. The linear discriminant analysis  
247 (LDA) effect size allows identifying statistically significant groups characterized by their

248 degree of consistency in relative abundance together with their effect relevance, in each  
249 sample class (Segata et al., 2011). A p-value of 0.05 was set as the significance level for all  
250 analyses.

## 251 **Results and discussion**

### 252 **1. Microplastics and seawater shared a high proportion of taxa**

253 After quality filtering and chimera checking of the initial 21,660,493 reads, 8,055,314 reads  
254 were retained (mean reads per sample = 136,530), ranging from 34,984 to 324,667 reads in  
255 samples MP004 (PE, 5 - 2 mm, station A1, December) and MP023 (PP, 1 - 0.3 mm, A1,  
256 December), respectively. In total, high-quality sequences were clustered into 1,548  
257 operational taxonomic units (OTU) with 1,395 for seawater samples and 1,540 for  
258 microplastic samples. Microplastic and seawater samples presented rarefaction curves with a  
259 stationary phase indicating sufficient depth of sequencing to account for most of the taxa  
260 amplified in both microplastic and seawater matrices (data not shown). The seawater samples  
261 (encompassing both free-living (FL) and natural particle-attached (PA) bacteria) were  
262 predominantly (around 84 %) composed of rare OTU (hereafter defined with a mean relative  
263 abundance per sample < 0.01 %) whereas abundant OTU (mean relative abundance per  
264 sample > 1 %) were rare in all sample types (around 4 %). Microplastics and seawater shared  
265 a high number of OTU:  $78 \pm 4$  % of the OTU recorded on microplastics were shared with  
266 seawater; and  $98 \pm 0.04$  % of the OTU identified in seawater were shared with microplastics  
267 (Figure 2).

268 The high proportions of shared OTU between MP and seawater (FL+PA communities)  
269 suggest that the local surrounding seawater has likely provided most of the bacterial  
270 communities identified on collected microplastics. The local environment was already  
271 suggested to serve as a bacterial source for plastic biofilm organisms for plastic sheets  
272 deployed in a coastal harbor (De Tender et al., 2017). The fraction of shared OTU between  
273 microplastics and surrounding seawater was however much lower (3.5 to 8.6 %) in a study  
274 conducted in the North Atlantic Ocean (Zettler et al., 2013). The low proportion of suspended  
275 matter in oligotrophic oceanic waters (as compared to eutrophic coastal waters studied here;  
276 Supplementary table 1) could partly explain this difference as the fraction analyzed on 0.2 $\mu$ m  
277 sterivex filters may be different between the two studies, i.e. mostly composed by FL  
278 communities in Zettler et al. (2013) vs. FL and PA communities in our study.

279 Microplastics presented a larger number of unique OTU ( $n = 335 \pm 60$  OTU; 10 to 25 % of  
280 the total identified OTU) than seawater, exhibiting few unique OTU ( $n = 27 \pm 1$  OTU; 2 % of  
281 the total identified OTU) (Figure 2). A unique OTU is defined as an OTU exclusively found  
282 in a single matrix (i.e. microplastic or seawater), as opposed to a shared OTU that is detected  
283 in both microplastics and seawater samples. Interestingly, among the unique microplastic  
284 OTU,  $94 \pm 4$  % were shared between PE, PP and PS, and 0.2 to 1.6 % were specific to each of  
285 the three polymer families (Figure 2). Fraction of shared OTU between polymers was higher  
286 than the 30 to 40 % found between PE ( $n=3$ ) and PP ( $n=3$ ) in the study of Zettler et al. (2013),  
287 which could be due to the microplastics life history upon their entrance in marine waters:  
288 microplastics collected closed to the source as in the bay of Brest and therefore more recently  
289 colonized, may exhibit more uniform assemblages. Overall, the high proportion of shared  
290 OTU among polymers observed here suggests a “core” of bacteria characterizing the plastic  
291 substrate, regardless of the polymer type, as reported in (Zettler et al., 2013).

## 292 **2. High bacterial diversity is observed on microplastics**

293 For both surveys, microplastic bacterial communities richness (number of observed OTU)  
294 appeared significantly higher than the one of seawater bacterial (FL+PA) community (p-value  
295 =  $<0.001$  and  $0.006$ , respectively; Supplementary table 2), consistent with previous studies  
296 (Bryant et al., 2016; De Tender et al., 2015, 2017; Debroyas et al., 2017; Dussud et al., 2018).  
297 This likely reflects the colonization process and biofilm formation, often characterized by  
298 complex microbial competition and increased species richness (Datta et al., 2016; Jackson et  
299 al., 2001). In October, microplastic bacterial communities showed a significantly higher  
300 number of observed OTUs and Shannon index than seawater communities (Supplementary  
301 table 2), presumably due to the high proportion of rare OTU on the plastic matrix. On the  
302 opposite, no significant difference in evenness (reflected by the Shannon and Simpson  
303 indexes) was observed between microplastics and seawater in December. Among the three  
304 polymer families (PE, PP and PS), no significant difference in alpha-diversity was observed in  
305 October in terms of species richness and evenness, while PS collected in December showed a  
306 significantly greater Shannon diversity index than PE (p-value =  $0.029$ ) (Supplementary table  
307 2).

