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1 **Characterization of the marine aquaculture microbiome: a seasonal survey in a seabass** 2 **farm**

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9 *Keywords:* seabass farm microbiomes, biofilms, tank water, 16S rRNA gene sequencing,
10 Vibrionaceae.

11 **Abstract**

12 Vibriosis is a recurrent disease in marine aquaculture environments. Monitoring the dynamics of
13 bacterial populations in several compartments of the aquaculture infrastructure is of paramount
14 importance to understand the occurrence of the disease. In the present study, the fish farm, located in
15 the north of France, is a flow-through system where the tanks are supplied by two water inlets. Water
16 from breeding tanks, and inlets and the biofilms in the tanks were sampled weekly in a seabass farm
17 for 7 months. DNA was extracted from 477 samples to sequence the variable V4-V5 region of the 16S
18 rRNA gene and analyze the sequences using the QIIME2 platform. Proteobacteria (54.52 %) and
19 Bacteroidetes (26.85 %) composed more than 80% of the phylum diversity. Alpha- and beta-diversity
20 indicators showed that bacterial communities were more diverse in samples from biofilms and tanks
21 than in samples from water inlets. Some families were specific to one compartment, e.g. ammonium-
22 oxidizing bacteria in biofilms, whereas Vibrionaceae family isolates were mostly found in tank water
23 samples. Taxa were determined up to the species level for the Vibrionaceae. Besides, all six
24 determined genera of Vibrionaceae were identified, with mostly *Vibrio* and *Photobacterium* isolates
25 detected in almost all samples. Vibrionaceae persisted over time in the biofilms. Our results suggest

26 that biofilms may be a potential reservoir in case of dysbiosis. This study helps better understand
27 microbial interactions, especially with Vibrionaceae in aquaculture environments.

28 **1. Introduction**

29 Over the past decade, aquaculture mass production has increased worldwide (Ahmed et al., 2019).
30 European seabass (*Dicentrarchus labrax*, L.) is one of the most farmed aquaculture species produced
31 with a production about 191,000 t in 2016 (FAO, 2005-2020). Aquaculture is a fast-growing sector
32 whose growth is limited by several challenges. There are geographic factors, such as the availability of
33 water and land, as well as environmental factors such as the pollution of coastal zones (Ahmed et al.,
34 2015). Disease outbreaks remain one of the main challenges, responsible for severe fish losses, leading
35 to the loss of billions of dollars every year (Lafferty et al., 2015).

36 Belonging to Gammaproteobacteria, the Vibrionaceae family is composed of six well-identified
37 genera and more than 140 species (Thompson et al., 2004). It is one of the most predominant bacterial
38 groups in marine environments with a worldwide distribution (Ina-Salwany et al., 2019, Thompson et
39 al., 2004). Several Vibrionaceae species have been identified as pathogens of farmed aquatic animals.
40 Specifically, the *Vibrio*, *Photobacterium* and *Aliivibrio* genera are responsible for vibriosis (Ina-
41 Salwany et al., 2019). Lethargy, skin lesions and hemorrhagic septicemia characterize affected fish
42 (Ina-Salwany et al., 2019). On the one hand, global warming stimulates the occurrence of the repeated
43 vibriosis outbreaks in farming systems (Travers et al., 2009, Vezzulli et al., 2015). On the other hand,
44 biofilm formation enhances the virulence and the persistence of Vibrionaceae and some other species,
45 partly due to horizontal gene transfer of potential virulence genes (Abe et al., 2020, Faruque et al.,
46 2006, Karunasagar et al., 1996). Michaud et al. (2009) found that most of the detected potential
47 pathogens (e.g. *Vibrio* spp., *Erwinia* spp. and *Coxiella* spp.) are associated with the biofilter biofilms
48 in a recirculating aquaculture system (RAS) for rearing seabass. Multispecies biofilms are therefore
49 considered as a reservoir for potential pathogenic and antibiotic-resistant bacteria (King et al., 2004).
50 Moreover, Kotob et al. (2016) indicated that the relationships between microorganisms determine
51 whether opportunistic pathogens proliferate and become virulent. Next-generation sequencing (NGS)

52 technologies are one way to consider the importance of all species for a better understanding of the
53 development of disease outbreaks and the fragile equilibrium within microbiomes.

