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1 **Metal resistance genes enrichment in marine biofilm communities selected by biocide-**  
2 **containing surfaces in temperate and tropical coastal environments**

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20

21 **Abstract**

22 Microorganisms able to form biofilms in marine ecosystems are selected depending on immersed  
23 surfaces and environmental conditions. Cell attachment directly on toxic surfaces like antifouling  
24 coatings suggests a selection of tolerant (or resistant) organisms with characteristics conferring  
25 adaptive advantages. We investigated if environment would drive metal resistance gene abundance  
26 in biofilms on artificial surfaces. Biofilms were sampled from three surfaces (a PVC reference and  
27 two antifouling coatings) deployed in three coastal waters with dissimilar characteristics: The  
28 Mediterranean Sea (Toulon) and Atlantic (Lorient) and Indian (Reunion) Oceans. The two coatings  
29 differed in metals composition, either Cu thiocyanate and Zn pyrithione (A3) or Cu<sub>2</sub>O (Hy). Metal  
30 resistance genes (MRG) specific to copper (*cusA*, *copA*, *cueO*) or other metals (*czcA* and *pbrT*)  
31 were monitored with qPCR in parallel to the microbial community using 16S rRNA gene  
32 metabarcoding. A lower  $\alpha$ -diversity on A3 or Hy than on PVC was observed independent on the  
33 site. Weighted Unifrac suggested segregation of communities primarily by surface, with lower site  
34 effect. Metacoder log<sub>2</sub> fold change ratio and LeFSe discrimination suggested *Marinobacter* to be  
35 specific of Hy and *Altererythrobacter*, *Erythrobacter* and *Sphingorhabdus* of A3. Likewise, the  
36 relative abundance of MRG (MRG/bacterial 16S rRNA) varied between surfaces and sites. A3  
37 presented the greatest relative abundances for *cusA*, *cueO* and *czcA*. The latter could only be  
38 amplified from A3 communities, except at Toulon. Hy surface presented the highest relative  
39 abundance for *copA*, specifically at Lorient. These relative abundances were correlated with LeFSe  
40 discriminant taxa. *Dasania* correlated positively with all MRG except *cueO*. *Marinobacter* found  
41 in greater abundance in Hy biofilm communities correlated with the highest abundances of *copA*  
42 and *Roseovarius* with *czcA*. These results prove the selection of specific communities with abilities  
43 to tolerate metallic biocides forming biofilms over antifouling surfaces, and the secondary but  
44 significant influence of local environmental factors.

45

46

47 **The capsule of no more than two lines that summarizes the main finding:**

48 Marine biofilm communities were selected by metal-releasing surfaces before environmental  
49 drivers, and enriched in metal resistance genes in temperate and tropical coastal environments

50

51 **Keywords:**

52 Complex communities, antifouling surface, copper tolerance, anthropogenic effect

53

## 54 **Introduction**

55 Submersion of a surface in aquatic ecosystems leads to microbial colonization within  
56 minutes. The microbial biofilm community formed is driven by environmental features as salinity  
57 and temperature (Briand et al., 2017; Lau et al., 2005; Oberbeckmann et al., 2018; Pollet et al.,  
58 2018), nutrients (Lawes et al., 2016), but also substrata (Catao et al., 2019; Celikkol-Aydin et al.,  
59 2016; Faÿ et al., 2018; Kirstein et al., 2018; Oberbeckmann et al., 2016; Pinto et al., 2019).  
60 Biocides-containing surfaces are used in maritime industry as well as in medicine to prevent the  
61 adhesion of microorganisms. To limit marine biofouling on submerged structures, the efficacy of  
62 antifouling coatings remains often related to high concentrations of metallic biocides, even though  
63 alternative strategies are being increasingly used (Lejars et al., 2012). Since 2012, in Europe, those  
64 biocides must be controlled by the EU Biocidal Products Regulation (BRP, Regulation EU  
65 528/2012) to reduce environmental contamination. Copper-based compounds became the main  
66 biocides, and zinc-based co-biocides are also often present in the coating formulation. These  
67 metals were reported to exert a selection on the naturally occurring organisms (Ancion et al., 2010;  
68 Coclet et al., 2018; Corcoll et al., 2019) and can co-select for resistant strains (Flach et al., 2017).  
69 In fact, coatings are still colonized by a reduced number of tolerant or resistant organisms (Briand  
70 et al., 2017; Catão et al., 2019; Corcoll et al., 2019; Muthukrishnan et al., 2014).

71 Biofilms are often referred as an ecological advantage that provide protection to the cells  
72 from a toxic environment (Hall-Stoodley et al., 2004; Hoyle and Costerton, 1991). Their ability to  
73 develop over toxic surfaces is considered dependent on tolerance mechanisms including  
74 exopolymeric production and/or resistance genes (Flemming and Wuertz, 2019). Regarding  
75 metallic biocides, bacteria can react to elevated concentrations by expressing metal resistance  
76 systems as: P-type ATPases, metallothioneins, resistance-nodulation-cell-division (RND) efflux  
77 pumps and/or CDF transporters (Bruins et al., 2000; Nies, 2003).

78 In microbial communities, the specific resistance to copper was studied on marine  
79 sediments (Besaury et al., 2016, 2014, 2013; Roosa et al., 2014), tailing dam (Chen et al., 2019)  
80 and in terrestrial samples as manure compost (Guo et al., 2019). This resistance seems to be  
81 mediated by 1) the efflux ATP-ase pump *copA* (Rensing et al., 2000); 2) the *cusA* protein related  
82 to chemo-osmotic copper extrusion and part of the RND family (Outten et al., 2001; Piddock,  
83 2006) and the 3) *cueO* encoding for a multicopper oxidase (Silveira et al., 2014). The genes above  
84 have been described from *Escherichia coli* and *Enterococcus faecium* and seem to be present in

85 certain *Bacteria* and few *Archaea*. Other genes can be monitored to detect resistance to other  
86 metals or co- and cross-resistance. As for example, the *czcA* gene which codes for the antiporter  
87 cation-anion for efflux of  $\text{Co}^{+2}/\text{Zn}^{+2}/\text{Cd}^{+2}$  is also part of the RND family (Nies et al., 1987).  
88 Together with this gene, the complex of proteins encoded by *pbr* have been identified from the  
89 indigenous plasmid pMOL30 of the bacteria *Ralstonia metallidurans* with the functions of uptake  
90 and efflux of Pb (Borremans et al., 2001).

91 Only one study on the Swedish west coast have reported the effect of metal released coating  
92 on the co-selection of antibiotic and metal resistance genes (MRG) using a metagenomics approach  
93 (Flach et al., 2017). Furthermore, MRG have been linked to sediment and biofilm microbial  
94 communities in freshwater lakes (Song et al., 2019). However, to our knowledge, no work has yet  
95 linked the specific MRG occurrence, using quantitative PCR, with the selection of specific taxa  
96 within marine biofilm communities, and most specifically related to human-made surfaces  
97 commonly deployed in the sea. The selection of specific communities on metal-containing surfaces  
98 in comparison to inert surfaces lead us to explore the hypothesis that the community selection was  
99 driven more strongly by metal containing surfaces than environmental conditions in three  
100 dissimilar coastal sites in both temperate and tropical ecosystems. Then, we wonder if correlation  
101 might be established more particularly between selected microbial taxa and the abundance of some  
102 of metal resistance genes in the different ecosystems.

103

## 104 **Material and methods**

### 105 *Experimental design and environmental parameters*

106 Immersions were performed in French harbors at the Toulon Bay (43°06'25" N;  
107 5°55'41" E) in the North-Western Mediterranean Sea, Lorient (Kernevel harbour,  
108 47°43'8.0178" N; 3°22'7.2048" W) in the south coast of Brittany in the Atlantic Ocean, and Le  
109 Port, Reunion Island (20°56'03" S; 55°17'0.6" E) in the Indian Ocean.