308 To our knowledge very few studies have investigated the alpha-diversity of plastic debris  
309 bacterial communities as a function of the polymer type and no data is available for the small  
310 microplastic size range we studied herein. The present study is the first to attempt the  
311 discrimination of potential differences in bacterial assemblages using metabarcoding in such

312 low size classes (0.3-1mm; 1-2mm; 2-5mm) for three distinct polymers (PE, PP, PS) collected  
313 in a coastal ecosystem. The difficulty of these analyses relies on the need to efficiently  
314 characterize the morphological and polymer nature of plastic particles down to a very small  
315 size in a very short term and in sterile conditions to avoid bacterial community shift. This is  
316 especially true for the lowest size class (0.3-1mm) for which DNA extraction on individual  
317 particle did not provide enough material for subsequent 16S amplicon sequencing analyses  
318 (data not shown), thus implying a pooling procedure per polymer type prior to DNA  
319 extraction. In addition the DNA extraction is destructive for microplastics due to the solvents  
320 used, thus requiring that the polymer characterization must be performed beforehand. Due to  
321 these constraints most studies discriminated the influence of polymer type using bigger  
322 particles (mainly pellets), small subsamples, or run their analysis in the whole microplastic  
323 pool without necessarily discriminating the polymer nature (Supplementary table 3). The  
324 strength of the present study lays in the comparison of different size classes and different  
325 polymers in a relatively large sample set (n=464 microplastics) sampled in one coastal  
326 ecosystem rich and diverse in terms of habitats, flora and fauna, and at the center of many  
327 human activities. The quantity of particles analyzed as well as the time required to process  
328 samples remained also often unknown, while these parameters are crucial for the evaluation  
329 of the protocol quality and representativeness of the sample size (Supplementary table 3).

330 No difference in bacterial species richness or evenness was observed as a function of particle  
331 size within the microplastic size range (300  $\mu\text{m}$ -5 mm, data not shown). For bigger plastic  
332 debris, surface area was shown to determine the abundance of fouling organisms (Fazey and  
333 Ryan, 2016). For instance, higher bacterial and eukaryotic richness were observed on  
334 mesoplastic sized PE (5mm-20cm) compared to 300 $\mu\text{m}$ -5mm microplastics primarily made of  
335 PE (Debroas et al., 2017), presumably due to differences in crystallinity and molecular weight  
336 between meso- and microplastics PE.

### 337 **3. Bacterial communities colonizing microplastics**

#### 338 3.1. Characterization of bacterial assemblages colonizing microplastics

339 For both sampling surveys, the bacterial communities structure were different between  
340 microplastics and the surrounding seawater as showed by separate clustering in the nMDS  
341 plot (Figure 3). This result was confirmed by statistical analyses using Bray-Curtis  
342 (PERMANOVA, p-value = 0.001 for October and December) (Supplementary table 4) as well  
343 as Jaccard similarity index (PERMANOVA, p-value = 0.001 for October and December)

344 (data not shown). Microplastics seemed to harbor different bacterial communities as  
345 compared to seawater (FL+PA) in terms presence/absence but also in terms of relative  
346 abundance of bacterial communities. Heterogeneity of variances was also observed  
347 (Supplementary table 5); however when the group with the largest dispersion also has the  
348 largest sample number (as it is the case here due to unbalanced sampling design) the  
349 PERMANOVA test becomes quite conservative and the observed significance differences can  
350 be confidently considered robust (Anderson & Walsh, 2013). The outputs of the PERMDISP  
351 analysis also showed differences between microplastics and seawater bacterial communities  
352 both in terms of centroid location and data dispersion (Supplementary figure 1A).  
353 Differences between water and plastic associated bacterial communities were demonstrated in  
354 urban freshwater ecosystems (Hoellein et al., 2014; McCormick et al., 2014, 2016), in North  
355 Atlantic and North Pacific oceans (Amaral-Zettler et al., 2015; Debroas et al., 2017; Zettler et  
356 al., 2013), in the North Sea (De Tender et al., 2015), in the Western Mediterranean Basin  
357 (Dussud et al., 2018) as well as for plastics incubated in coastal waters and sediments (De  
358 Tender et al., 2017; Harrison et al., 2014; Oberbeckmann et al., 2014). However, such  
359 distinction cannot be rigorously ascertained in the present study) because (i) a clear distinction  
360 between FL and PA bacterial assemblages present in the collected seawater was not  
361 performed here and (ii) a dilution effect may have masked the relative importance of PA  
362 communities. Indeed, collected seawater was filtered on 0.22  $\mu\text{m}$  filters which concentrated  
363 both free-living (3 - 0.22  $\mu\text{m}$ ) and particle-associated bacterial communities (> 3  $\mu\text{m}$ ), making  
364 the discrimination of both fractions not possible. In addition, even though the SOMLIT data  
365 confirmed the presence of suspended organic and particulate matter (Particulate Organic  
366 Carbon (POC) = 125-134  $\mu\text{g L}^{-1}$ ; Suspended Particulate Matter (SPM) = 1.3-2.5  $\text{mg L}^{-1}$ ) in  
367 the seawater at the time of sampling (Supplementary table 1) we cannot exclude a potential  
368 dilution effect considering the difference between the quantity of natural particle analyzed per  
369 filter (estimated to 1.3 - 2.5 mg) and the quantity of microplastic used for DNA extraction  
370 (maximum mass estimated at 9.8 – 61.5 mg per pool). Thus, an appropriate “particle” control,  
371 well characterized in terms of particle matter quantity and quality, and distinct from a “free-  
372 living” control is lacking in the present study to confirm the specificity of microplastic  
373 bacterial communities. For instance, Oberbeckmann et al. (2016) demonstrated that even  
374 though PET bottles-attached bacterial communities were distinct from free-living seawater  
375 communities, they were similar to other types of particle-associated or glass-attached  
376 communities collected in the surrounding seawater (with the exception of some unique OTU  
377 identified on PET). However, investigations conducted in a larger sample set of various PMD

