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The marine intertidal zone shapes oyster and clam digestive bacterial microbiota.

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8 **One sentence summary:** structuration of bivalve digestive microbiota is shaped by intertidal position
9 after a four months field implantation.

10

11 Abstract

12 Digestive microbiota provides a wide range of beneficial effects on host physiology and are
13 therefore likely to play a key role in marine intertidal bivalve ability to acclimatize to the intertidal
14 zone. This study investigated the effect of intertidal levels on the digestive bacterial microbiota of
15 oysters *Crassostrea gigas* and clams *Ruditapes philippinarum*, two bivalves with different ecological
16 niches. Based on the 16S rRNA region sequencing, digestive glands, seawater and sediments harbored
17 specific bacterial communities, dominated by OTUs assigned to the *Mycoplasmatales*,
18 *Desulfobacterales* and *Rhodobacterales* orders, respectively. Field implantation modified digestive
19 bacterial microbiota of both bivalve species according to their intertidal position. *Rhodospirillales* and
20 *Legionellales* abundances increased in oysters and clams from low intertidal level, respectively. After
21 a 14-day depuration process, these effects were still observed especially for clams, while digestive
22 bacterial microbiota of oysters were more subjected to short-term environmental changes.
23 Nevertheless, 3.5 months stay on intertidal zone was enough to leave an environmental footprint on
24 the digestive bacterial microbiota, suggesting the existence of autochthonous bivalve bacteria. When
25 comparing clams from the three intertidal levels, 20% of the bacterial assemblage was shared among
26 the levels and it was dominated by OTU affiliated to the *Mycoplasmataceae* and *Spirochaetaceae*
27 families.

28 **Keywords:** microbiota; oysters; clams; intertidal zone; digestive gland; metabarcoding

29 INTRODUCTION

30 Host-associated microbiota play a key role in host homeostasis and health, by (i) promoting
31 development (McFall-Ngai 2002), (ii) providing protection against pathogens (Offret *et al.* 2018)
32 and/or (iii) improving adaptation to environmental modifications (Torda *et al.* 2017). It is hypothesized
33 that microbiota modification may strongly impact its host in terms of physiology, immunology and
34 nutrient uptake (McFall-Ngai *et al.* 2013; Baker *et al.* 2018; Clerissi *et al.* 2018; Dubé *et al.* 2019).
35 Host-associated microbiota consist of more or less complex communities of microorganisms, some of
36 which are more adapted to their host, others generalist, or transient, representing a wide range of
37 potential contributions (Shapira 2017). It is well known that bivalves harbor their own microbiota (as
38 for other organisms), whose characteristics and functions are still poorly understood, but cannot be
39 ignored (Desriac *et al.* 2014; Offret *et al.* 2019).

40 Microbial community composition and diversity associated with oysters (Trabal *et al.* 2012;
41 Trabal Fernández *et al.* 2014; King *et al.* 2019b) and clams (Romalde *et al.* 2013; Meisterhans *et al.*
42 2015) are beginning to be described with culture-independent methods from different tissues, such as
43 hemolymph (Lokmer and Wegner 2014; Lokmer *et al.* 2016b, 2016a), mantle (Lokmer *et al.* 2016b;
44 King *et al.* 2020), gills (Wegner *et al.* 2013; Lokmer *et al.* 2016b; King *et al.* 2020), adductor muscle
45 (King *et al.* 2019c, 2020) or digestive gland (King *et al.* 2012, 2019a, 2020; Lokmer *et al.* 2016b; Milan
46 *et al.* 2018; Vezzulli *et al.* 2018). The digestive gland (DG) is one of the most colonized tissue of
47 bivalves with the highest concentrations of bacteria (Kueh and Chan 1985). Digestive microbiota
48 generally supplies the host with exogenous nutrients and extracellular enzymes, fatty acids and
49 vitamins (Dhanasiri *et al.* 2011), thus contributing to nutrient degradation and uptake (Harris 1993;
50 Simon *et al.* 2019). The establishment and structuring of the DG microbiota depend on physiological,
51 genetic and immune characteristics of the host, the environment, the type of food ingested, as well as
52 the interactions between microorganisms (Hacquard and Schadt 2015). For that reason, structuring of
53 DG microbiota may play a key role in the metabolic condition of bivalves by influencing their growth
54 capacity, immunity, energy load, nutrition process and digestive enzyme activities (Harris 1993;
55 Röszer 2014).

56 Microbiota structuration and composition are affected by both host and habitat factors
57 (Kvennefors *et al.* 2010), such as intertidal position. Marine intertidal zones represent a heterogeneous
58 environment (Harley *et al.* 2006) structured by different gradients of biotic and abiotic factors including
59 temperature, salinity, nutrients, UV and rainfall variations (Connell 1972; Helmuth and Hofmann
60 2001). Marine organisms are facing different physiological challenges based on their position within

61 the intertidal zone, which further result in physiological differences (Soudant *et al.* 2004; Fernández-
62 Reiriz, Irisarri and Labarta 2016; Yin *et al.* 2017). The Pacific oyster, *Crassostrea gigas*, and the
63 Manilla clam, *Ruditapes philippinarum*, are epifaunal and infaunal bivalves, respectively, with
64 important economic value worldwide. Both species are subject to different biotic and abiotic factors
65 due to their distribution within the marine tidal zone. Oysters living in the intertidal zone, attach and
66 feed on planktonic microalgae, while clams burrowing in sediment mainly ingest benthic microalgae
67 and sedimented phytoplankton (Simons *et al.* 2018). To date, previous studies have shown that the
68 oyster microbiota could change under a multitude of different stressful treatments, such as
69 translocation, starvation, temperature, infection and antibiotic treatment (Green and Barnes 2010;
70 Wegner *et al.* 2013; Lokmer and Wegner 2014; Lokmer *et al.* 2016b, 2016a; Green *et al.* 2019).
71 However, to our knowledge no study has ever investigated the effect of different intertidal levels on
72 bivalve microbiota along a transect between the upper and lower limits of their distribution from shore.
73 The aim of this study was to investigate consequences of intertidal position on DG microbiota in two
74 bivalve species with different ecology, the Pacific oyster *C. gigas* and the Manilla clam *R.*
75 *philippinarum*. To this end, individuals of both species were deployed at one site in the Bay of Brest
76 (Brittany, France) at three contrasted intertidal levels (high, middle and low) for 3.5 months. Bacterial
77 microbiota from the DG were explored for composition and structuration by metabarcoding analysis.
78 To evaluate the environmental footprint on the DG microbiota, a cohort of bivalves was placed in
79 depurated conditions to analyze the intertidal level-specific microbiota that remains in the animals.

80

81 **MATERIALS AND METHODS**

82 **Biological samples**

83 *C. gigas* oysters' families were produced in February 2017 using a developed methodology that allowed
84 the production of pathogen-free juveniles, called "Naissain Standardisé Ifremer" (NSI). In larval and
85 post-larval stages, the oysters were maintained in controlled condition at the laboratory (Argenton,
86 France). The clams *R. philippinarum* were provided by a commercial exploitation (SATMAR, France).
87 They were descendants of clams families (around 1000 families) born in April 2016. Before
88 deployment in the field, mean shell length was 47.1 ± 5.2 mm for oysters and 20.3 ± 2.3 mm for clams.