110 Biofilm was studied over panels (Figure S1) immersed in the euphotic zone (from 0.5 to 2  
111 m deep) for 30 days in the summer season, from 15<sup>th</sup> June to 15<sup>th</sup> July 2016 at Lorient and Toulon,  
112 and 1<sup>st</sup> to 29<sup>th</sup> December 2016 at Reunion.

113 Water chemistry was analysed in the three sites during the immersion period according to physical,  
114 nutritional and trace metals (Catão et al., submitted, Table S1). Briefly, water quality in the three  
115 coastal sites differed significantly. At Reunion, water presented high signals of contamination in

116 terms of phosphate and Pb concentrations. Lorient was also considered eutrophic with the highest  
117 levels of nitrate between the three sites. Toulon was characterized by the highest salinity but lowest  
118 phosphate and silicate concentrations. Toulon also presented the highest contamination of Cu and  
119 Zn. Due to sample loss, Lorient could not be evaluated regarding trace metal contamination.

120

### 121 *Surface parameters*

122 PVC panels of 5 × 5 cm were either sandblasted or painted with one of the two  
123 commercially available antifouling paints (in triplicates): a solvent-based Nautix A3 white self-  
124 polishing paint (hereafter named A3) and a water-based Boero Hydrocoat blue paint (hereafter  
125 abbreviated as Hy). A3 and Hy are chemically-active paints as they contain metallic biocides that  
126 are continuously released during immersion to hamper formation of biofouling on human-made  
127 surfaces deployed in the sea. Furthermore, A3 and Hy differed in biocides composition and  
128 formulation, as the former contained 219 g.L<sup>-1</sup> (15%) copper thiocyanate (CuSCN; 114.5 g Cu)  
129 and 58.4 g.L<sup>-1</sup> of Zn (4% of Zn pyrithione – ZPT, and 3% ZnO) and Hy had 844.8 g.L<sup>-1</sup> (749.3 g  
130 Cu) of copper oxide (Cu<sub>2</sub>O) (40.23%), but no Zn. Information on the polymer and metals used  
131 were obtained directly from the providers or in technical sheets.

132

### 133 *Biofilm sampling*

134 After scraping the biofilms on the 5 x 5 cm panels with a sterile scalpel, samples were  
135 frozen in liquid nitrogen and kept at –80 °C until DNA extraction. For flow cytometry analysis,  
136 biofilms were also scraped but fixed in 10 mL solution of artificial seawater (ASW; Sigma-  
137 Aldrich) containing 0.25% glutaraldehyde (Sigma-Aldrich). Cells were enumerated as previously  
138 described (Pollet et al., 2018).

139 DNA extracted with PowerBiofilm DNA isolation kit (Qiagen, Courtaboeuf, France) was  
140 used for 16S rRNA gene amplification with the primers 515F-Y/926R (Parada et al., 2016) as  
141 previously described (Pollet et al., 2018). Picogreen (Quant-iT™ PicoGreen® dsDNA Assay kit,  
142 ThermoFischer Scientific) quantified PCR products were pooled in equimolar mixes before  
143 Illumina MiSeq 2 × 250 pb sequencing or diluted to 1 ng/μL for qPCR quantification. Raw  
144 sequences can be found under the project PRJNA608293 in NCBI database, and under the samples  
145 number SAMN14169847, SAMN14169848, and SAMN14169852 to 58.

146

147 *Bioinformatic and statistical analyses*

148 A total of 429,545 paired-end raw sequences were treated in R v3.5.3 with DADA2 default  
149 pipeline for 16S rRNA (Callahan et al., 2017) (Supp informations). ASV taxonomy assignment  
150 was performed against SILVA database 132.  $\alpha$ - and  $\beta$ -diversity, as well as significance tests  
151 (PERMANOVA) were calculated in QIIME2 after matrix rarefaction to smallest sample (2399  
152 sequences). All statistical analysis or graphical representation were performed in R (version 3.5).  
153 *Metacoder* was used to display Log2 fold change of relative abundance of each taxon in pairwise  
154 comparisons between the surfaces. For each taxa level, metacoder calculates the abundance per-  
155 taxon.

156 Furthermore, biomarkers were detected for each site between the three surfaces with LEfSe  
157 (Segata et al., 2011). The discriminant taxa were filtered from the *biom* table and their relative  
158 abundance was correlated (Spearman) with metal resistance genes abundance (calculated as the  
159 ratio between targeted gene over 16S rRNA copies, for each gene).

160

161 *Quantitative PCR (qPCR)*

162 Ribosomal (archaeal and bacterial 16S rRNA genes) and metal resistance genes (*cusA*,  
163 *copA*, *cueO*, *czcA*, *pbrT*) were quantified with BioRad CFX Connect System. Each reaction (20  
164  $\mu$ L) contained 1X SSO Advanced Universal Sybr Green Supermix, 0.3  $\mu$ M of each primer and 4  
165  $\mu$ L of diluted DNA (1ng/  $\mu$ L). Primers, gene size and amplification cycles are presented in Table  
166 S2. Fluorescence was read after each cycle at 72 °C. A melting curve (95°C for 10s and incremental  
167 increase of 0.5 °C from 65 to 95 °C) was performed at end of each run to ensure specificity. The  
168 standard curves (details on Table S3) were obtained by a 10-fold dilution of linearized plasmids  
169 containing the reference gene. Copy number was estimated from the concentration of linearized  
170 plasmid (ng) multiplied by the Avogadro number and divided by the size of the vector summed to  
171 the partial gene times 660 (bp weight  $\text{g}\cdot\text{mol}^{-1}$ ) times  $10^9 \text{ ng}\cdot\text{g}^{-1}$ .

172 The origin of each gene fragment is described in Table S3. Both 16S rRNA genes and *czcA*  
173 were amplified from environmental samples and cloned in PGEM T easy. *copA* was synthesized  
174 by Eurofins Genomics and delivered within a pEX-A128 vector. The remaining genes (*cusA*, *cueO*  
175 and *pbrT*) were synthesized by ThermoFischer Scientific. All vectors contain ampicillin gene  
176 resistance for clone selection.

177 All qPCR were performed in technical replicates to ensure reproducibility, especially in  
178 low-gene abundance quantification. We accepted only technical replicates with less than 15% of  
179 relative standard deviation and only samples that had number of copies above the detection limit.  
180 Diluted DNA was estimated to have no significant PCR inhibition as indicated by assays on  
181 serially diluted samples for bacterial 16S rRNA amplification (data not shown).

182

## 183 **Results**

### 184 *Density and diversity within prokaryotic biofilm communities*

185 Cell density for heterotrophic prokaryotes and phototrophic microorganisms varied from  
186  $2.6 \times 10^5$  to  $2.6 \times 10^7$  and  $6.4 \times 10^3$  to  $1.1 \times 10^8$  cells  $\text{cm}^{-2}$ , respectively (Figure S2). In all three  
187 sites, PVC presented significantly higher abundances of both cellular communities. However, at  
188 Reunion, biofilms grown on Hy surfaces presented similar heterotrophic cell density as found for  
189 PVC.

190 Across the 27 samples, 175,747 paired-end Illumina reads remained after treatment  
191 according to DADA2 pipeline. *Archaea* were present in only one sample (one of the replicates of  
192 PVC immersed in Toulon) and corresponded to an ASV of the family of *Nitrosopumilaceae*.  
193 Archaeal sequences were consequently removed for further analyses. Similar trends were found  
194 for  $\alpha$ -diversity indexes (Chao1, PD or Pielou) as for densities. PVC exhibited the highest richness  
195 and diversity and no significant difference could be noticed between the two coatings in all the  
196 three sites (ANOVA;  $p < 0.05$ ) (Figure S3). However, we detected a significant difference between  
197 sites only for Hy, with Reunion exhibiting the highest phylogenetic diversity and Toulon the  
198 lowest.