54 Despite the development of aquaculture, most studies still mainly focus on microbiota of the reared
55 species' organs (Larsen et al., 2014). One study investigating the microbiome of the shrimp gut and
56 tank water samples from two shrimp farms showed that microbial diversity was lower in the shrimp
57 gut than in the tank water (Md Zoqratt et al., 2018). Another recent study investigated the skin and gill
58 microbiomes of seabass and seabream from a fish farm (Rosado et al., 2019) and attested that the
59 diversity of microbiomes was tissue-specific within and between species. Nevertheless, both studies
60 detected potential pathogens such as *Vibrio* spp., which can lead to disease outbreaks in case of a shift
61 in bacterial abundance (e.g. dysbiosis). Studies dealing with the microbial composition from biofilms
62 and water in the tanks mainly involve RAS (Michaud et al., 2009, Wang et al., 2018, Wietz et al.,
63 2009), but other studies have investigated temporal variations of bacterioplankton and the seasonal
64 dynamics of bacterial and microeukaryotic plankton communities, e.g. in a semi-intensive European
65 seabass aquaculture system (Duarte et al., 2019, Martins et al., 2018). The seasonal studies showed
66 that changes in environmental variables influence the overall microbial communities.

67 In this context, we conducted a seasonal survey from April to October 2018 in a seabass farm in the
68 North of France. Every year, this farm undergoes economic losses due to vibriosis outbreaks during
69 summer. The aim of this study was to characterize the marine fish farm microbiomes and to determine
70 the dynamics of bacterial populations in the different compartments of the aquaculture infrastructure.
71 The objective was particularly to understand if *Vibrio* species get into the tanks during summer
72 through the water inlets or if they persist throughout the year as biofilms. Studying the microbial
73 composition can help understand the recurrence of vibriosis during summer. We therefore sampled
74 biofilms and the water from the tanks and the inlets. The V4-V5 variable region of the 16S rRNA gene
75 was amplified and then sequenced using an Illumina MiSeq platform. To our knowledge, this is the
76 first study to carry out a weekly seasonal survey for 7 months on a fish farm with a comparison of
77 microbial composition between water inlets, tank water and biofilms in the tanks.

78 **2. Materials and methods**

79 **2.1. Sampling campaign**

80 The experiments were conducted from April to October 2018 for 31 weeks, in Aquanord Ichthus sea
81 farm (Gravelines, France). The fish farm installation and process have been described in the year 2018
82 IFREMER annual report (Antajan et al., 2019). Briefly, the fish farm is a flow-through system where
83 the tanks are supplied by two water inlets. The “cold-water” inlet supplies tanks with water from the
84 surrounding local seawater. The “hot-water” inlet supplies tanks with seawater coming from the hot-
85 water discharge canal of a nuclear power system. Several parameters, such as temperature, salinity,
86 conductivity, pH and dissolved oxygen, were measured in the upper surface of the water of each
87 compartment (Table S1). During the sampling campaign, no acute vibriosis outbreak occurred and fish
88 losses remained low and stable. Fishes had been vaccinated with an autovaccine, as per annual routine,
89 to fight against vibriosis. Fish density fluctuated from 20,000 to 30,000 individuals per breeding tank.
90 The water capacity of each tank was 75 m³, with a water-column of 1.3 m. The tanks had a mean fish
91 load of 48.13 kg of fish.m⁻³. Every week, 1 L of water was collected 20 cm below the water surface, in
92 a sterilized bottle, in triplicate from the three tanks and the two water inlets. The 15 water bottles were
93 kept on ice during the transport to the laboratory and filtered within 2 h. Nitrocellulose filters of 0.45
94 µm pore size (Sartorius, Goettingen, Germany) were used and then frozen at -80°C in Luria-Bertoni
95 (LB) broth medium supplemented with NaCl (LBS)/20% glycerol. The filtration stage has been
96 optimized for maximum recovery of the bacterial community using a larger volume of water and
97 larger pore size. The pore size was determined based on the high turbidity of the tank water due to
98 suspended organic material. Moreover, to mimic the biofilm formation occurring on the walls of
99 aquaculture tanks, we used the same concrete material to manufacture the pieces of concrete that were
100 immersed (20 cm below the surface of the water), collected and brought back to the laboratory. Three
101 pieces of concrete were not immersed and were considered as blanks (hereafter named B0), whereas
102 samples immersed 2 h in the tanks were named 0B. Every two weeks, pieces of concrete were
103 collected in triplicate from the three same tanks as the water samples. The nine pieces of concrete, still
104 immersed in the water, were maintained on ice during the transport and until processing at the
105 laboratory. Each piece of concrete was swabbed on a defined surface (of 2.34 cm²) with two cotton