89

90 **Experimental design on intertidal site**

91 Oysters and clams were transferred in mid-October 2017 to a farming area (surface of ca. 200m²)
92 located in the Bay of Brest at Pointe du Chateau (48° 20' 06.19" N, 4° 19' 06.37" W, Brittany, France).
93 The chosen implantation period (water temperature < 16°C) is a period without oyster mortality events
94 (Petton *et al.* 2015), without phytoplanktonic bloom (Lessin *et al.* 2019), with low growth rates
95 (Menzel 2018) and no breeding, which facilitated access to the DG. Animals were deployed at three
96 rearing heights (1, 2.8 and 4 meters above sea level) corresponding to 20%, 56% and 80% of
97 exondation time (Fig. 1). Similar to cultivation practices, animals were placed in two duplicated mesh
98 bags of 190 individuals for oysters (2 × 190 = 380) and 250 individuals for clams (2 × 250 = 500).
99 Oyster bags were attached to an iron table, whereas clam bags were directly placed in the sediment.
100 Sediment was collected next to the clams in October at each level in triplicate to evaluate the bacterial
101 community.

102 An initial sampling was performed just before deployment in the field. Digestive gland (DG) of
103 oysters (n=15) and clams (n=15) were sampled in RNase-DNase free conditions. Dissected DG were
104 rinsed using sterilized filtered (0.22µm) and autoclaved seawater and were frozen in liquid nitrogen
105 before being stored separately in cryotubes at -80°C. Sediments were collected next to the clams in
106 October at each level in triplicate to evaluate the bacterial community.

107 In February 2018, oysters and clams were removed from the three intertidal levels over three
108 consecutive days at spring low tides (31/01, 01/02 and 02/02). For each level, collected animals were
109 either directly dissected (n=15; clams 19.6 ± 3.4 mm; oysters 49.1 ± 7.1 mm) or brought to the
110 laboratory to be placed in depuration (n=15; clams 18.9 ± 4.1 mm ; oysters 45.7 ± 5.7 mm). In the
111 present study, the purpose of depuration was to empty the digestive glands and to reduce the
112 environmental microorganisms (Romero *et al.* 2002; Lee *et al.* 2008), in order to evaluate the
113 persistence of environmental conditioning on bivalve DG microbiota.

114 Depurated oysters and clams were grouped by sampling day in a bag and placed for 14 days in
115 30L-tanks (one by intertidal level) containing filtered seawater (10- and 5-µm sand filters and UV
116 treatment before two 1-µm filters and a second UV treatment) renewed at 3L minutes⁻¹. Temperature
117 of seawater in tanks was similar to temperature variations of the natural seawater. Tanks were cleaned
118 every second day to avoid biofilm formation and no feed was added. Sediments (25g on triplicate)
119 were collected at each level, while seawater was sampled (1 L in triplicate) 2 hours before low tide,
120 close to animals. Sediments collected next to clams were directly stored at -80°C, while the seawater
121 samples were successively passed through 8- and 0.22-µm polycarbonate filters (Whatman, USA),

122 before being stored at -80°C until DNA extraction. During this period, no mortality events occurred
123 for both bivalves, no brown ring disease nor *Vibrio tapetis* were detected in clams, suggesting that this
124 study was realized on healthy bivalves.

125

126 **DNA extraction**

127 The extraction of bacterial genomic DNA (gDNA) from the DG of oysters and clams combined
128 the use of phenol-chloroform-isoamyl alcohol (PCI) extraction with a DNA extraction kit (PowerLyser
129 Powersoil DNA Isolation, Qiagen, USA). Briefly, after homogenization of the DG, 40 mg were
130 collected to be digested at 45°C for 30 minutes in a lysis buffer (178 µL) consisting in TNE (Tris-HCl
131 1M at pH 8, NaCl 5M, EDTA 0.5M at pH 8), SDS 20% and proteinase K (20 mg mL⁻¹). After
132 centrifugation (10 min, 10,000 g), supernatant was recovered and stored at 4°C. A second digestion
133 was carried out on the pellet by adding 100 µL of lysis buffer at 45°C for 1 hour. After centrifugation
134 (10 min, 10,000 g) the recovered digestate was then pooled with the first one. This digestion product
135 (200 µL) was then mechanically lysed in PowerBead tubes (0.1 mm) from the PowerLyser kit, to which
136 Beads (650 µL) and the C1 solution (60 µL) were added, before being shaken in the FastPrep24TM (2
137 x 45 seconds). Supernatant (750 µL) was transferred to a new tube. One volume of phenol-chloroform-
138 isoamyl alcohol (25:24:1) was added. After mixing (45 sec) and centrifugation (15 min, 16,000 g at
139 4°C), 1 vol of chloroform was added to the supernatant, mixed again (45 sec) and centrifuged (15 min,
140 16,000 g at 4°C). Isopropanol (0.7 vol, -20°C) was added to the supernatant to precipitate DNA. The
141 tubes were placed overnight at -20°C. Precipitated DNA was centrifuged (30 min, 16,000 g at 4°C).
142 Pellet was solubilized in 500 µL of TNE buffer and PowerLyser Powersoil DNA Isolation kit was used
143 according to manufacturer's protocol. Finally, DNA was precipitated by centrifugation (30 sec, 10,000
144 g at room temperature), eluted in 100 µL ultra-pure water (Gentrox, UK) and stored at -80°C.

145 gDNA from sediment samples (250 mg) was extracted using PowerLyser Powersoil DNA
146 Isolation kit (Qiagen, USA), exclusively, according to the manufacturer's instructions. gDNA from
147 seawater filters (0.22 µm and 8µm) were extracted using PCI extraction according the same steps used
148 for tissue samples as described above. After precipitation, DNA was washed with ethanol 75% (500
149 µL) and dried before being hydrated with 100 µL ultra-pure water (Gentrox, UK).

150 To check for bacterial contamination of reagents, additional blank extractions were included. gDNA
151 concentrations from tissues and environmental samples were determined by spectrofluorometric
152 quantification using Quantifluor kit (Promega, USA) according to manufacturer's protocol.

153

154 **Microbiota analyses**

155 For each sample, 16S rRNA amplicon libraries were generated using the 341F (5'-
156 CCTACGGGNGGCWGCAG -3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') primers
157 targeting the variable V3V4 region (Herlemann *et al.* 2011). Paired-end sequencing with a 300-pb read
158 length was performed at McGill University (Génome Quebec Innovation Centre, Montréal, Qc,
159 Canada) on a MiSeq system (Illumina).

160 The sequencing data obtained were processed via the FROGS pipeline (Find Rapidly OTU with
161 Galaxy Solution, v2.0.0) developed in the Galaxy environment ([http://sigenae-
162 workbench.toulouse.inra.fr/galaxy/](http://sigenae-workbench.toulouse.inra.fr/galaxy/)). This pipeline groups sequences by similarities into OTUs
163 (Operational Taxonomic Units) and calculates taxonomic affiliations (Escudié *et al.* 2018). Briefly, the
164 "pre-process" step allowed to join the paired ended reads together using FLASH with a mismatch of
165 0.1 (Magoč and Salzberg 2011) and to remove both primers and adapters using cutadapt (Martin 2011).
166 A *de novo* clustering was carried out using the SWARM method, which groups the sequences into
167 clusters from a local clustering threshold with an aggregation distance $d = 3$ (Mahé *et al.* 2014).
168 Chimeras were removed using VSEARCH, a method dividing each sequence into four fragments and
169 then searching for possible parent sequences in all OTUs (Rognes *et al.* 2016). An abundance filter
170 with an optimal threshold of 0.005% was applied on OTUs (Bokulich *et al.* 2013), except for α -
171 diversity. Finally, the OTUs were assigned using Blast+ and the Silva 132 16S database containing
172 known sequences of bacterial 16S rRNA. The multi-affiliated sequences were corrected by indicating
173 for each of them an affiliation at a higher taxonomic rank. A phylogenetic tree of the OTUs and a table
174 of abundances of affiliated OTUs were then produced in the standard BIOM format.