199 Concerning their taxonomic structure, bacterial communities present within the biofilms  
200 were firstly segregated by surface and then by site (Figures 1 and S3). The first axis indicated  
201 segregation of A3 and Hy and the differences between sites within each surface. With smaller  
202 contribution, the second axis pointed at the greater distinction between the communities over the  
203 control (PVC) and the metals-containing surfaces (A3 and Hy). Surface presented greater pseudo-  
204 F values, indicating a greater effect-size of the differences between communities in each surface  
205 (Table S4). In all four metrics used to detect the similarity between communities, Hy biofilms were  
206 the most distinct (Figure S4). However, the phylogenetic signal incorporated in Unifrac (weighted  
207 and unweighted) distances indicated greater proximity of the biofilms developed over the two

208 biocidal surfaces. Likewise, Bray-Curtis and Jaccard separated the communities present at  
209 Reunion, while Lorient and Toulon seemed more similar. Nevertheless, Unifrac suggested lower  
210 influence of site on biofilm communities, as PERMANOVA indicated no significant difference  
211 between sites with Weighted Unifrac distances (Table S4). Site differentiation within each surface  
212 seemed to be more due to presence/absence (Jaccard) of certain ASVs, or even their relative  
213 abundance (Bray-Curtis), rather than communities phylogenetically closeness.

214  $\beta$ -diversity differences between surfaces and sites were also apparent in the relative  
215 abundance of certain families (Figure S5). Flavobacteriaceae (27.2%  $\pm$  13.3) and  
216 Rhodobacteriaceae (13.8%  $\pm$  5.1) were the most abundant families except on A3 from both  
217 Reunion and Toulon, respectively. Sphingomonadaceae and Marinobacteriaceae completed the  
218 top three most abundant families on A3 (19.9%  $\pm$  2.5) and Hy (13.2%  $\pm$  2.7) respectively.  
219 Saprospiraceae was abundant on biocidal in Lorient (8.9%  $\pm$  1.3) and Toulon (7.1%  $\pm$  1.9).

220

#### 221 *Site specific distinct taxa on biocide-containing surfaces*

222 Over 1.5 thousand taxa have been parsed by metacoder from which only 9 were singletons  
223 (remaining 1551 taxa). Due to the taxon redundancy in higher levels of taxonomic hierarchy, 889  
224 taxon\_id remained for relative abundance calculation. *Metacoder* graphical representation of the  
225 log<sub>2</sub> fold change of taxon proportions between surfaces provided symmetrical results to compare  
226 the surfaces two by two and to display the differences over the taxonomic hierarchy.

227 The family *Marinobacteriaceae* (Gamma-proteobacteria), and most specifically the genus  
228 *Marinobacter*, were significantly more present on Hy than on PVC or A3 surfaces.  
229 *Sphingomonadaceae* (Alpha-proteobacteria) were also more abundant on Hy than on PVC, but  
230 less abundant than on A3, especially the genus *Altererythrobacter* (Figure 2 and S6). Both the  
231 genera *Erythrobacter* and *Sphingorhabdus* had greater fold change on A3 when compared to PVC.  
232 Some other genera among Bacteroidetes showed greater fold change on Hy as *Fabibacter*,  
233 *Psychroflexus* and *Owenweeksia*.

234 Despite greater proportion of *Rhodobacteriaceae* in PVC, the genus *Roseovarius* was more  
235 present on A3. Similarly, *Aquimarina* and *Dokdonia* were specific of A3 within the  
236 *Flavobacteriaceae*. Furthermore, *Micavibrionaceae*, *Dasania* (Spongiibacteraceae), *Litorimonas*  
237 (Hyphomonadaceae) were characteristic of A3 (compared to either Hy or PVC). Taken together,

238 biocidal surfaces have selected *Sphingomonadaceae*, *Caedibacteraceae*, *Peredibacter* and  
239 *Dasania*.

240 Most of the taxa described above were also detected as biomarkers by LeFSe (LDA > 3)  
241 when comparing the three surfaces regardless of site. For example, *Aquimarina* was detected as  
242 biomarker for Hy, and *Alteromonas* was considered a Hy biomarker but did not present significant  
243 log<sub>2</sub> fold change against either of the two other surfaces. Lastly, families detected as PVC  
244 biomarkers were *Rhodobacteriaceae*, *Saprospiraceae*, *Pirellulaceae*, *Rubritaleaceae* and  
245 *Hyphomonadaceae*

246 However, many of those surface biomarkers were detected specifically in one or two sites  
247 (Figure S7). Only *Micavibrionaceae* was distinctly found on A3 in all three sites, and  
248 *Marinobacter* and *Psychroflexus* on Hy. PVC could only be analysed between Lorient and Toulon,  
249 since we had data only in two replicates from PVC at Reunion (one sample presented too few  
250 sequences). *Lewinella*, *Blastopirellula*, *Loktanella*, *Granulosicoccus*, *Rhodobacteraceae* and  
251 *Rubritaleaceae* were specifically detected on PVC at those two sites.

252 Otherwise, *Hyphomonadaceae* and *Litorimonas*, *Sphingomonadaceae*, and  
253 *Cycloasticaceae* were biomarkers for A3 at Toulon and Lorient. *Methyloligellaceae* and the  
254 *Rhodobacteraceae* *Roseovarius*, *Cellvibionales* and *Dasania* were biomarkers for A3 in Toulon  
255 and Reunion. For Hy, Lorient and Toulon had in common the genus *Fabibacter*. Toulon and  
256 Reunion both presented *Alteromonadales* and *Cellvibrionales* as biomarkers.

257

#### 258 *Metal resistance genes (MRG) abundance*

259 Bacterial abundance, estimated by the abundance of 16S rRNA gene, was not different  
260 between sites, except for A3 at Reunion. The bacterial density ranged between  $3.0 \times 10^6$  and  $1.1$   
261  $\times 10^8$  copies.cm<sup>-2</sup> (Figure S8). Archaeal 16S rRNA gene quantification indicated between  $5.5 \times$   
262  $10^{-5}$  and  $8.1 \times 10^{-4}$  less *Archaea* than *Bacteria*, but with no significant variation of archaeal density  
263 between surfaces or sites. In addition, it is noteworthy that *Archaea* were not amplified from all  
264 PVC at Reunion or from two of three PVC at Lorient. Considering MRG, *cusA* gene presented an  
265 average density ( $3.8 \times 10^7 \pm 5.3 \times 10^7$  copies.cm<sup>-2</sup>) similar to bacterial 16S rRNA gene. The other  
266 metal resistance genes (*copA*, *cueO*, *czcA*, *pbrT*) ranged from  $2.1 \times 10^2$  to  $4.9 \times 10^5$  copies.cm<sup>-2</sup>.

267 Due to slight differences in 16S rRNA densities among samples, we have analysed  
268 functional genes abundance as a ratio of each MRG by the prokaryotic ribosomal gene abundance

269 (Archaea+ Bacteria abundances of 16S rRNA). The relative abundance of MRG (MRG/16S rRNA  
270 gene) varied between sites and surfaces (Figure 3). A3 presented the greatest relative abundances  
271 (irrespective of the site) for *cusA*, *cueO* and *czcA*. Otherwise, A3 immersed at Reunion presented  
272 a community with significantly higher relative abundance of *cusA* and *czcA*, when compared to  
273 A3 at Lorient and Toulon. In fact, *czcA* could only be amplified from A3 communities, except in  
274 Toulon.