378 (n=72) at a large spatial scale (Western Mediterranean Basin) provided contrasted results with  
379 a significant distinction between FL, PA and PMD-attached bacterial communities. Despite  
380 the presence of a large proportion of OTUs being able to colonize indifferently PMD or PA,  
381 and to subsequently free themselves (Dussud et al., 2018) FL bacteria were dominated by  
382 Alphaproteobacteria (mainly *Pelagibacter* sp.). PA bacteria, on the other hand, were  
383 dominated by Alphaproteobacteria (mainly *Erythrobacter* sp.) and Gammaproteobacteria  
384 (mainly *Alteromonas* sp.) while PMD was predominantly colonised by Cyanobacteria and  
385 Alphaproteobacteria. Plastic debris exhibiting different bacterial communities than other  
386 marine substrate was also demonstrated in previous studies conducted in freshwater  
387 (McCormick et al., 2014). This was however not necessarily consistently observed when  
388 comparing different type of hard substrates including plastic (Hoellein et al., 2014).

389 At the phylum level, bacterial communities of all sample types (microplastics and seawater)  
390 were dominated by Proteobacteria ( $60.72 \pm 5.41$  %), Bacteroidetes ( $20.58 \pm 4.64$  %) and  
391 Cyanobacteria ( $9.09 \pm 7.40$  %) representing major bacterial classes colonizing substrate in  
392 marine ecosystems (Keswani et al. 2016). Microplastics were mainly colonized by Alpha- and  
393 Gammaproteobacteria ( $17.67 \pm 5.28$  % and  $40.76 \pm 8.43$  %, respectively), which were shown  
394 to act as primary colonizers, and Flavobacteria (Bacteroidetes,  $16.83 \pm 2.64$  %), which  
395 appeared to act as secondary colonizers (Lee et al., 2008; Oberbeckmann et al., 2015).

396 At the family level, Flavobacteriaceae and Rhodobacteraceae were identified in relatively  
397 high abundance in all sample types (microplastics and seawater) with  $16.06 \pm 1.23$  % and  
398  $13.40 \pm 4.03$  %, respectively. The families of Vibrionaceae ( $9.88 \pm 8.27$  %) and  
399 Pseudoalteromonadaceae ( $8.24 \pm 6.95$  %) were commonly found in microplastic samples but  
400 rarely observed in seawater community encompassing both FL and PA bacteria (Figure 4).  
401 However, the low relative abundance of these families in seawater could simply be due to a  
402 dilution effect, despite the fact that these bacteria may densely populate natural particles. .  
403 Both Vibrionaceae and Pseudoalteromonadaceae families were similarly found on marine  
404 plastic litter collected along the Belgian coast while rarely observed in surrounding seawater  
405 and sediments (De Tender et al., 2015). Very few information focusing on the  
406 Pseudoalteromonadaceae family is available in the microplastic literature, while several  
407 studies reported that the genus *Pseudoalteromonas* was previously detected on plastics  
408 (Zettler et al., 2013) or as a dominant genus on PET bottles (Oberbeckmann et al., 2014,  
409 2016). This genus is known as a hydrocarbon degrader (Lin et al., 2009) and has often been  
410 observed associated with marine algae.



411 At the genus level, *Litoreibacter* and *Vibrio* were commonly detected on microplastics, while  
412 *Candidatus Actinomarina*, *Synechococcus*, *Owenweeksia*, NS3 marine group and NS5 marine  
413 group appeared as biomarkers of seawater. Interestingly, the genus *Vibrio* has been very  
414 frequently detected in association with plastic debris for the past few years (Dussud et al.,  
415 2018; Kirstein et al., 2016; Oberbeckmann et al., 2016; Schmidt et al., 2014) and even  
416 represented up to 24% on PP pellet collected in the North Atlantic (Zettler et al., 2013).  
417 However, this observation is not consistent as Bryant et al. (2016) and Oberbeckmann et al.  
418 (2017) did not observe any enrichment of *Vibrio* on microplastics. *Vibrios* are ubiquitous  
419 marine bacteria belonging to diverse ecological populations that are ecologically and  
420 metabolically different and pursue different lifestyles in the water column (free living, particle  
421 and animal-associated) (Le Roux et al., 2016). The vibrio genus comprises numerous  
422 pathogenic species for human, fish and shellfish, and some of which (*V. coralliilyticus*, *V.*  
423 *harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fluvialis*) that have been  
424 detected on microplastics (Dussud et al., 2018; Kirstein et al., 2016; Schmidt et al., 2014),  
425 thus there is some concerns that the ever increasing microplastic contamination in marine  
426 environment may influence their population dynamics, and ultimately pathogen emergence.  
427 For instance, rapid growth of vibrios has been observed in association with diatom bloom or  
428 algae proliferation (Gilbert et al., 2012; Nealson and Hastings, 2006) suggesting the  
429 importance of substrate and habitat occurrence on particle-associated vibrios dynamic in the  
430 marine environment. In our study, the genus *Vibrio* was recovered in high abundance in  
431 microplastic samples (1.5 to 18.6 %) but the specificity of *Vibrio* genus to colonize plastic as  
432 compared to other natural particulate matter has yet to be clarified. . To further investigate the  
433 presence of potential pathogenic *Vibrio* strains on microplastics, we assayed two *Vibrio*  
434 species *V. splendidus*-related and *V. aestuarianus* that have frequently been associated with  
435 massive mortality events in Pacific oyster in France alongside with the herpesvirus OsHV-1  
436 (Le Roux et al., 2002; Saulnier et al., 2009). Some strains of these bacteria are known to  
437 exhibit virulent abilities in experimental infection trials (De Decker and Saulnier, 2011) and it  
438 was recently demonstrated that these agents can act solely or in concert (polymicrobial  
439 disease) in the field (Lemire et al., 2015; Petton et al., 2015). *V. splendidus* related species  
440 were commonly detected on 77% of the MP samples (36 out of 47 pools exhibited Ct values  
441 comprised between 19.4 and 34.9) while *V. aestuarianus* strain was never detected at the  
442 threshold defined above (supplementary Table 6). None of these species were ever detected in  
443 seawater samples by qPCR. This result raises concern about the transport of potential

444 pathogens by microplastics, as recently demonstrated for *Aeromonas salmonicida* (Virsek et  
445 al., 2017).