175

176 **Statistical analyses**

177 Niche-wise (oyster, clam, sediment, seawater fractions) microbial communities α -diversity was
178 assessed at the OTU level after assigning OTUs to the lowest possible taxonomic level using Simpson's
179 inverse and Shannon entropy. Indices were computed for each individual bivalve microbiota and
180 Kruskal-Wallis tests were used to investigate mean differences between experimental conditions
181 (*ecological niche, sampling period, depuration impact, intertidal level effect and sampling day*).

182 Variation in microbiota composition and structure between individual bivalves, β -diversity, was
183 first visualized with principal component analysis (PCA) of Hellinger transformed OTU abundances.
184 The Hellinger transformation does not give excessive weight to rare categories and may therefore help
185 to overcome differences in sequencing depth (Legendre and Gallagher 2001). The effects of *ecological*

186 *niche, sampling period, depuration, intertidal level and sampling day* (and their interactions) were
187 tested using permanova (McArdle and Anderson 2001). Homogeneity of multivariate dispersion to
188 group medoid was first assessed in order to satisfy assumptions.

189 To assess whether presence/absence based β -diversity in DG microbiota between tidal levels was
190 predominantly driven by changes in species identity or fluctuations in species richness, Jaccard
191 dissimilarity between each pair of samples was partitioned in species replacement ($\beta_{\text{Replacement}}$) and
192 richness difference (β_{RichDiff}) following protocols described by Legendre 2014. Calculation and
193 decomposition of the Jaccard dissimilarity was performed for each DG microbiota from non-depurated
194 or depurated clams (separately), between levels (pairwise comparisons). Venn diagrams based on the
195 Jaccard dissimilarity (presence/absence of OTUs) gave access to shared or specific OTUs of the
196 different intertidal levels. All analyses were carried out using R3.5.2 (Team 2018), with all β -diversity
197 analysis conducted with functions from the *vegan* package (Oksanen *et al.* 2019).

198

199 **RESULTS**

200 **Data analysis information**

201 A total of 3,193,963 sequences were kept from all samples (n=245) after processing via the
202 FROGS pipeline (Supplementary Data). These sequences represented about 51% of all reads
203 sequenced from the V3V4 hypervariable region of the 16S rRNA gene. The average Quality Score of
204 amplicons was 33 (Sogin *et al.* 2006). After removing clusters representing less than 0.005% of all
205 sequences, the swarm clustering produced 1,322 different OTUs divided into seawater fractions (987
206 OTUs), sediments (705 OTUs), DG of oysters (1129 OTUs) and clams (1197 OTUs).

207

208 **α -diversity of OTUs from bivalve DG and their environment**

209 The number of most abundant OTUs was higher in DG than in seawater or sediments. Shannon
210 and inverse Simpson's indices (Supplementary Table 1) indicated that total bacterial diversity
211 (considering singletons and rare OTUs) was lower in the DG of both bivalve species (Kruskal Wallis,
212 Shannon: d.f. = 3; $p = 9.65e-13$ and Simpson's inverse: d.f. = 3; $p = 4.20e-08$) compared to their
213 immediate environment (Supplementary Figure 1). Bivalve digestive microbiota were dominated by
214 few OTUs accounting for the majority of reads, whereas rare OTUs were determinant for structuring
215 bacterial community composition of environmental samples. The implantation on the intertidal zone
216 led to a significant increase of both α -diversity indices (Kruskal Wallis, $p < 0.001$) for oysters and

217 clams. Depuration significantly reduced the α -diversity of clams DG microbiota (Kruskal Wallis,
218 Shannon: d.f. = 1; $p = 2.53e-11$ and Simpson's inverse: d.f. = 1; $p = 7.68e-05$). In depurated oysters,
219 only the Shannon index was significantly reduced, indicating a loss of rare OTUs. On the other hand,
220 whatever indices, α -diversity were not impacted by intertidal position for both bivalve species.

221

222 **Microbiota specific structure according to the host ecological niche**

223 Principal component analysis (PCA) of the Hellinger-transformed abundances (Fig.2) identified
224 a separation of microbiota which were significantly different between sample types (permanova test,
225 $R^2 = 27\%$, $F = 30.28$, $p = 0.001$), in terms of both OTUs richness and relative abundances (Fig. 3).
226 Bacterial community composition was represented on two principal component (PC) axes, separating
227 DG microbiota of bivalve species on PC1 (15.2%) and environmental from animal samples on PC2
228 (10.4%). PCA showed that environmental microbiota were closer to DG microbiota of oysters
229 compared to that of clams. The observed patterns were mostly associated to the contribution of OTUs
230 affiliated to the orders of *Mycoplasmatales* and *Rhodospirillales* for oysters, *Spirochaetales*,
231 *Rickettsiales* and *Oceanospirillales* for clams, and *Rhodobacterales* for environmental samples
232 (Supplementary Figure 2). Seawater fractions appeared to be clearly separated on both axes, with the
233 8-0.22 μ m fraction closer to animals than the >8 μ m fraction which was confounded with bacterial
234 communities from sediments. Interestingly, a lower similarity of bacterial communities was established
235 between clams DG and their closest environment, the sediment.

236

237 **Taxonomical composition of DG microbiota and environmental samples**

238 Microbiota (OTUs > 0.5% of total sequences), whatever from bivalve or environmental samples,
239 were dominated by 8 phyla, *Proteobacteria*, *Tenericutes*, *Actinobacteria*, *Spirochaetae*, *Chlamydiae*,
240 *Fusobacteria*, *Bacteroidetes* and *Planctomycetes* (Fig. 3). Before implantation in the field in October,
241 oyster DG microbiota were mainly dominated by *Mycoplasmatales* (87%), while *Mycoplasmatales*
242 (43%), *Chlamydiales* (23%), *Rickettsiales* (21%) and *Spirochaetales* (10%) were dominant in clams.
243 After 4 months of implantation in the intertidal zone, the DG microbiota diversity of both bivalves was
244 significantly modified (Fig.4). Indeed, the relative abundance of *Mycoplasmatales* decreased in non-
245 depurated oysters, while that of *Rhodospirillales* and *Campylobacterales* increased. Moreover,
246 *Chlamydiales*, *Legionellales* and *Planctomycetales* were detected in February but not in October. The
247 same trend was observed in clams, with a decreased abundance of *Mycoplasmatales* and *Chlamydiales*

248 in February compared to October in favor of *Rickettsiales*, *Spirochaetales* and *Oceanospirillales* as
249 well as, to a lesser extent, *Legionellales*, *Corynebacteriales* and *Planctomycetales*. Bacterial
250 communities from bivalves DG microbiota were clearly different from those in environmental samples
251 (Fig. 3), which were mainly dominated by *Desulfobacterales* (36%), *Campylobacterales* (30%),
252 *Acidimicrobiales* (25%) and *Fusobacteriales* (7%) for sediments, and *Rhodobacterales* (99%) for the
253 seawater small particles fraction (8-0.22 μ m); and *Rhodobacterales* (66%), *Campylobacterales* (14%),
254 *Desulfobacterales* (10%), *Acidimicrobiales* (5%) and *Fusobacteriales* (3%) for the large particles
255 fraction (> 8 μ m). This later fraction harbored several taxa similar to those found in sediments
256 (Supplementary Data).