275 The A3 ratio of *cueO*/16S rRNA did not differ from Hy at Reunion, was similar to Hy and  
276 PVC at Toulon, but was the highest at Lorient. Also, in this site, the Hy surface presented the  
277 highest ratio for *copA*. Finally, *pbrT* relative abundance showed no difference between the  
278 communities developed on the two antifouling surfaces and was only amplified in one PVC sample  
279 from Toulon.

280 Concerning the sites, we could detect all MRG at Toulon only. Besides, regarding the  
281 abundance of MRG, Toulon was the only site where there was no difference in MRG relative  
282 densities between the biofilms grown on the antifouling surfaces and on PVC. Lastly, in Toulon,  
283 *cusA* was significantly higher on PVC compared to the other sites.

284

#### 285 *Specific taxa positively correlated to resistance genes*

286 Relative abundance for each MRG was used to calculate their correlation in each site and/or  
287 surface against taxa found discriminant for each surface in each site by LeFSe (LDA > 2) (Figure  
288 4). *Dasania* (Spearman's  $\rho = 0.73$ , p-value < 0.001, n=27) and *Alteromonas* ( $\rho = 0.75$ , p-value <  
289 0.001, n=27) showed the strongest correlation at the genus level with *cusA* whatever the site and  
290 coatings.

291 *Marinobacter*, a discriminant taxon for Hy, had the greatest positive correlation with the  
292 abundance of *copA* ( $\rho = 0.64$ , p-value < 0.001, n=27). Other genera found on Hy also correlated  
293 but to a lesser extent with this gene, namely *Fabibacter* ( $\rho = 0.50$ , p-value < 0.01, n=27) and  
294 *Owenweeksia* ( $\rho = 0.49$ , p-value = 0.01, n=27). *czcA* – mainly detected on A3 – exhibited a  
295 correlation with *Roseovarius* ( $\rho = 0.61$ , p-value < 0.001, n=27), OM60\_NOR5 clade ( $\rho = 0.60$ , p-  
296 value < 0.01, n=27) and BD1-7 clade ( $\rho = 0.60$ , p-value < 0.01, n=27). Even though with a lower  
297 coefficient, *czcA* was the only gene to correlate with *Altererythrobacter* ( $\rho = 0.41$ , p-value < 0.05,  
298 n=27).

300 *pbrT* correlated with genera present on A3, either exclusively or in common with Hy and  
301 PVC. Noteworthy, *Sphingorhabdus* presented a correlation of 0.53 (p-value < 0.01) and *Dasania*  
302 of 0.49 (p-value = 0.01). As a matter of fact, *Dasania* correlated positively with all MRG except  
303 *cueO*. This latter gene correlated with few genera, all discriminant of A3 or A3 and PVC, namely  
304 two from the most abundant families *Flavobacteriaceae* and *Rhodobacteraceae* – *Maritimimonas*  
305 and *Roseobacter* cladeNAC11 (both,  $\rho = 0.55$ , p-value < 0.01, n=27).

## 306 **Discussion**

307 Our study of antifouling surfaces immersed in temperate and tropical harbor waters aimed  
308 to provide providing new ecological insights to the selection of biofilm communities according to  
309 their potential resistance to metals on those surfaces. These findings might also contribute to  
310 further grasp the mechanisms that allow the colonization of such coatings depending on the local  
311 water characteristics.

### 313 *Communities were selected by surface, and by site to a lesser degree*

314 AF coatings contain metallic biocide embedded in an organic matrix, which both differ for  
315 the two coatings here. Hy contained greater concentration of Cu<sub>2</sub>O within an aqueous-based resin,  
316 whereas A3 was conceived as an organic solvent matrix containing CuSCN and ZnO. These  
317 differences could probably explain the variability in community composition as well as in MRG  
318 relative abundance. We have chosen three sites with contrasting environmental conditions but with  
319 a common factor: the anthropogenic pressure. Human effect in coastal waters is often regarded as  
320 contamination either by nutrients or pollutants and can potentially exert selective pressure over  
321 microbial communities (Nogales et al., 2011). Microbial communities are influenced by trace  
322 metal contamination, whether in freshwater (Ancion et al., 2010), seawater (Coclet et al., 2019;  
323 Webster and Negri, 2006) or sediments (Gillan et al., 2005; Misson et al., 2016). Even though  
324 planktonic communities in our sites might thus present already a tolerance to metals as shown in  
325 the Toulon Bay (Coclet et al 2019; 2020), the contribution of harbour relevant trace metals  
326 concentrations to microbial biofilm structuration, among other covarying factors, is less evident  
327 (Briand J.-F., pers. com, Catao et al submitted). Furthermore, as one explanation to the relatively  
328 low impact of seawater metals concentrations on biofilm community structure could be the ability  
329 of EPS to trap metals, hence preventing the contact with biofilm organisms and consequently their

330 selection, the actual level of tolerance to metals in biofilm community on inert surface, like  
331 plastics, remains to be compared to biofilm community from non-contaminated ecosystems.

332 Significant effect on periphyton biofilm diversity was only reported on higher copper  
333 concentration ( $>0.32 \mu\text{M}$ ), rarely measured in natural seawater (Corcoll et al., 2019) and at least  
334 ten times concentrations measured in our sites (20 to 50 nM for dissolved copper, (Catão et al.,  
335 submitted)). However, we assume that the amount of copper and zinc directly in contact with  
336 microorganisms trying to colonize a biocidal substrate should be much more considerable as we  
337 could see significant differences between biofilm communities over the inert PVC and the  
338 antifouling coatings – A3 and Hy. That means that especially copper and zinc released by the  
339 coatings applied a strong selection pressure on the potentially already tolerant microbial  
340 community that will colonise substrates in each harbor.

341 Toxicity and consequently community selection by metal-enriched surfaces depend on the  
342 gradual release of toxic metals. Our observations were made in communities developed after one-  
343 month immersion, which can be long in terms of biofilm succession, but is coherent with the  
344 highest release rates observed from metals-containing coatings (Valkirs et al., 2003). Leaching of  
345 these compounds varies with as well hydrodynamics (Valkirs et al., 2003) and environmental  
346 factors as the increased Cu leaching and lower Zn release in sites with increased salinity (Singh  
347 and Turner, 2009). Despite significant differences in  $\beta$ -diversity between all three surfaces, A3  
348 and Hy did not differ from each other in terms of cell density (phototrophs and heterotrophic  
349 prokaryotes) nor regarding  $\alpha$ -diversity, but both selected for lower cell number and diversity than  
350 PVC. However, within each biocidal surface, we could detect sites with higher or less cell density,  
351 richness and diversity. The variation in metals composition between these two coatings did not  
352 seem to affect significantly their diversity or cell number, contrary to other trends observed before  
353 of greater effect of Cu than Zn in microfouling density inhibition (Agostini et al., 2019).

354 As seen before, the selection of certain organisms by lifestyle, ie planktonic vs biofilm  
355 (Catão et al., submitted.; Oberbeckmann et al., 2018; Song et al., 2019) or metal releasing surface  
356 (Briand et al., 2017; Catão et al., 2019; Oberbeckmann et al., 2018; Tagg et al., 2019) prevails over  
357 environmental factors. Slight variations observed within communities from the same surface  
358 between the three sites might be related to the different ranges of nutrients, metal contamination,  
359 physical characteristics or even exposure of the immersed surfaces to the hydrodynamics found in  
360 each one of the three ports (Catão et al., submitted.). Toulon and Reunion sites are semi-enclosed

361 and more protected from marine currents with also higher temperatures, salinity and lead  
362 concentrations. On the other hand, Lorient site exhibits fluctuant salinity and dissolved organic  
363 carbon as influenced by the mixing of freshwater in the sea, which might lead as well to dilution  
364 of metallic contamination (Briand et al., 2017). In this site there was no difference of chao1  
365 richness between PVC and A3, which can be a sign of lower diffusion of copper from A3 surface  
366 due to water characteristics, as lower temperature and salinity.