### 446 3.2. Bacterial assemblages were influenced by polymer nature but not by particle size

447 Size and therefore surface area did not appear as a main factor in shaping microplastic  
448 bacterial communities at the microscale (0.3-1 vs. 1-2 vs. 2-5mm) as no significant difference  
449 in terms of OTU composition and structure was detected among microplastic size ranges ( $p >$   
450 0.05 for both PERMANOVA and PERMDISP; Supplementary Tables 4 and 5;  
451 Supplementary Figure 1B). This is not necessarily true when comparing bigger plastic debris  
452 as difference in community structures was recently demonstrated between mesoplastic sized  
453 PE (5mm-20cm) as compared to the microplastics (300 $\mu$ m-5mm) primarily made of PE  
454 (polymer nature identified on a separated subsample; Debroas et al. 2017).

455 On another hand, the microplastic bacterial community composition was significantly  
456 influenced by the polymer family with PS presenting a distinct bacterial community to those  
457 of PE and PP in December 2015 (PERMANOVA,  $p$ -value = 0.013 and  $p$ -value = 0.017,  
458 respectively) (Supplementary table 4). It is noteworthy that a great heterogeneity in dispersion  
459 was observed for the PE communities while PS and PP displayed more tightly clustered  
460 groups (PERMDISP,  $p$ -value = 0.004; Supplementary table 5 and Supplementary figure 1C).  
461 As most PS collected in the bay of Brest was found in the form of foam fragments, the distinct  
462 bacterial communities may be related to difference in terms of physical structure and/or  
463 chemical load. Similarly, PS was also found to be distinct from PE and PP in terms of  
464 community assemblage and structure in earlier studies conducted in the Atlantic and Pacific  
465 oceans (Amaral-Zettler et al. 2015). Difference in structural and/or chemical (plasticizers,  
466 dyes) properties observed among polymer families is likely to influence bacterial  
467 communities and dynamics (De Tender et al., 2015) even though studies specifically  
468 addressing this point are still lacking (Oberbeckmann et al., 2015). For instance, PE ropes and  
469 sheets deployed at the same coastal location quickly exhibited distinct bacterial structures  
470 while being made by the same polymer and incubated in the same habitat (De Tender et al.,  
471 2017) suggesting that particle shape (and/or unknown additive compounds) is a determining  
472 factor influencing bacterial colonisation of PMD for a given polymer.

### 473 3.3. Spatial and temporal influence in bacterial communities composition

- 474 ● Temporal variability

475 Bacterial assemblages were different across surveys (October and December 2015) for all  
476 sample types (microplastics and seawater) (Figure 3, PERMANOVA, p-value = 0.004 for  
477 seawater, p-value = 0.001 for microplastics; Supplementary table 4). While homogeneity of  
478 dispersion was observed in seawater communities collected in October and December, a  
479 significant heterogeneity of dispersion was demonstrated in the microplastics communities  
480 from these two surveys (PERMDISP, p-value = 0.302 and 0.001, for seawater and  
481 microplastics, respectively; Supplementary table 5 and Supplementary figure 1D-E). As a  
482 consequence, change in the microplastic community structure between both sampling times is  
483 likely due to both location and dispersion effects). Temporal variability in bacterial  
484 assemblages associated to microplastics was also found in previous studies conducted in  
485 freshwater (Hoellein et al., 2014) and coastal ecosystems (De Tender et al., 2017; Hoellein et  
486 al., 2014; Lee et al., 2008; Oberbeckmann et al., 2014). As a consequence, different  
487 taxonomic group significantly discriminated the microplastic matrix and the seawater  
488 bacterial communities according to sampling date. The Sphingomonadales order and  
489 *Psychoserpens* genus were biomarkers of microplastics in October samples whereas *Bacilli*  
490 (Firmicutes) and *Tenacibaculum*, *Leucothrix*, *Oleibacter* and *Psychomonas* genera were  
491 biomarkers in December samples (Figure 5). The phylum *Firmicutes* is typically related to  
492 sewage associated bacteria (e.g. *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Staphylococcus*  
493 and *Streptococcus* genera) (Oberbeckmann et al., 2015) and was previously detected on MP  
494 collected in the North Adriatic Sea (Viršek et al., 2017).

495 In terms of polymer differences, the Pseudomonadales order was detected as a PE biomarker  
496 in October 2015. In December 2015, the Oceanospirillales order (Gammaproteobacteria),  
497 which was shown to play a role in oil spill degradation (Mason et al., 2012), and the  
498 *Propionispira* genus, described by Ueki et al. (2014), were found as biomarkers of PE. The  
499 Alphaproteobacteria class and the Holophagae order (Acidobacteria) were biomarker of PP.  
500 Alphaproteobacteria constitute early colonizers commonly found on plastic debris, while  
501 Acidobacteria was previously found significantly associated with PET (and in a lesser extent  
502 PS) mesoplastics (Debroas et al., 2017) and in plastic marine debris in North Sea samples (De  
503 Tender et al., 2015). Finally, Rhodospirillaceae family (previously detected on PP and PE;  
504 Zettler et al., 2013), *Roseovarius* (belonging to the Roseobacter group common in coastal and  
505 open oceans) and *Nitrosomonas* genera (known to oxidize ammonia) were biomarkers of PS in  
506 this study.