257 For both bivalves, depuration decreased the abundance of *Mycoplasmatales*. This decrease, in
258 favor of *Spirochaetales* in clams and *Chlamydiales* in oysters, was associated with a decrease of less
259 abundant OTUs, such as *Desulfobacterales* and *Rhodobacterales* in both bivalves. In non-depurated
260 oysters, a negative correlation (Pearson correlation: -0.34, $p = 0.020$) was observed between the three
261 intertidal levels and relative abundance of *Rhodospirillales*, which were more abundant at the lowest
262 level. In non-depurated clams, the relative abundance of *Oceanospirillales* was positively correlated
263 (Pearson correlation: 0.33, $p = 0.025$) with a higher position on the intertidal zone, whereas this
264 correlation was negative for *Legionellales* (Pearson correlation: -0.39, $p = 0.008$).

265

266 **Consequences of implantation on digestive microbiota and inter-individual variability**

267 OTU abundances from non-depurated oysters or clams highlighted two separate groups (Fig. 4)
268 according to the sampling period (permanova test, oyster: $R^2 = 17\%$, $F = 11.48$, $p = 0.001$ and clam:
269 $R^2 = 15\%$, $F = 15.59$, $p = 0.001$). These results demonstrated that bivalve DG microbiota were
270 drastically modified after three-and-a-half-month implantation in the intertidal zone. These changes in
271 DG microbiota were mainly explained by OTUs affiliated to *Mycoplasmatales* and *Rhodobacterales*
272 for oysters (Supplementary Figure 4A), and to *Chlamydiales*, *Legionellales*, *Mycoplasmatales*,
273 *Oceanospirillales*, *Rhodospirillales* and *Rickettsiales* for clams (Supplementary Figure 4B).
274 Additionally, sample dispersion, measured by average of the distances to the median, was significantly
275 higher (ANOVA, d.f. = 1, $p = 2, 51e-05$) for oysters sampled in February (d = 0, 73) than in October
276 (d = 0, 59). These results reflected an increase in the inter-individual variability during implantation.
277 Unlike oysters, clams' inter-individual variability was not modified during implantation, indicating
278 different responses in both species.

279

280 **Short spatial and temporal scales as shapers of bacterial community of bivalve DG**
281 **microbiota and environmental samples**

282 Bivalve position on the intertidal zone significantly modified their DG microbiota (Table 1). The
283 "*Sampling day*" factor only affected DG microbiota composition in oysters (permanova test, $p = 0.013$).
284 No interaction between "*level*" and "*sampling day*" factors was observed, regardless of the species.
285 Oysters DG microbiota were impacted both by short spatial and temporal scales, while that of clams
286 was only impacted by the position on the intertidal zone. Bacterial communities from sediments
287 differed significantly regarding the intertidal level (permanova test, $p = 0.001$). Bacterial community
288 found in the 8-0.22 μ m seawater fraction was the only water fraction impacted by the sampling day
289 (permanova test, $p = 0.01$).

290 In order to evaluate the persistence of intertidal level impact on bivalve DG microbiota, oysters
291 and clams were placed in controlled laboratory conditions for depuration. After 14 days of depuration,
292 significant dissimilarities were observed between DG microbiota of depurated and non-depurated
293 bivalves for both species (Supplementary Figure 5). Moreover, depuration induced an increase of the
294 inter-individual variability in both species, compared to non-depurated bivalves (ANOVA, oysters: d.f.
295 = 1, $p = 1.624e-10$ and clams: d.f. = 1, $p = 0.017$). Both species DG microbiota were still significantly
296 impacted after depuration by their intertidal levels (Table 1). Moreover, even if the sampling day did
297 not significantly impact oysters DG microbiota after depuration, this factor significantly interacted with
298 the intertidal levels factor (permanova test, $p = 0.005$), suggesting that oysters DG microbiota were
299 fairly unstable from one day to the next.

300

301 **Decomposition of OTU variations in DG microbiota of clams placed at three different**
302 **intertidal levels**

303 Calculation and decomposition of the Jaccard dissimilarity between the three intertidal levels
304 provided information on the percentage of inter-level similarity (Fig. 5). Inter-levels comparisons
305 highlighted that non-depurated clams DG microbiota shared on average 25% of their OTUs between
306 the three levels (Fig. 5A), with the most pairwise similarities lying between 20% and 40% of shared
307 OTUs. For all level comparisons, dissimilarities between clams DG microbiota were mainly the
308 consequence of OTU replacement (43%-48%), while richness difference ranged from 26% to 32%.
309 Compared to similarity, values of the two dissimilarity components were much less uniform, with
310 replacement and difference richness lying respectively between 10%-75% and 5%-80%. If
311 dissimilarity seemed to be distributed in a consistent manner in high-middle and low-middle level

312 comparisons, replacement was higher (48% vs 43%) and richness difference lower (27% vs 33%) for
313 high-low level comparison. These results indicated a global microbiota composition shifting (led by
314 OTUs replacement) between DG microbiota from clams placed on these two extreme levels.

315 Comparatively to non-depurated clams, depuration led to a drastically decrease of richness
316 difference (19% on average) for all inter-level comparisons, while similarity (22% on average) was
317 also reduced by 3% (Fig. 5B). These richness-difference and similarity decrease were offset by a higher
318 replacement (59% on average), indicating that some OTUs were removed and that depurated clams
319 DG microbiota were more homogeneous in term of OTUs numbers. Compared to non-depurated
320 bivalves, amplitudes of pairwise comparison values (for all intertidal levels comparisons) of both
321 replacement and richness difference were lower, ranging respectively between 25%-80% and 5%-60%.
322 This lower amplitude of pairwise comparisons induced by depuration highlighted that dissimilarities
323 of DG microbiota between intertidal levels were mainly explained by a replacement of OTUs rather
324 than a richness difference.

325

326 **Identification of shared and specific OTUs among clams DG microbiota according to** 327 **their intertidal level**

328 Non-depurated clams from the all three intertidal levels shared a total of 737 common OTUs out
329 of 1102 identified (Fig. 6A and Supplementary Material). These common OTUs were mainly affiliated
330 to the orders *Planctomycetales* (13%), *Legionellales* (9%) and *Rhodobacterales* (8%), with the most
331 abundant OTU affiliated to the *Mycoplasmataceae* family, representing 11% of total sequences. Clams
332 from the high and middle levels shared the highest number of OTUs (110) compared to high-low (66)
333 and middle-low (32) levels. For level-specific OTUs, clams placed on high and middle intertidal levels
334 exhibited respectively 68 and 63 specific OTUs. OTUs specifically found in clams placed on the high
335 level were affiliated to the orders *Flavobacteriales* (18%) and *Rhodobacterales* (15%), while those
336 from the middle level were affiliated to the orders *Planctomycetales* (16%) and *Rhodobacterales* (8%).
337 By contrast, only 23 OTUs were specific to clams placed on the low intertidal level and were dominated
338 by *Planctomycetales* (12%).