106 swabs as previously done by Mougín et al. (2019) and swabs were stored at -80°C in an LBS/20%
107 glycerol medium.

108 **2.2. DNA extraction**

109 Filter and swab samples were thawed and vortexed for 3 min to retrieve bacteria. Filters and swabs
110 were discarded and the samples were centrifuged at 8000 × g for 10 min. Supernatants were removed
111 and 25 mL or 3 mL of physiological water were added for samples from water or biofilm,
112 respectively. After a brief vortex, the samples were centrifuged again (8000 × g for 10 min) and
113 supernatants were removed. The pellets were resuspended in 2 mL of physiological water, and briefly
114 vortexed. Samples were then centrifuged (8000 × g for 10 min) for the third time and the supernatants
115 were discarded.

116 The DNA from each pellet was extracted using the DNeasy PowerBiofilm Kit (Qiagen, Hilden,
117 Germany) following the manufacturer's protocol. The DNA purity was assessed on a Denovix DS-11
118 spectrophotometer (Clinisciences, Nanterre, France) and DNA concentration was measured using the
119 Qubit™ dsDNA HS Assay Kit on a Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The
120 quantity of extracted DNA was normalized to 5 ng·μL⁻¹ in Tris HCL (10 mM, pH 8.5) for the
121 subsequent PCR assays and stored at -20°C. The 477 aliquots and the three negative control samples
122 (composed of nuclease-free water) were used for metagenomic 16S rRNA gene sequencing. Hereafter
123 samples were named tank water (W), cold-water inlet (CW), hot-water inlet (HW) and biofilm (B)
124 samples depending on their compartment of origin. In the results, the sampling week is indicated
125 before the compartment letter, e.g. sample named 8B came from swabbed biofilms at week 8.

126 **2.3. PCR amplification, library preparation, 16S rRNA gene amplicon sequencing**

127 A two-step PCR was performed according to 16S metagenomic sequencing library preparation
128 protocol from Illumina. The first PCR step was carried out in duplicate. The amplicon primers 515F-
129 Y/926R targeted the V4-V5 variable region of the 16S rRNA gene and were purchased from TIB
130 MOLBIOL (Syntheselabor GmbH, Germany). The primers coupled with Illumina Reads adapters
131 515F-Y 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-
132 3' and 926R 5'-

133 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGYCAATTYMTTTRAGTTT-3'
134 resulted in an amplicon of around 480 bp (Parada et al., 2016, Quince et al., 2011). PCR reactions
135 were carried out in a final volume of 25 μL using 0.5 μL of each amplicon primer (10 μM), 2.5 μL of
136 DNA (5 $\text{ng}\cdot\mu\text{L}^{-1}$) and 12.5 μL of 2X PlatinumTM Green Hot Start Master Mix (Life Technologies SAS,
137 Thermofisher Scientific, Villepinte, France). The PCR program was as follows: 94°C for 2 min
138 followed by 30 cycles of a 94°C denaturation step for 45 s, a 50°C annealing step for 60 s and a 72°C
139 elongation step for 90 s (Raut et al., 2018).