507 • Spatial variability

508 PERMANOVA analyses showed a significant difference in bacterial communities  
509 composition between A1 and M1 in December. The M1 station was dominated by the  
510 Moraxellaceae family (42.3 %) and the *Psychrobacter* genus (41.7 %) while station A1 was  
511 dominated by the 34P16 order (Gammaproteobacteria, unknown family and genus) (16.4 %).  
512 No reliable spatial analysis can be performed here due to the low MP sample size in M1 (n=4  
513 pools containing a total of 40 MP) as compared to A1 (n=43 pools containing 424 MP)  
514 (Table 1), but spatial variability in plastic-associated communities were more rigorously  
515 assessed in previous studies conducted in the North Sea (Oberbeckmann et al., 2015; De  
516 Tender et al., 2017) and in the North Pacific and North Atlantic Oceans (Amaral-Zettler et al.,  
517 2015).

518 While temporal differences were only supported by two sampling times herein and must  
519 therefore be considered with cautious, these results open up a relevant issue for understanding  
520 the temporal and spatial variability of microplastics's microbial communities at the scale of a  
521 bay taking into account the sources and consequences for human activities, both being major  
522 points for decision support.

## 523 **Conclusion**

524 The efficient colonisation of microplastics floating at sea emphasizes the fact that this new  
525 man-made habitat may facilitate the persistence and long range dispersal of microorganisms.  
526 As a consequence plastic bacterial communities are likely to be dynamic and able to quickly  
527 adapt to their changing environment. For instance, hydrodynamics modeling work  
528 demonstrated that 60% of the floating microplastics present in the bay of Brest are expelled  
529 from the bay after 10 days (Frère et al., 2017), and the fate of the associated bacterial  
530 communities remains unknown in the Iroise Sea and the Atlantic Ocean.

531 The *Vibrio* genus was commonly found on the collected microplastics and *V. splendidus*  
532 related species harboring potential oyster pathogens were detected on most microplastic pools  
533 (77%). This raises questions about the role of microplastics on pathogenic *Vibrio* species  
534 transport and potential disease emergence and much work has to be done on clarifying the  
535 specificity of these bacteria for the plastic substrate. To investigate the ecological effects of  
536 microplastic pollution on pathogens emergence and virulence, proper 'natural particulate  
537 matter' controls must be considered in field surveys to avoid any misinterpretations. In  
538 addition, risk evaluation based on bacterial identification should be completed by more in-  
539 depth studies involving RNA sequencing of pathogenicity markers coding for instance for

540 toxins, adhesins, or invasins (Goudenège et al. 2015) and experimental testing of virulence in  
541 laboratory trials (Labreuche et al., 2006).

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## Tables

**Table 1.** Description of the microplastic pools used for bacterial communities analysis. Each pool contained n=20 fragments in the 1-0.3 mm range, 8 for the 2-1mm range, and 4 for the 5-2 mm range. The pooling rate was adapted to each size class to ensure the recovery of enough DNA quantity for subsequent extraction and sequencing analyses. The number of pool processed per polymer type and size class was dependent on the number of particles collected and correctly identified by micro-spectrometry Raman. PE: polyethylene, PP: polypropylene, PS: polystyrene.

Survey	Station	Polymer	Size class	Nb MP per pool	Nb pool processed	Total nb MP processed
October (n=200 MP)	A1 (n=172 MP)	PE	0.3-1 mm	20	2	40
			1-2 mm	8	4	32
			2-5 mm	4	4	16
		PP	0.3-1 mm	20	1	20
			1-2 mm	8	2	16
			2-5 mm	4	2	8
	PS	0.3-1 mm	20	1	20	
		1-2 mm	8	2	16	
		2-5 mm	4	1	4	
	M1 (n=28 MP)	PE	1-2 mm	8	1	8
		PS	0.3-1 mm	20	1	20
	December (n= 264 MP)	A1 (n=252 MP)	PE	0.3-1 mm	20	4
1-2 mm				8	5	40
2-5 mm				4	5	20
PP			0.3-1 mm	20	2	40
			1-2 mm	8	1	8
			2-5 mm	4	2	8
PS		0.3-1 mm	20	2	40	
		1-2 mm	8	1	8	
		2-5 mm	4	2	8	
M1 (n=12 MP)		PE	5-2 mm	4	1	4
			1-2 mm	8	1	8

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## Figures

**Figure 1.** Map of the Bay of Brest indicating sampling locations A1 and M1 and the main anthropogenic pressures. The red cross indicates the sampling station used for surface water collection for the monitoring of physical, chemical, biogeochemical and biological parameters within the scope of the SOMLIT (Service d'Observation en Milieu Littoral), the French Coastal Monitoring Network (<http://somlit.epoc.u-bordeaux1.fr/fr/>).

**Figure 2.** Shared and specific OTU in all sample types (PE, PP, PS and seawater) in October and December 2015.

**Figure 3.** nMDS plot comparing OTUs of bacteria in all sample types (PE, PP, PS and seawater) in October and December 2015 (Bray-Curtis dissimilarity index). For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

**Figure 4.** Heatmap of the 20 dominants bacterial families in the different sample types (PE, PP, PS and seawater) at October (A) and December 2015 (B).