339 Depuration led to a major overhaul of the specific and common clams DG microbiota (Fig. 6B).
340 Compared to non-depurated clams, depuration decreased by 42% the number of total identified OTUs
341 (636). Common OTUs (240) were mainly affiliated to *Legionellales* (16%), *Chlamydiales* (11%),
342 *Rickettsiales* (7%) with the most abundant OTU affiliated to the *Spirochaetaceae* family representing
343 19% of total sequences. Nevertheless, the general pattern remained the same as for non-depurated

344 clams, with DG microbiota originating from high and middle levels sharing the highest number of
345 OTUs (88), and DG microbiota from low intertidal level showing the lowest number of specific OTUs
346 (55), which were mainly affiliated to *Flavobacteriales* (15%) and *Planctomycetales* (15%). Clams from
347 the high-low and middle-low levels shared respectively 44 and 37 OTUs. Clams placed on high and
348 middle intertidal levels exhibited respectively 91 and 81 specific OTUs, those placed on the high level
349 were dominated by *Rhodobacterales* (16%) and *Planctomycetales* (11%), while those from the middle
350 level were mainly affiliated to *Planctomycetales* (12%). At the same time both absolute and
351 proportional values of level-specific OTUs increased for all intertidal levels after depuration compared
352 to non-depurated clams (high: 6% to 14%, middle: 6% to 12% and low: 2% to 9%). In that respect, the
353 depuration emphasized clams DG microbiota differences observed between the three intertidal levels.

354 **DISCUSSION**

355 In the present study, we investigated the structuration of the DG microbiota of the Pacific oyster
356 *C. gigas* and the Manilla clam *R. philippinarum*, in response to their location on the intertidal zone
357 during a three-and-a-half-months period (October 2017 to February 2018). We found that location on
358 the intertidal zone shaped DG microbiota of both bivalve species, in terms of taxonomical composition
359 and structuration. The footprint of the intertidal position on bivalve DG microbiota persisted after
360 depuration at the laboratory. The DG microbiota of oysters were unstable and fluctuated on a daily
361 basis, while that of clams appeared to be more stable in the short-term.

362 *DG microbiota specificity depends on its ecological niche*

363 OTUs present in the DG microbiota of oysters were mainly associated to the orders
364 *Mycoplasmatales*, *Rhodospirillales*, *Campylobacterales* and *Chlamydiales*, while those of clams were
365 associated to *Mycoplasmatales*, *Chlamydiales*, *Rickettsiales*, *Spirochaetales* and *Oceanospirillales*.
366 These taxa, including the predominance of *Mycoplasmatales*, are commonly described in oysters (King
367 *et al.* 2012; Lokmer *et al.* 2016b) and clams (Milan *et al.* 2018) DG microbiota, as well as in the gut
368 of other invertebrates (Tanaka *et al.* 2004; Meziti *et al.* 2010; Hollants *et al.* 2011; King *et al.* 2012;
369 Cleary *et al.* 2015). Bacteria assigned to the *Spirochaetales* order have often been associated to the
370 crystalline style of bivalves (Bernard 1970), whereas *Chlamydiales* and *Rickettsiales* are known as
371 intracellular bacteria found in digestive cells of oysters and clams (Harshbarger and Chang 1977; Fryer
372 and Lannan 1994). OTUs belonging to the orders *Mycoplasmatales* and *Rickettsiales*, and OTUs
373 assigned to the family *Spirochaetaceae*, were recently identified as core members of the Manila clam

375 and Pacific oyster microbiota, respectively (Milan *et al.* 2018; King *et al.* 2020). Although large intra-
376 species differences for relative abundances of different taxa have previously been described in bivalves,
377 mostly associated to location, age and sampling period, microbiome host-specificity is widely accepted
378 (Pierce and Ward 2018).

379 As in all filter feeding bivalves, DG of these two bivalve species are indirectly linked to their
380 surrounding environment (seawater, sediment) through the gills that pump water into the pallial cavity
381 to capture, process and transport food particles (Rosa *et al.* 2018). Nevertheless, DG microbiota of both
382 bivalves were clearly different from the bacterial communities of sediments and seawater, confirming
383 the existence of a gut-specific microbiota in clams (Meisterhans *et al.* 2015; Milan *et al.* 2018) and
384 oysters (Lokmer *et al.* 2016b; Vezzulli *et al.* 2018; Dubé *et al.* 2019). The majority of microorganisms
385 present in seawater were affiliated to the orders *Rhodobacterales*, *Campylobacterales*, and
386 *Desulfobacterales* previously found in seawater from the Atlantic ocean (Celikkol-Aydin *et al.* 2016;
387 Papadatou and Harder 2016). Bacterial communities from the two seawater fractions presented
388 dissimilarities that could be linked to the presence of free-living bacteria in one fraction (0.22-8 μm
389 fraction) and particle associated bacteria in the second one ($> 8 \mu\text{m}$) as previously described by Milici
390 *et al.* (2017). This later fraction ($> 8 \mu\text{m}$) showed a high similarity with sediment bacterial
391 communities, suggesting a sinking capability of the bigger particles which may be also found on the
392 seabed. Bacterial communities from environmental samples, including sediments and water; and more
393 specifically the 0.22-8 μm seawater fraction, were closest from oyster DG microbiota than clams. This
394 is probably the result of the differences in feeding behavior between oysters and clams, an important
395 factor that can contribute to specific microbial differences across bivalve species (Murphy *et al.* 2019).
396 Oysters filter particulate matter from pelagic zone, while clams use two siphons to ingest deposition
397 (Rosa *et al.* 2018). Despite the absence of a clear trend in the composition of major taxa, several OTUs
398 belonging to the major orders observed in oysters such as *Mycoplasmatales* (genus *Mycoplasma*),
399 *Campylobacterales* (genus *Arcobacter*), and *Planctomycetales* (genera *Blastopirellula* and
400 *Singulisphaera*) were also present in the water fraction (0.22-8 μm fraction). This proximity suggests
401 that oysters may have preferentially fed on small particles during the sampling period, as previously
402 described by Wisely and Reid (1978) where they identified an optimal particle ingestion size ($< 5\mu\text{m}$)
403 in the oyster *Saccostrea glomerata*. The daily changes in seawater bacterial community (0.22-8 μm
404 fraction) were previously observed and expected here (Yung *et al.* 2016). Similarities between the
405 oysters DG and seawater (0.22-8 μm fraction) bacterial communities, suggest a direct relationship
406 between environmental changes (mostly seawater) and oysters DG microbiota. Lokmer *et al.* (2016a)

407 previously showed the impact of short-term environmental fluctuations on oysters hemolymph
408 microbiota. This study emphasizes this point showing the same tendency for the DG, an external tissue,
409 which is more likely to reflect short-term environmental fluctuations.

410 Clams DG microbiota and their surrounding environment exhibited low similarities, especially
411 for sediments, which were closer to oysters DG microbiota. OTUs present in the sediments belonged
412 to the orders *Desulfobacterales* (*Desulfosarcina*, *Desulfobulbus*, *Desulfococcus*, *Desulforhapalus*,
413 *Desulfovibrio*) and *Campylobacterales* (*Sulfurovum*, *Sulfurimonas*, *Arcobacter*) that are common
414 sulphure cycle-associated bacteria present in marine sediments (de Wit 2008; Colin *et al.* 2013).
415 *Acidimicrobiales* are generally observed in marine sediments with low salinity (Wu *et al.* 2009), and
416 *Fusobacteriales* (*Psychrilyobacter* & *Propionigenium*) are involved in denitrification processes (Otte
417 *et al.* 2019). Oysters possess a higher filtration rate (3.92 μg carbon consumed $\text{L}^{-1} \text{g}^{-1}$) than clams (3.03
418 μg carbon consumed $\text{L}^{-1} \text{g}^{-1}$) with a lower trophic efficiency (18.38% for oysters and 23.69% for clams)
419 (Tenore, Goldman and Clarner 1973). This suggests that oysters ingested more bacteria from the
420 environment than clams, and that the transit of these microorganisms through the digestive gland was
421 therefore more important.