140 Dual indices were attached during the second step PCR, using the Illumina Nextera XT Index Kits v2
141 (Illumina, Paris, France). PCR reaction was carried out in a final volume of 50 μL using 5 μL of
142 previously purified amplicon, 5 μL of each Nextera XT Index and 25 μL of 2X PlatinumTM Green Hot
143 Start Master Mix. PCR program started with 94°C for 2 min followed by 8 cycles with a denaturation
144 step of 94°C for 45 s, an annealing step of 59°C for 60 s and an elongation step of 72°C for 90 s. After
145 each PCR step, PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter,
146 Life Sciences, Villepinte, France) and were verified using electrophoresis on a 1% agarose gel. The
147 expected amplicon size was around 500 bp long. Final product purity was assessed with a Denovix
148 Spectrophotometer and final product concentration was measured using the QubitTM dsDNA HS Assay
149 Kit on a Qubit 3.0 fluorometer. The quantity of each sample was normalized to 4 nM in TrisHCL
150 (10mM, pH 8.5) and then 5 μL of diluted DNA from each library were mixed.

151 Following the Illumina protocol, pooled library was denatured, diluted to a final concentration of 4
152 pM, combined with 10% PhiX (Illumina) and loaded using MiSeq reagent v3 kits (600 cycles, 2 \times 300
153 bp) as recommended by the manufacturer. Finally, five sequencing runs of 96 samples each were
154 sequenced as paired-end reads using an Illumina MiSeq platform (Illumina).

155 **2.4. Sequence analyses**

156 Sequences were generated in demultiplexed fastq files and analyzed using the quantitative insights
157 into microbial ecology 2 (QIIME2; release 2019.10) open-source platform (Bolyen et al., 2019,
158 Caporaso et al., 2010). In each run, a total of 47,746,767 sequences were processed using DADA2
159 pipeline (Callahan et al., 2016, Martin, 2011). Briefly, paired-end sequences were trimmed to 240 bp,

160 filtered, denoised, merged and chimeras were discarded, resulting in 20,134,001 dereplicated
161 sequences (42.2%), (Table S2). The DADA2 pipeline gave five amplicon sequence variant (ASV)
162 tables, which listed the number of times each exact ASV was observed in each sample (Callahan et al.,
163 2017). The five tables were merged and technical triplicates grouped using the sum function. The
164 combined table was filtered to exclude singletons and technical negative control samples. Taxonomy
165 was assigned using a naive Bayes classifier trained on the 99_otus SILVA 132-release database
166 (Altschul et al., 1990, Bokulich et al., 2018, Edgar, 2010, Pedregosa et al., 2011, Quast et al., 2012,
167 Wang et al., 2007). The table with taxonomic assignments was filtered a second time to exclude
168 sequences assigned with mitochondria and chloroplast identification. Thus, analyses were
169 subsequently performed on 159 samples, with 17,243,983 sequences clustered into 147,640 ASVs.
170 Then, we chose a rarefaction depth of 29,725 sequences (27.41% of total retained sequences), which
171 was the lowest read number. All samples were included in the beta-diversity analysis, without need to
172 favor the rarest sequences. The values of the alpha-diversity indicators are detailed in Table S3. Chao1
173 and Shannon indices were selected to evaluate richness and evenness across sampling weeks and
174 compartments (Chao, 1984, Shannon, 1997). Two phylogenetic measures of beta-diversity were also
175 selected and visualized in EMPEROR (Vázquez-Baeza et al., 2013). The unweighted UniFrac distance
176 was used as a qualitative measure based on the presence/absence of different considered taxa. The
177 weighted UniFrac distance was used as a quantitative measure based on the relative abundance of taxa
178 (Lozupone and Knight, 2005, Lozupone et al., 2011). The phylogeny pipeline was previously applied
179 to the data using MAFFT (Kato and Standley, 2013), MASK (Lane, 1991) and FastTree (Price et al.,
180 2010) algorithms. Alpha-diversity statistical significances were tested using a Kruskal-Wallis test for
181 pairwise comparisons (Kruskal and Wallis, 1952). Statistical significance of beta-diversity distances
182 was analyzed using a PERMANOVA test (999 permutations).

183 **2.5. Data availability**

184 All sequences generated in this study are available in the NCBI sequence read archive under the
185 Bioproject accession number PRJNA613386.