**Figure 5.** Cladogram of LEfSe results according to sample types (microplastics and seawater) for samples collected in October 2015 (A) and December 2015 (B)



Figure 1.

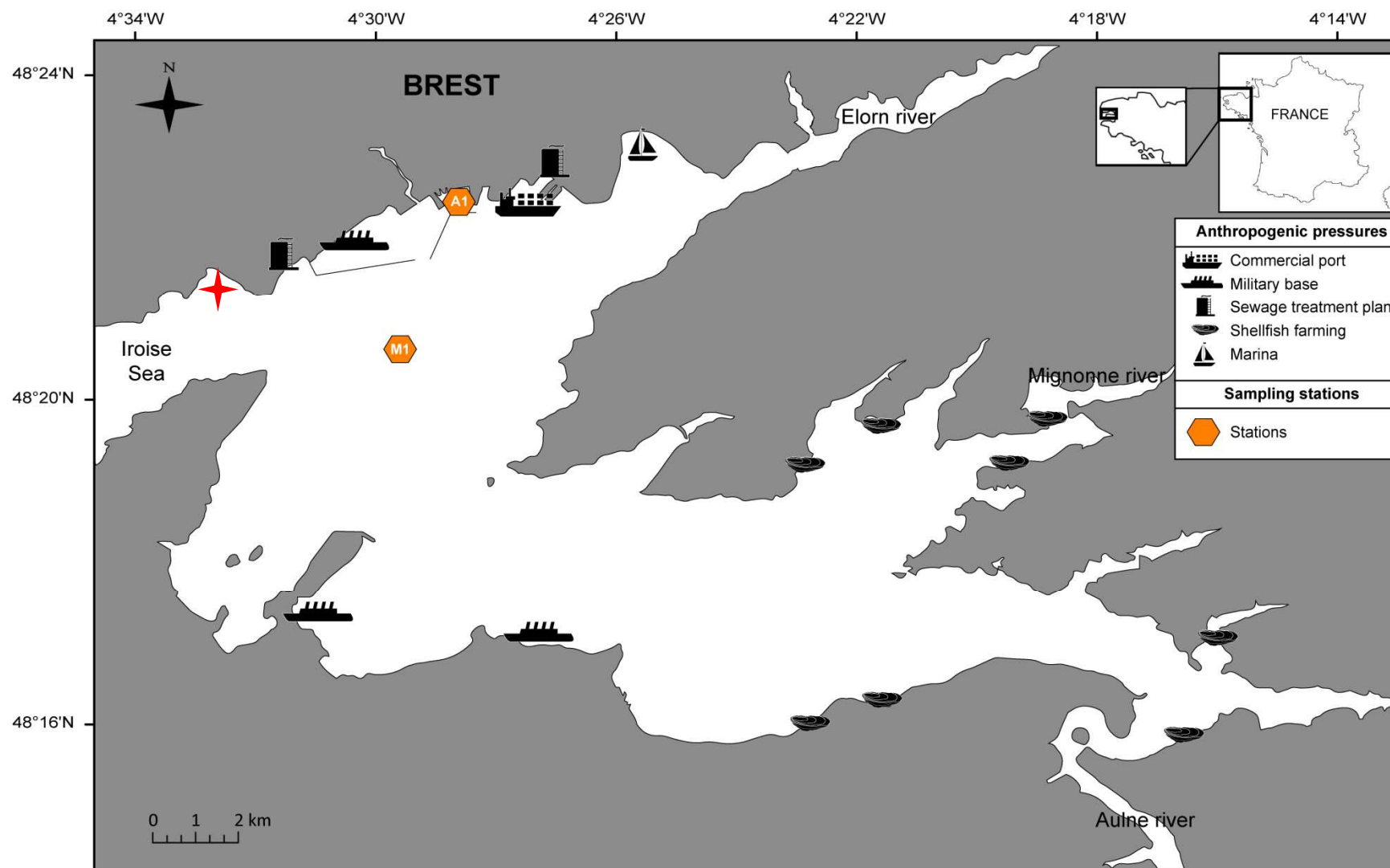


Figure 2

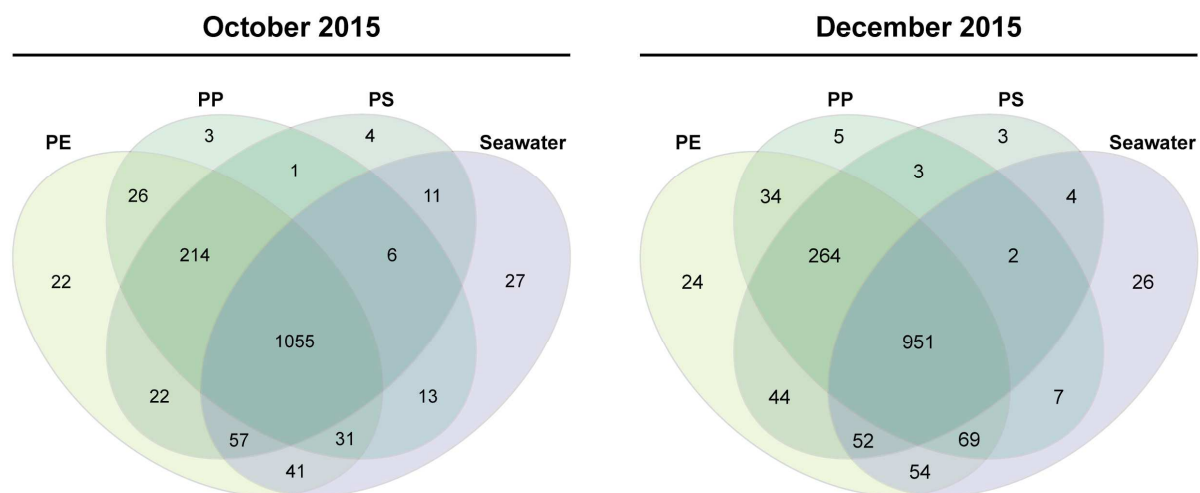


Figure 3