422

423 ***Spatial trends for non-depurated DG microbiota***

424 The 3.5 months spent on the intertidal zone led to significant and differential changes in the DG
425 microbiota of both bivalves, confirming that they were highly influenced by site of implantation, as
426 already observed in oysters (Clerissi *et al.* 2018). However, in this study, microbiota of oysters DG
427 seemed to be highly sensitive to small-scale environmental fluctuations, whereas that of clams was
428 more stable at the same scale of observation. Implantation on the intertidal zone led to an increase of
429 inter-individual heterogeneity of DG microbiota in non-depurated oysters but not in clams. This may
430 be explained either by a different environmental impact on each individual due to genetics (Wegner *et*
431 *al.* 2013; Clerissi *et al.* 2018) and/or the presence of micro-environmental heterogeneity (Lokmer *et al.*
432 2016a). The different intertidal positions, localized within a small area, impacted the relative
433 abundance of major OTUs of DG microbiota, with a predominance of OTUs related to *Rhodospirillales*
434 and *Legionellales* orders for oysters and clams, respectively, placed at the low level on the intertidal
435 zone. Previous studies have shown that the oyster microbiota are influenced by large and small spatial
436 location (< 1m), engendering heterogeneity in microbial composition (Wegner *et al.* 2013; Lokmer *et*
437 *al.* 2016a; King *et al.* 2019a).

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Environmental footprint on depurated DG microbiota

In order to evaluate how deeply the implantation in different intertidal positions influenced their DG microbiota, bivalves were placed in depuration for 14 days without feeding. Mostly, depuration is used to remove environmental contaminants such as microplastics (Paul-Pont *et al.* 2016), heavy-metal (Freitas *et al.* 2012) or human pathogens (El-Shenawy 2004), during a short period (few hours) (Vezzulli *et al.* 2018). In the present study, it was used to empty the DG and to reduce transient environmental microorganisms (Romero *et al.* 2002; Lee *et al.* 2008).

The α -diversity of the DG microbiota was reduced in clams and oysters following depuration, and a restructuring of OTU abundances was observed, as it was highlighted in oysters and mussels tissues (Lokmer *et al.* 2016a; Vezzulli *et al.* 2018). These modifications were probably related to the new environmental niches made available for other bacteria following depuration. Depuration also induced a drastic OTU reduction, which mostly affected OTUs common to all the three levels, leading to a strong increase in level-specific OTUs for clams, and inter-individual variability for both species. In oysters, the intertidal position effect observed after depuration was in interaction with the sampling day, supporting the hypothesis that oysters DG microbiota were susceptible to seawater variations on a daily basis.

For clams, persistence of intertidal position effect on depurated animals could be related to bacteria closely associated to the clams DG. This environmental footprint coupled with the increase of level-specific OTUs (belonging to *Rhodobacterales*, *Planctomycetales* and *Flavobacteriales*), suggest the existence of autochthonous bacteria in clams DG microbiota. The notion of autochthonous and allochthonous microorganisms has already been evocated for the hemolymph microbiota of Pacific oysters, where seawater-associated OTUs are transient within the microbial community (Lokmer and Wegner 2014). Based on similarities comparisons between depurated or non-depurated clams placed on the three intertidal levels, autochthonous bacteria of the DG represented around 20% of bacteria present in the microbiota. Although it has not been possible yet to make a clear distinction between non-native and indigenous microorganisms in bivalves (King *et al.* 2012), microbiota associated with clams internal organs, may have been made up of indigenous populations despite the strong environmental influence (Meisterhans *et al.* 2015).

471 **CONCLUSION**

472 The present study confirmed that the DG bacterial microbiota of the Pacific oyster and the
473 Manilla clam clearly differed from their surrounding environments. It highlighted that small
474 differences in the spatial distribution of oysters and clams, along the intertidal zone, induced significant
475 changes in their DG bacterial microbiota after three-and-a-half-month of winter implantation. We
476 currently do not know whether these changes, which persisted after 14 days of depuration, were likely
477 to affect the nutrient absorption capacity or other physiological traits of the two bivalves. While the
478 DG microbiota of oysters were unstable and fluctuated on a daily basis, that of clams seemed to be
479 more stable in the short-term, suggesting a better ability to regulate its DG microbiota. The depuration
480 process revealed the presence of 20% identical OTUs shared among the three intertidal levels in clams.
481 The exact roles of these resident bacteria on clams physiology are currently unknown, but they may
482 play a key role by maintaining specific metabolic functions within the DG bacterial microbiota that
483 was otherwise subjected to a wide influence of transient bacteria.

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486 **SUPPLEMENTARY DATA**

487 Supplementary data are available at FEMSEC online.

488

489 **AUTHOR CONTRIBUTIONS**

490 Experiments, microbiota analyses, results interpretation, writing-original draft preparation, C.O.;
491 microbiota analyses, results interpretation, S.P.; Experiment preparations, K.C.; Expertise for statistical
492 analyses, O.G.; Project and experimental design, oyster and clam dissections, A.B., P.M., B.P., C.C.,
493 F.P., C.F., and C.P.; Project supervision and experimental design, field monitoring, oyster and clam
494 dissections, results interpretation, writing-review and funding acquisition, G.L.B.

495

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510

511 **Conflict of Interest.** The authors declare that the research was conducted in the absence of any
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513

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707

708 **Figure 1.** Representation of experimental design detailing the deployment of bivalves on the three intertidal levels (low,
709 middle and high, corresponding to 20%, 56% and 80% of exondation time, respectively) and the sampling of digestive
710 glands and environmental samples.

711
712 **Figure 2.** Principal component analysis of the Hellinger-transformed OTUs abundances for the bacterial community of
713 sediment (yellow circles), 8µm seawater fraction (blue squares), 0.22-8µm seawater fraction (blue triangles), and DG of *C.*
714 *gigas* (red circles) and *R. philippinarum* (green circles) sampled in October and February. Ellipses represent standard
715 deviation (99%) of data.

716
717 **Figure 3.** Relative abundance of majoritarian OTUs (OTUs representing 0.5% of total sequences) summarized at the order
718 taxonomic rank, found in sediments, seawater (SW), and DG of *C. gigas* (OYSTER) and *R. philippinarum* (CLAM)
719 sampled in October (OCT) or February (FEB) from non-depurated (ND) or depurated (D) animals placed at different
720 intertidal levels (H, high ; M, middle ; L, low).

721 **Figure 4.** Principal component analysis of the Hellinger-transformed OTUs abundances for non-depurated DG bacterial
722 communities of *C. gigas* (A) and *R. philippinarum* (B) sampled in October (violet) and February (yellow). The first two
723 axes of PCA explain 28.4% and 27.4% of total variation of bacterial communities for oyster and clam respectively. Ellipses
724 represent standard deviation (99%) of data.

725
726 **Figure 5.** Triangular plots illustrating the variations of the Jaccard dissimilarity between OTU composition
727 (presence/absence data) of non-depurated (A) and depurated (B) *R. philippinarum* sampled on the three intertidal levels
728 (high, middle, low), and its decomposition into similarity, richness difference (variation in OTU richness) and OTU
729 replacement (variation in OTU identity). Legend information are provided in the box. Contributions were calculated for
730 each group of depurated or non-depurated animals separately, and for pairwise comparisons between each sample belonging
731 to one level with all samples from a different level. Due to the high number of pairwise comparisons, the density of points
732 was estimated by two-dimensional kernel estimations and was represented with dark blue for higher numbers of
733 comparisons. Red lines indicate the centroid value for each graph with its associated mean values for the three components
734 of dissimilarity.