367 The comparison of communities that colonized a same type of surface in three different  
368 sites indicated the presence of deterministic factors (according to niche-based theory) as judged  
369 by the greater site-to-site similarity (low  $\beta$ -diversity) (Chase et al., 2009). These factors can be  
370 interspecies tradeoffs or competition, and/or the environmental conditions. We showed here that  
371 surface characteristics imply selection of certain taxa independently from the site.

372

### 373 *Taxonomic indication of competitive fitness over antifouling surfaces*

374 Copper contamination affected microbial community structure in marine sediments (Gillan  
375 et al., 2005), and promoted changes in bacterial community composition and modification of the  
376 EPS in *in vitro* groundwater treatment for Cu removal (Vilchez et al., 2007).

377 Flavobacteriaceae seems to be widespread and core species in marine biofilms (Pollet et  
378 al., 2018; Salta et al., 2013), with few specific taxa found on antifouling surfaces. Similarly,  
379 Planctomycetes, Verrucomicrobia and *Saprospiraceae*, groups often found in marine biofilms  
380 (Catão et al., 2019; Dang and Lovell, 2016; Oberbeckmann et al., 2016; Pinto et al., 2019), were  
381 only detected on PVC, which reflects the apparent lack of organisms resistant to metals in these  
382 phyla and family. However, counter-examples could be noticed in each of these groups with the  
383 genera *Aquamarina* and *Maritimimonas* (Flavobacteriaceae), identified as biomarkers on A3, and  
384 previously detected on paint particles (Tagg et al., 2019), *Roseovarius* (Rhodobacteraceae) or  
385 BD7-11 (Planctomycetes), underlining that bioindication remains difficult at high taxonomic  
386 levels.

387 On the other hand, the organisms enriched on A3 and Hy compared to PVC were mainly  
388 from  $\alpha$ - and  $\gamma$ -Proteobacteria. The genera *Altererythrobacter* and *Erythrobacter* were already  
389 discriminant for biocidal coatings, including under shear stress in Toulon Bay (Dobretsov et al.,  
390 2018, Catão et al., 2019). However, when comparing the three different coastal waters, we could  
391 observe variations with lower values for these genera at Reunion. Furthermore, another

392 *Sphingomonadaceae* genus was found specific of biocidal surfaces at Lorient – *Sphingorhabdus*.  
393 These findings are important to highlight the variability of lower rank taxa depending on site, even  
394 if site was less relevant than surface type, as shown before between two biocidal coatings  
395 immersed in different locations in the Mediterranean Sea (Catão et al., 2019). Among  $\gamma$ -  
396 Proteobacteria, *Alteromonas* or *Dasania* were already reported on AF coatings in dissimilar  
397 environments as the Swedish coast (Flach et al., 2017) or the Sea of Oman (Dobretsov et al., 2018).

398 Lastly, in terms of *Archaea*, sequencing with prokaryotic universal primers (Parada et al.,  
399 2016) detected *Archaea* (Thaumarchaeota) only in one biofilm (0.06 %), and archaeal-specific  
400 primers for qPCR (Einen et al., 2008) confirmed little abundances in our marine biofilms (10 to  
401 100 thousands less than *Bacteria*), without detectable enrichment on metal-releasing coatings  
402 whatever the site.

403

404 *MRG were enriched on A3 and Hy, and varied from site to site*

405 The lack of metals on PVC surface lead to greater cell number (using flow cytometry) and  
406 bacterial diversity in the biofilms formed as already noticed at two of the three sites (Toulon and  
407 Lorient) with copper releasing coatings (e.g. Camps et al., 2014, Briand et al., 2017). However, no  
408 significant difference was observed for the copy number of the bacterial 16S rRNA gene on PVC  
409 compared to AF coatings, except A3 at Reunion. Three hypotheses can be drawn and contribute  
410 together to these results : (i) a lower coverage of the qPCR primers for the communities developed  
411 on the PVC, leading to underestimation of the community, (ii) a greater copy number of the 16S  
412 rRNA gene within the organisms that were found on A3 and Hy, which would explain the  
413 difference found between cytometry and qPCR, or, (iii) the much greater number of eukaryotes on  
414 PVC panels (Figure S1) which lead to less efficient prokaryotic DNA amplification by the qPCR  
415 primers. However, a higher actual copy number of the bacterial 16S rRNA gene on PVC would  
416 induce a lower relative MRG abundance on this coating, amplifying the difference between metal-  
417 releasing coatings and PVC.

418 Specific copper resistance within the marine biofilms was detected for the first time by the  
419 relative abundance of three genes, *copA*, *cusA* and *cueO*. They were identified on all the coatings  
420 except *copA* on PVC at Lorient and Reunion, with an expected global increase on copper-releasing  
421 surfaces, except at Toulon where PVC showed surprising high abundances of MRG as well.  
422 Certain organisms possess multiple genes to cope with environments rich in Cu. *Acidithiobacillus*

423 *ferrooxidans* is an example, portraying *cop* and *cus*, and Cu-specific periplasmic chaperones  
424 (Almárcegui et al., 2014). This differential distribution of MRG according to environment was  
425 expected, as found in copper contaminated Chilean marine sediments, with a greater abundance of  
426 *copA* with respect to *cusA* (Besaury et al., 2013) or along an urban stream (Roberto et al., 2019).  
427 Despite the fact that MRG (*copA*) can be found as well in Crenarchaeota (Orell et al., 2013),  
428 archaeal abundance in biofilm community remained low, as already reported (Pollet et al., 2018,  
429 Catao et al., 2019), limiting the possibility to discuss their role in metal resistance in the biofilm  
430 community.

431 Even though *copA* gene is the most used to monitor copper resistance, *czcA* gene was also  
432 well correlated with Cu availability in sediments (Roosa et al., 2014). In addition, even if we  
433 focused on copper due to its massive utilization as a biocide for antifouling coatings, it was  
434 important in this study to try to cover mechanisms of resistance for additional metals, especially  
435 in waters containing high concentrations of other trace metals as Zn or Pb. Interestingly, A3, that  
436 included Zn unlike Hy, showed a higher *czcA* relative ratio than Hy at Lorient and Reunion,  
437 consistent with its definition of RND antiporter cation-anion for efflux of  $\text{Co}^{+2}/\text{Zn}^{+2}/\text{Cd}^{+2}$ . MRG  
438 have been described regarding one or more specific metals. However, these genes can also  
439 correlate with non-target metals (Roosa et al., 2014), as the proteins by them encoded can have a  
440 broad range of action (Conroy et al., 2010). Moreover the tolerance may vary depending on the  
441 organisms (Nishino et al., 2006) and not only on the metal concentration. *pbrT* was not specifically  
442 enriched on biocidal coatings at Lorient and Reunion, despite dissimilar environmental Pb  
443 concentrations in these two sites. This could indicate either a co-selection or a non-specific action  
444 for this gene. Hence, quantification of multiple MRG might be used to detect cross-resistance in  
445 the selected communities over antifouling surfaces despite the presence of only one or two metals.  
446 Bacteria were reported to carry more than one resistance system in their genomes, and even though  
447 most have been described from chromosomes, RND efflux systems were also detected in  
448 conjugative plasmids (Gutiérrez-Barranquero et al., 2013; Norberg et al., 2014). This could explain  
449 greater numbers of *cusA* within the described biofilms. A greater abundance of *cusA* (10x higher)  
450 than *copA* was also observed for intertidal sediments (Besaury et al., 2016).