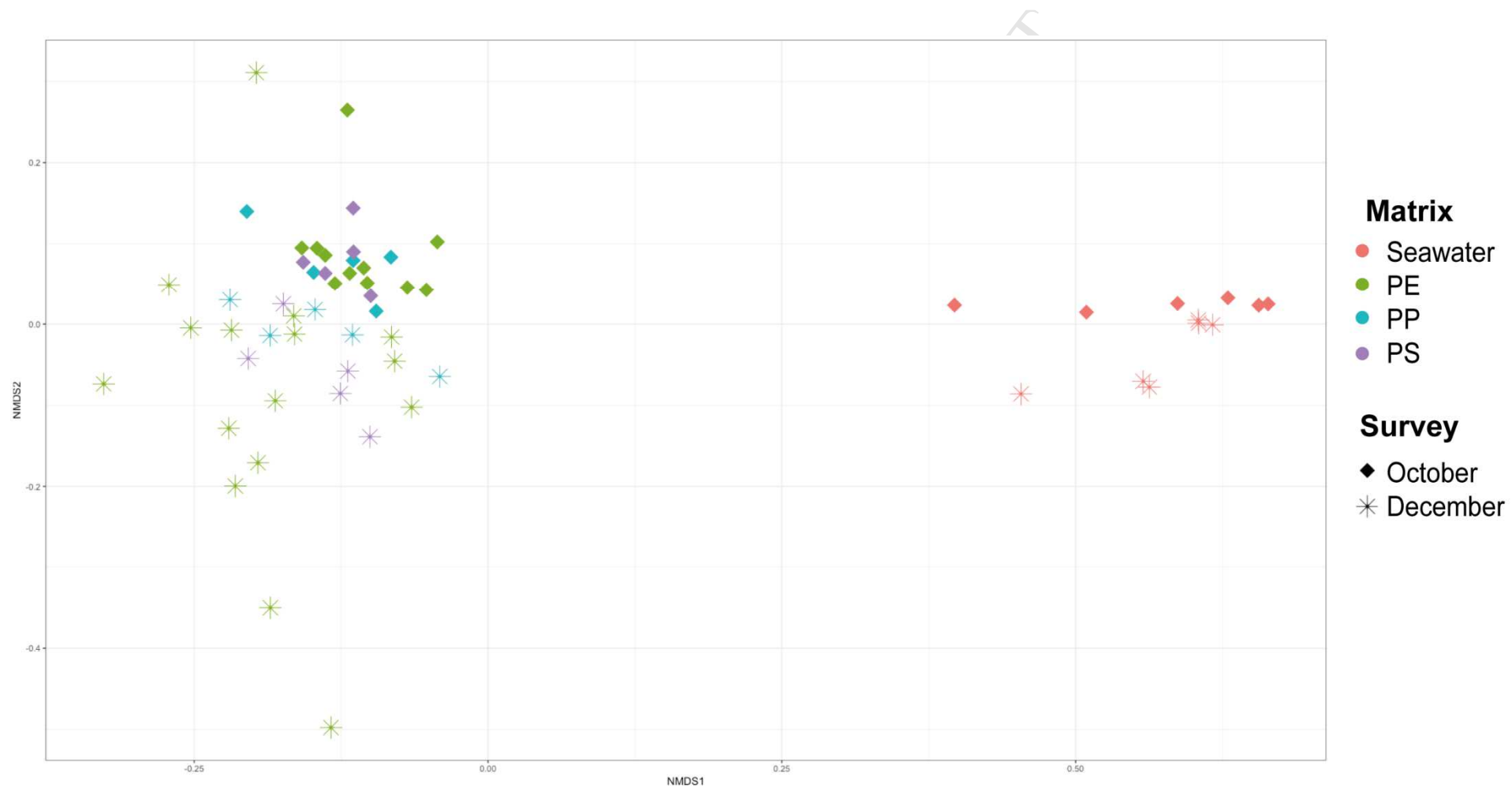


Figure 4

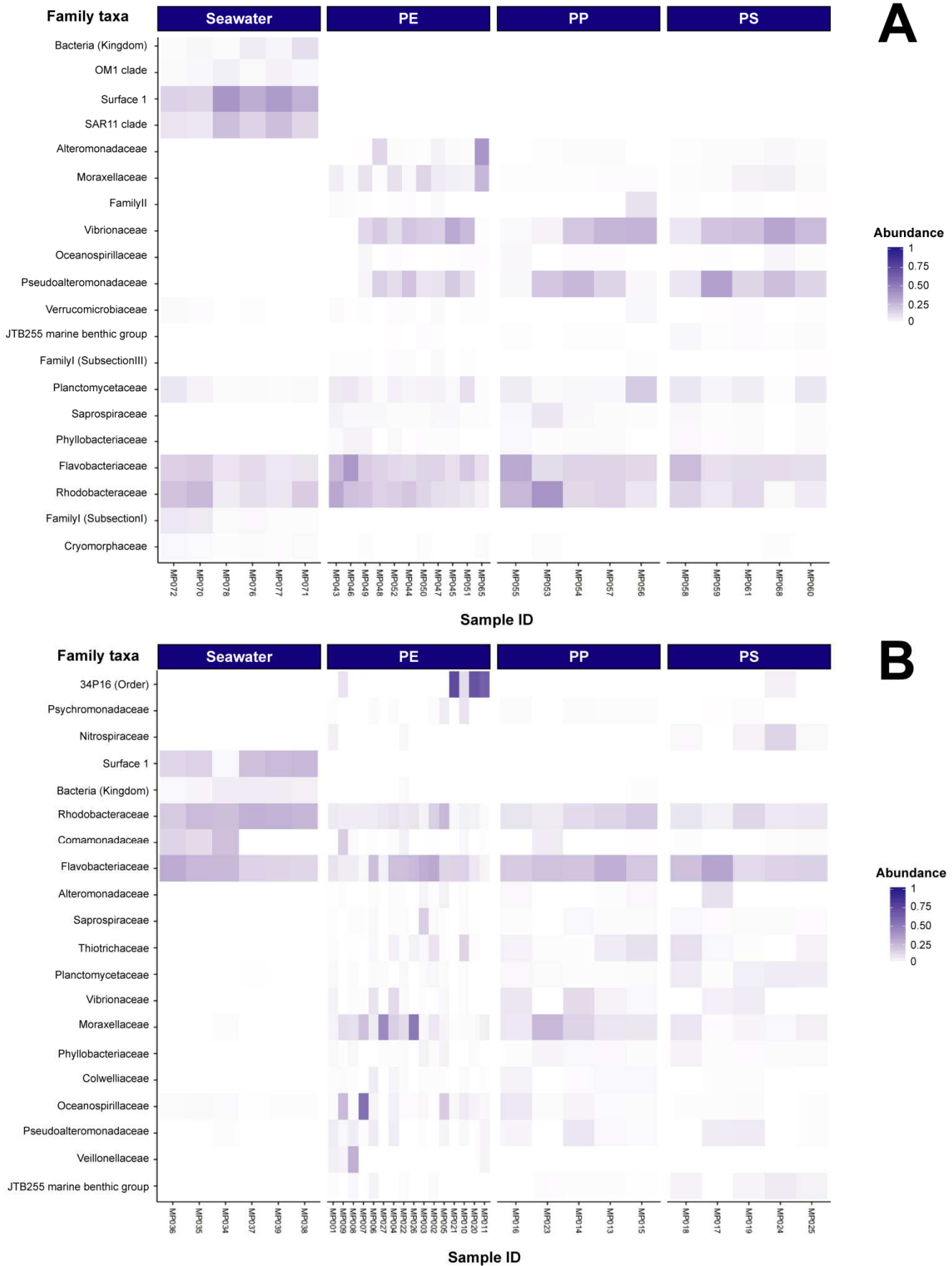
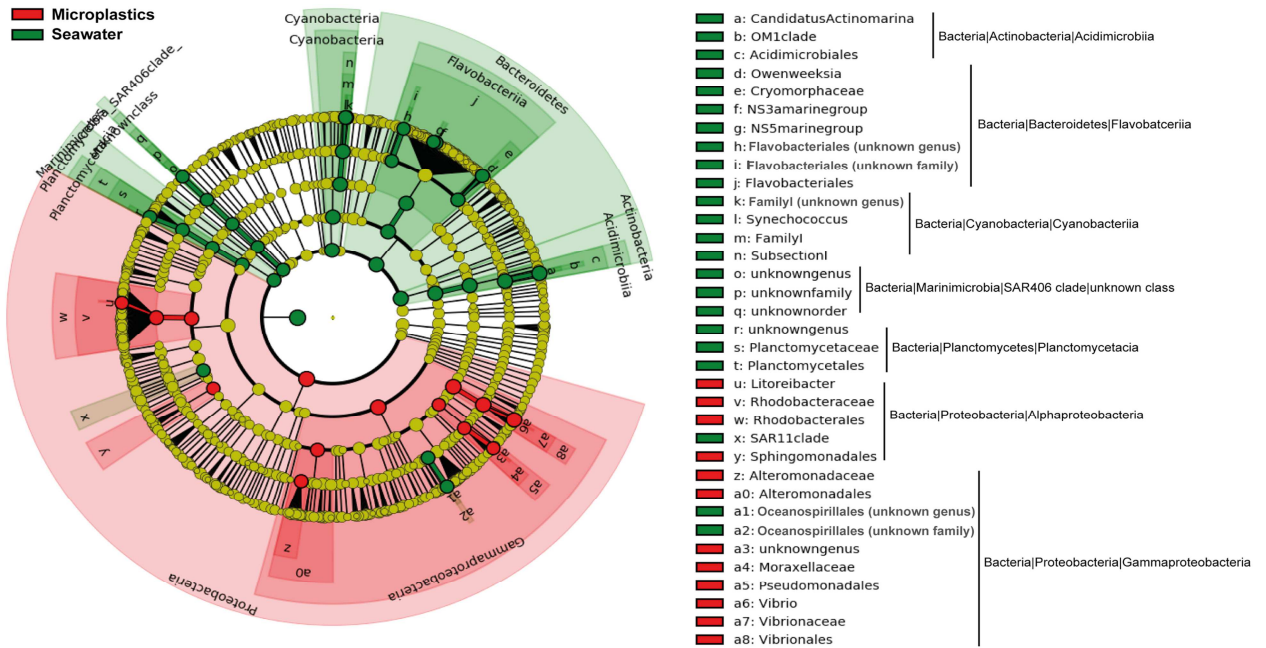


Figure 5

A



B