735
736 **Figure 6.** Venn diagrams representing shared OTUs (based on presence/absence data) between DG bacterial communities
737 of non-depurated (A) and depurated (B) *R. philippinarum* that had been placed on high (red), middle (yellow) or low (blue)
738 intertidal level.

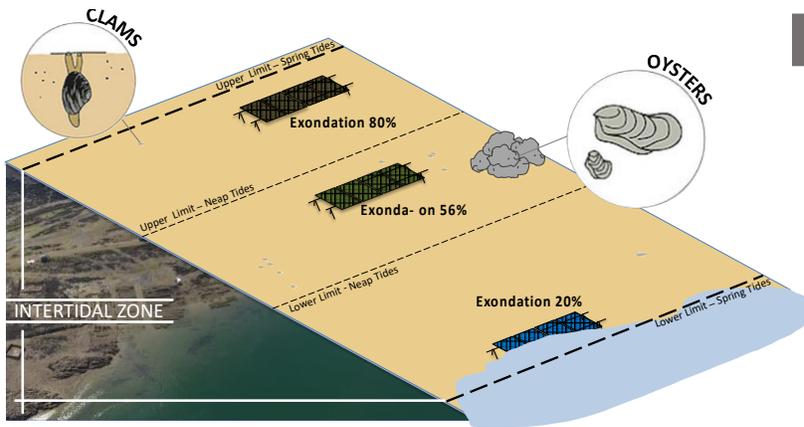
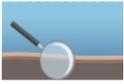
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OCTOBER 2017