451 In this study, we have compared two antifouling coatings that varied in metal composition  
452 and concentration, as well as in solvent base. We assumed that aqueous matrix present in Hy was  
453 less prone than the organic one of A3 to select for certain organisms. Therefore, the differential

454 taxa selection in Hy compared to A3, and their different behaviors towards MRG abundance, are  
455 most likely related to the greater concentration of Cu used in Hy. This surface had significantly  
456 higher *copA* abundance in Lorient, well correlated to the higher abundance of *Marinobacter* in Hy  
457 surfaces, and *Dasania* and *Aquimarina* in Hy at Lorient. Overall, in addition to the possible specific  
458 actions of these organisms, the biofilm structure can possibly affect the abundance of MRG, in  
459 terms of oxygen availability. As such, greater abundance of *copA* could be related to its ability to  
460 translocate Cu(I) in both aerobic and anaerobic conditions (Besaury et al., 2016; Rensing and  
461 Grass, 2003), contrary to *cueO*, which is unable to oxidize Cu in the absence of O<sub>2</sub> (Outten et al.,  
462 2001).

463 All three sites present coastal waters contaminated by anthropogenic marine activities,  
464 leading to the hypothesis that organisms undergo already from a selection for resistance systems  
465 to metal contamination. The overall absence of trends in the distribution of MRG between sites on  
466 coatings could be related to the similar copper and zinc concentrations, although a lower Pb  
467 contamination at Lorient was noticed. This also implies that seawater characteristics that  
468 discriminate microbial communities between sites, especially planktonic ones (Catão et al.,  
469 submitted), did neither trigger the MRG type nor their relative abundances, as was reported for  
470 urban stream (Roberto et al., 2019). Interestingly, the number of all the MRG detected on the  
471 communities developed over PVC at Toulon, thus also including *czcA* and *pbrT*, was as high as  
472 on biocidal coatings. Despite there was not huge phylogenetic differences between sites on this  
473 plastic surface, biofilm communities at Toulon appeared highly adapted to metal resistance,  
474 probably related to the historical contamination of the Bay and responsible of a chronicle metal  
475 exposure history (Tessier et al., 2011, Coclet et al., 2020). In addition, as the immersion site in the  
476 bay of Toulon is a semi-enclosed pond, we assume the residence time was much higher than in  
477 Lorient or Reunion, which could have also led to an increased adaptation of the whole community.  
478 This could also be related to a differential adsorption of metals onto the PVC surface (Brennecke  
479 et al., 2016) which would vary depending on site because dissimilar environmental conditions lead  
480 to different organic matter and consequently distinct surface conditioning. Additionally, we cannot  
481 exclude that similar communities can produced dissimilar EPS, also depending on environmental  
482 parameters, which would possess higher metal binding capacity at Toulon. Finally, site specific  
483 taxa could further synthesize particular EPS maximizing metal binding at Toulon.

484

485 *Sphingomonadaceae* or *Alteromonadales* are selected in metals-rich environments

486 Primers targeting functional genes are prone to bias for certain taxonomic groups. In  
487 sediments, sequenced *copA* genes were found only in  $\beta$ -Proteobacteria, and *czcA* covered this class  
488 as well as  $\alpha$ -,  $\gamma$ - and  $\delta$ -Proteobacteria, and Cyanobacteria (Roosa et al., 2014). Here, most  
489 organisms presenting resistance to Cu contamination, measured by the abundance of *copA* and  
490 *czcA*, belonged to Proteobacteria. Others have also detected increased abundance of  $\gamma$ -  
491 Proteobacteria in biofilms on antifouling surfaces (Flach et al., 2017), class where we can find  
492 cultured organisms possessing metal RND efflux systems (Conroy et al., 2010).

493 Within the  $\alpha$ -Proteobacteria, *Sphingomonadaceae* organisms have been described more  
494 than once from copper contaminated environments (Balkwill et al., 2006; Vilchez et al., 2007).  
495 Biofilms rich in *Sphingomonas* (78% relative abundance) were observed as dense aggregates  
496 induced by the presence of Cu(II), suggesting EPS as the main form of cellular detoxification  
497 (Vilchez et al., 2007). The ability of Sphingomonads to form biofilms is most likely related to their  
498 production of abundant EPS (Balkwill et al., 2006) as well as their mobility, hydrophobic cell  
499 surface due to glycosphingolipids and their ability to metabolize several substrates (de Vries et al.,  
500 2019). Most specifically, *Erythrobacter* was found colonizing several types of microplastics  
501 immersed in an environmental gradient (Oberbeckmann et al., 2018) and was discriminant of other  
502 biocidal coatings under increased shear stress (Catão et al., 2019). Besides *Erythrobacter*, the  
503 Rhodobacteraceae *Roseovarius*, here shown to be correlated with the *czcA* gene, was also detected  
504 as one of the most abundant genera in biofilms formed on zinc-coated surfaces (Ding et al., 2019).  
505 The same study has detected an increase in KEGG protein families for transposase and MRG in  
506 those biofilms compared to seawater microbial communities.

507 Similarly to *Sphingomonadaceae*, the resistance of the  $\gamma$ -Proteobacteria *Marinobacter* to  
508 Cu toxicity has been linked to its sorption by the EPS (Bhaskar and Bhosle, 2006). Moreover,  
509 *Alteromonas* and *Alteromonadaceae* have been shown to be early colonizers of Cu-based surface  
510 (Catão et al., 2019; Chen et al., 2013), but also on PVC (Pollet et al., 2018). Two strains were  
511 recently reported to possess multiple megaplasmids with MRG including Cop, Cus and Cue systems  
512 (Cusick et al., 2020). That family were finally found as indicators of tolerance as they were  
513 positively correlated with increasing Cu concentrations in biofilms formed in microcosms with  
514 sea water (Corcoll et al., 2019).

515

516

## 517 **Conclusions**

518 Our study allowed the detection of specific taxa that are present on metal-releasing surfaces  
519 independently from the site, but depending on metallic biocide composition. Surface composition  
520 had great influence on cell density, bacterial diversity and composition as well as on relative  
521 abundance of MRG. This evaluation performed on biofilms grown in temperate and tropical  
522 contaminated waters, provided evidences that cell tolerance/resistance to metals may be based on  
523 several MRG with possible co-selection and not specific mechanisms. Furthermore, local  
524 contamination history may influence the basal level of MRG enrichment in biofilm community.

525

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534

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806

807 **Figure legends**

808

809 **Figure 1.** Principal Coordinates Analysis (PCoA) analysis on weighted Unifrac distances within  
810 bacterial communities. Colors correspond to the three surfaces and shapes to the three sites.

811

812 **Figure 2.** *Metacoder* heatmap plotted to genus level where each connected node represents a  
813 different taxonomic rank (Domain to genus from center to outwards, respectively). Nodes' size is  
814 proportional to the number of ASVs that constitute that taxonomic level. Nodes were colored  
815 according to Log2 ratio of mean proportion of each rang between A3 or Hy surfaces against PVC  
816 as control surface. Sites were not distinguished. Due to PVC higher richness, taxa that were present  
817 only on PVC were filtered out before graphical display.

818

819 **Figure 3.** Quantification of metal resistance genes in the sampled biofilms over the three surfaces  
820 and in the three sites. Ratios were calculated with each MRG over the sum of archaeal and bacterial  
821 16S rRNA genes abundance. Whiskers represent the smallest and the largest values. One-way  
822 ANOVA analysis was used to detect differences between sites or between surfaces, indicated  
823 respectively by upper and lower letters (Tuckey's HSD *posthoc* groupings, p-value<0.05).

824

825 **Figure 4.** Lefse discriminants (LDA>2) Spearman correlation with genes abundance (gene/16S  
826 rRNA). Dotplot corresponding to the site & surface combination for which the genera were found  
827 discriminant. Heatmap of only positive correlations (with p-value < 0.05) between those genera  
828 and the MRG. Genera are ordered according to the surface(s) for which they were detected as  
829 discriminant.