Digestive Gland



Sediment

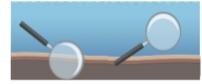


FEBRUARY 2018

Digestive Gland

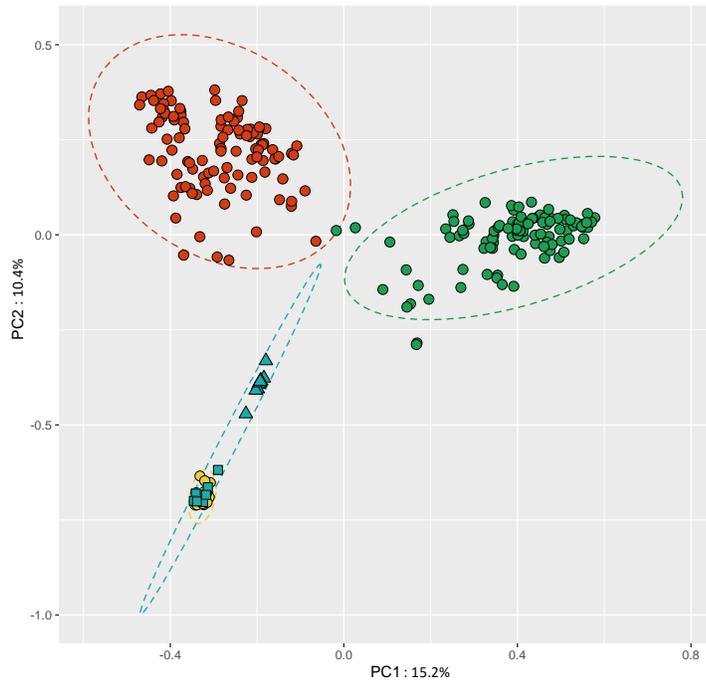


Sediment and Seawater



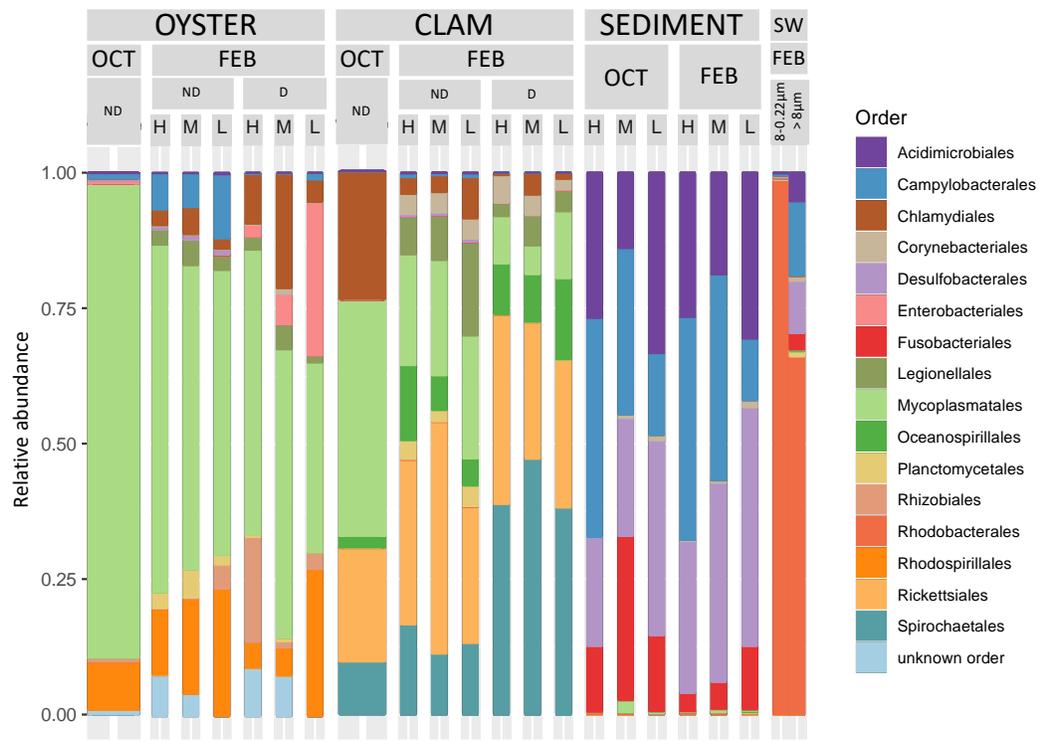
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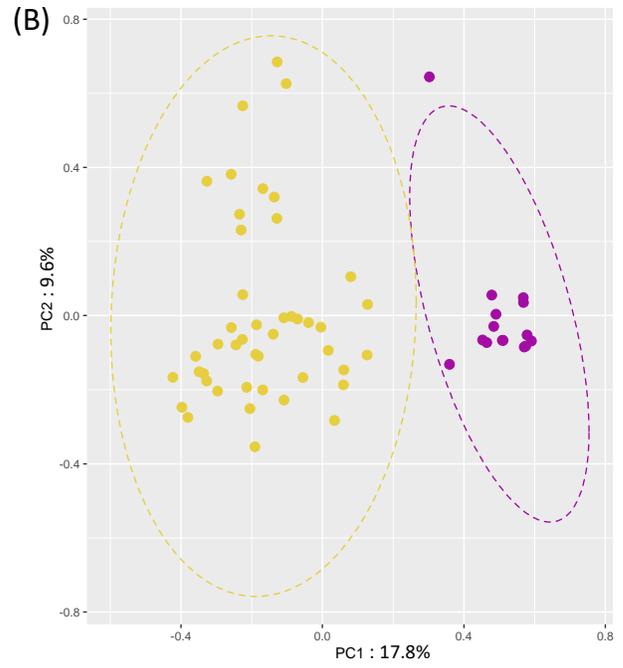
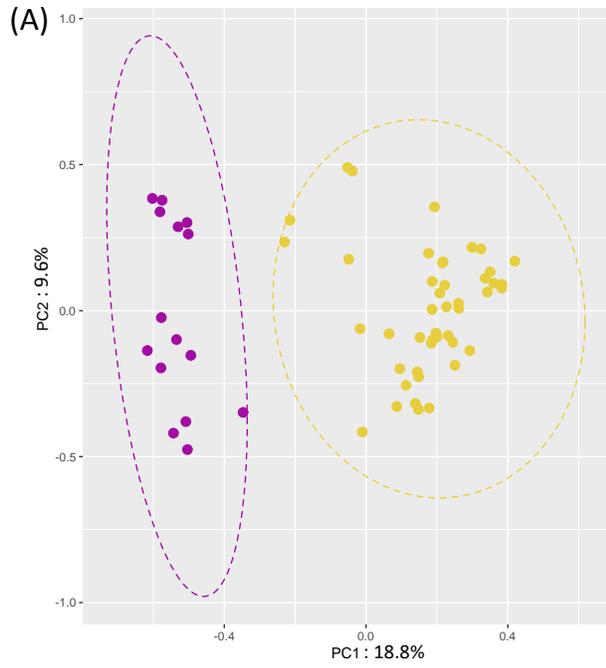
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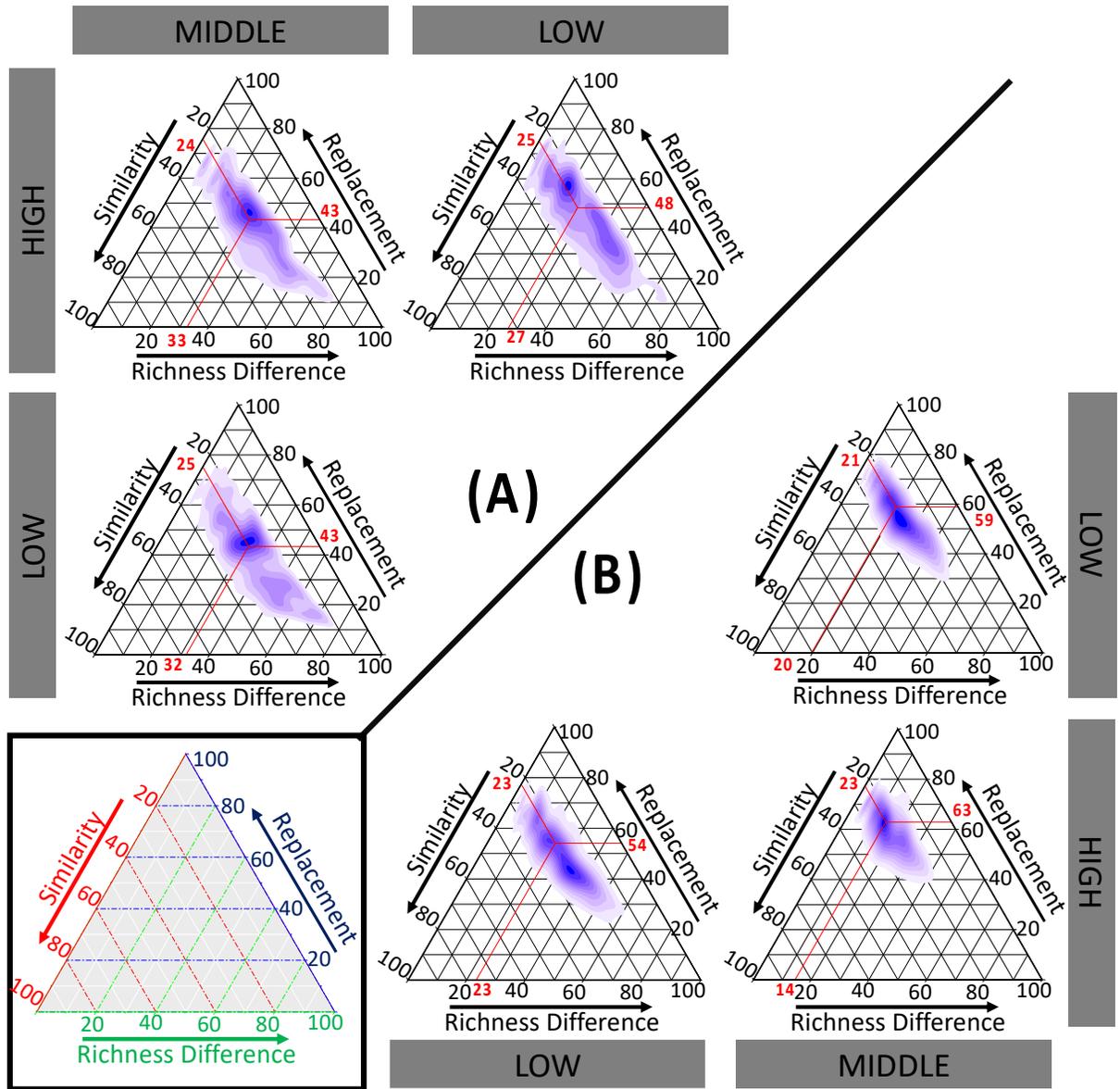


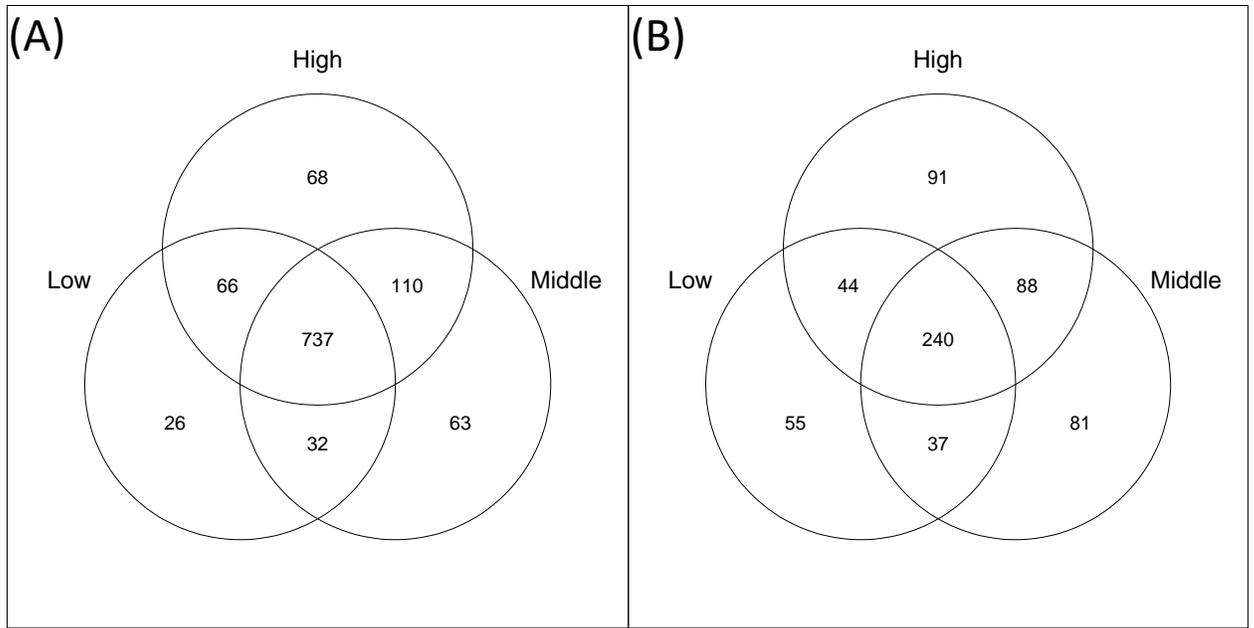
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