830

Figure 1

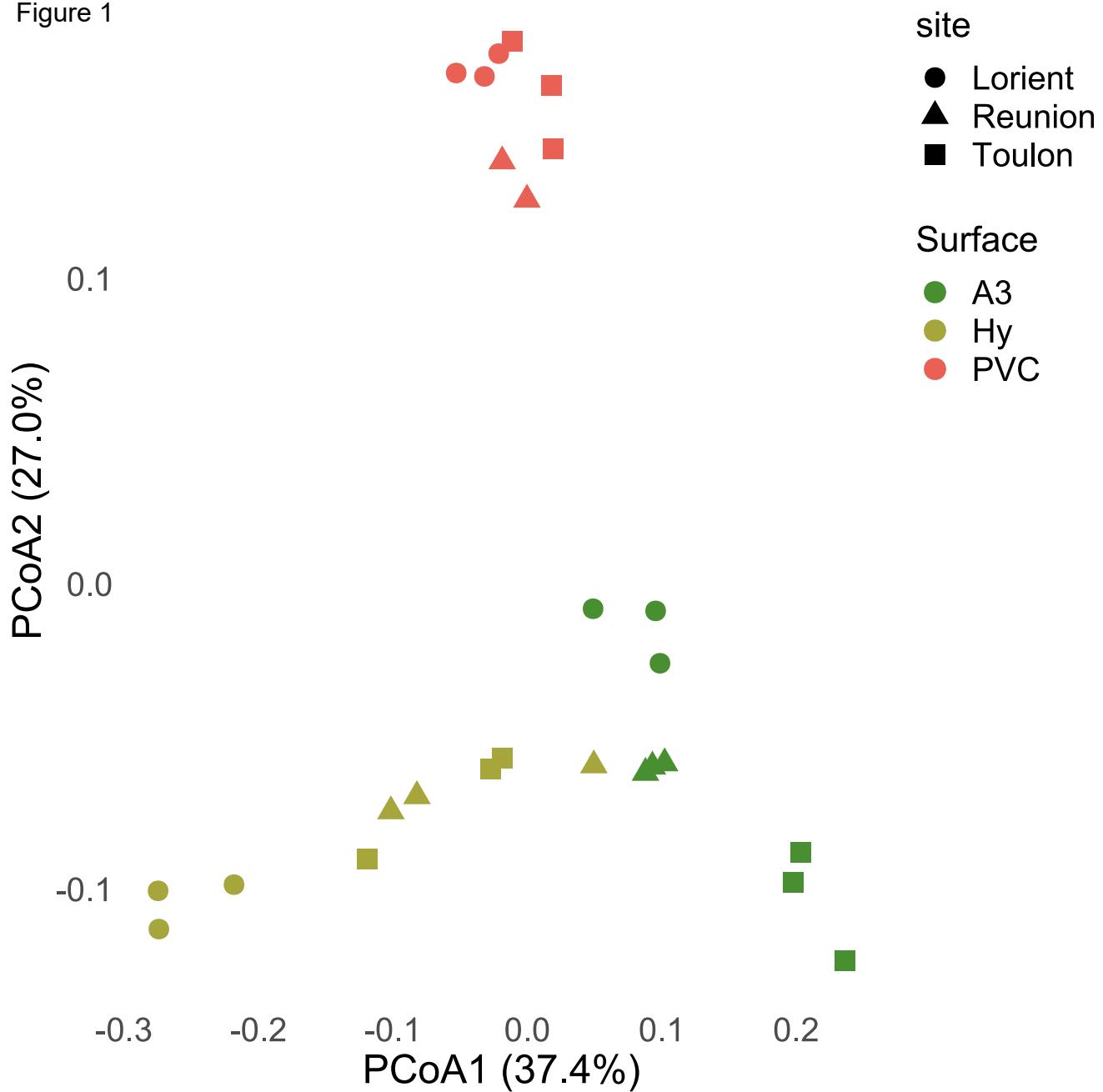


Figure 2

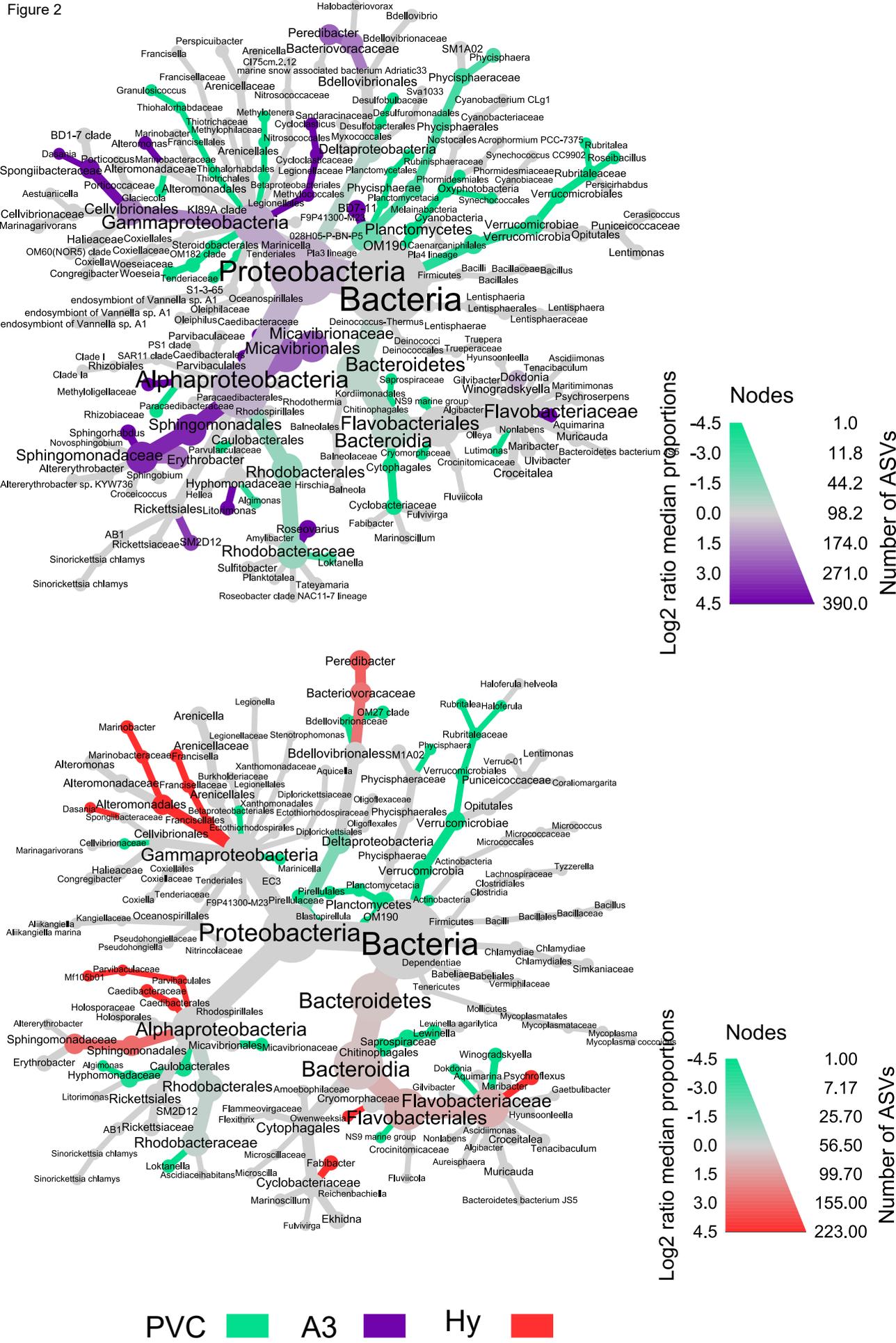


Figure 3

Relative abundance of MRG per 16S rRNA gene

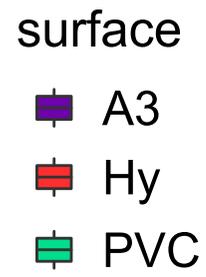
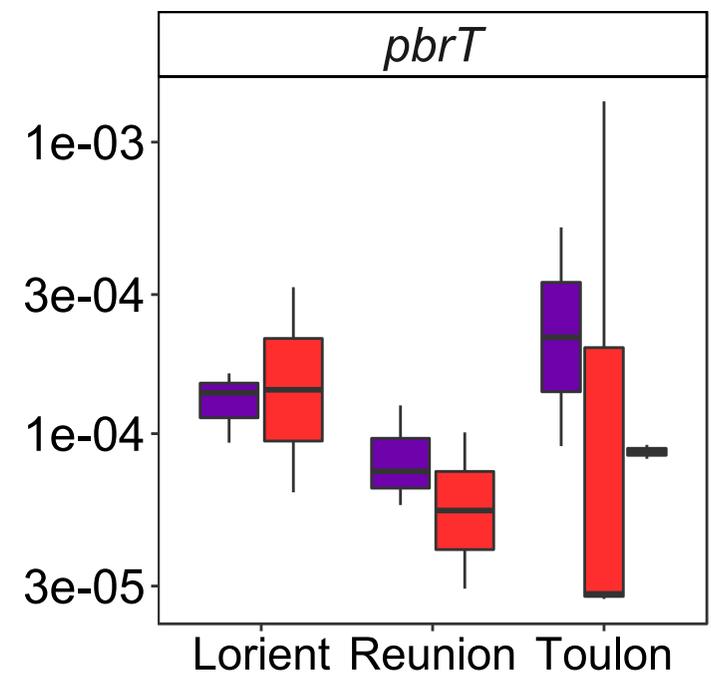
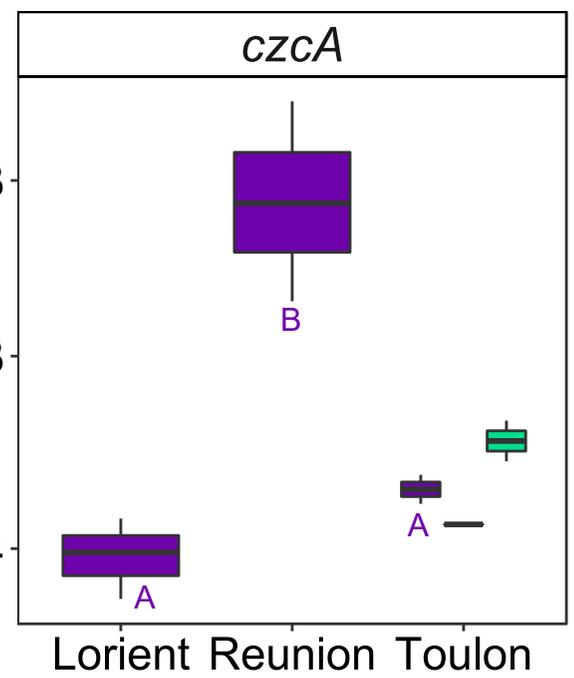
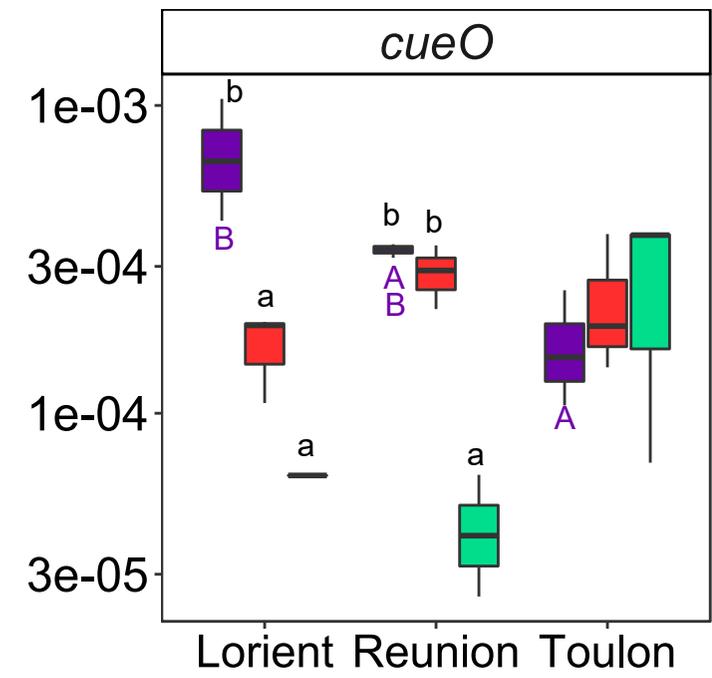
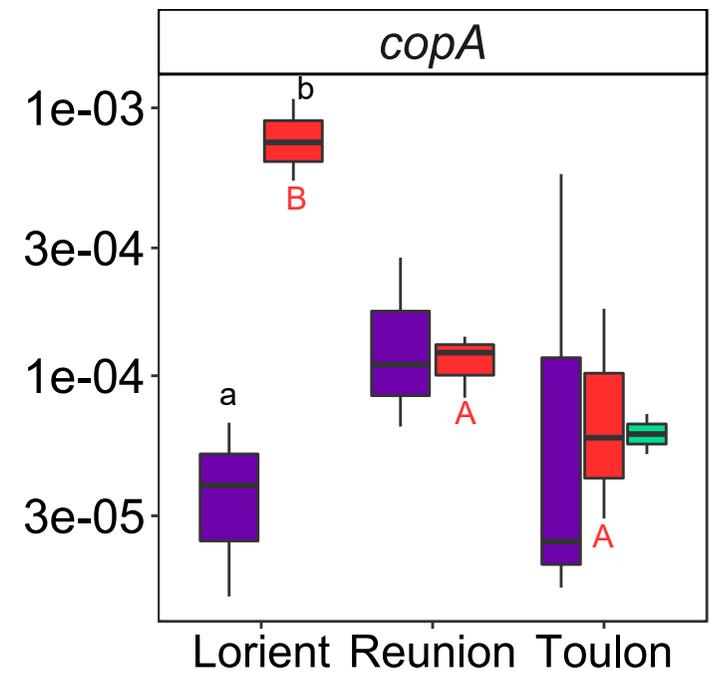
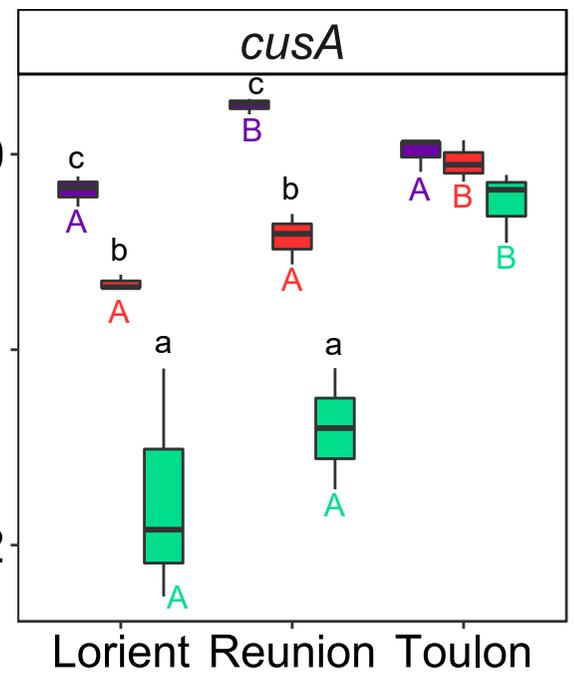


Figure 4