186 **3. Results**

187 **3.1. Sequencing and classification**

188 During the weekly sampling campaign from April to October 2018, total DNA from 477 samples from
189 water or biofilm samples were extracted. Illumina MiSeq sequenced amplicons of the V4-V5 variable
190 region of the 16S rRNA gene using the paired-end method and high quality sequences were obtained
191 and processed. All technical triplicates were combined, leading to 159 samples subsequently analyzed
192 and split into two datasets (Table S4). The water dataset was composed of 110 water samples from
193 hot- and cold-water inlets and from the three breeding tanks for 22 weeks. The second dataset was
194 composed of 48 biofilm samples from the three breeding tanks for 16 weeks and the non-immersed
195 blank (B0). The mean number of sequences per sample obtained in biofilm samples was 140,286 and
196 94,271 in water samples. The number of sequences ranged from 29,725 for the hot-water inlet sample
197 at week 4 (4HW), to 315,298 for the biofilm sample from the second tank at week 24 (24B2). More
198 than 17 million high quality sequences were thus processed with a mean of 108,453 sequences per
199 sample. Each sequence was labeled, generating 147,640 ASVs. After a rarefaction process, taxonomy
200 assignment was applied using the SILVA 132 database and the bacterial compositions were studied at
201 different taxonomic levels.

202 **3.2. Bacterial composition**

203 *3.2.1. Taxonomic identification*

204 At the phylum level (Fig. 1), results attributed 94.38% of microbial diversity, all sampling dates and
205 compartments (biofilm or water samples) combined, to six major phyla: Proteobacteria, Bacteroidetes,
206 Verrucomicrobia, Planctomycetes, Chloroflexi and Thaumarchaeota. Proteobacteria and Bacteroidetes
207 represented more than 80% of the diversity with 54.52% and 26.85%, respectively. Moreover,
208 Gamma-, Alpha- and Deltaproteobacteria were the three main classes composing the Proteobacteria
209 phylum with 55.88%, 37.20% and 6.82%, respectively. Secondly, because the phylum taxonomic level
210 did not distinguish among samples, we preferred the family taxonomic level. Thus, the taxonomy of
211 water samples was assigned based on 1,291 ASVs at the family level, whereas biofilm samples were
212 taxonomically identified from 1,009 ASVs (Table S4).

213 *3.2.2. Composition across both datasets across time*

214 3.2.2.1. *Water dataset*

215 The samples from the water dataset were separated into three compartments: the cold-water inlet (Fig.
216 2A), the hot-water inlet (Fig. 2B) and tank water samples (Fig. 3). The samples of the three tanks at
217 the same sampling date for tank water were combined. No significant differences were observed
218 between tanks at each week according to the pairwise comparisons of the diversity indices with the
219 Kruskal-Wallis test. From April to October, bacterial compositions were obtained from 22 sampling
220 weeks. The major phyla represented in water dataset were Proteobacteria, Bacteroidetes and
221 Verrucomicrobia. Among them, Vibrionaceae, Rhodobacteraceae, Flavobacteriaceae and
222 Rubritaleaceae were the most frequently observed families. The relative abundance of the bacterial
223 families seemed more similar between cold- and hot- water inlets samples than with the tank water
224 samples, particularly for Vibrionaceae. In fact, the relative abundance of Vibrionaceae barely reached
225 5% in either water inlet sample (Fig. 2A, 2B), whereas it reached 50% in tank water samples, with the
226 highest abundances for 18W, 24W and 28W (Fig. 3). Inversely, the relative abundance of
227 Rhodobacteraceae and Flavobacteriaceae accounted for around 20% of the bacterial communities for
228 each water inlet across time, and their abundance fluctuated more drastically in tank water samples
229 from around 20% at week 0 to barely 10% at week 30. To a lesser extent, the SAR, Halieaceae,
230 Desulfobulbaceae, Pirellulaceae and Cyanobiaceae families were specifically detected in samples from
231 hot- and cold-water inlets and Colwelliaceae were mostly observed in tank water samples.

232 3.2.2.2. *Biofilm dataset*

233 As in the tank water samples, the biofilm samples from the three tanks at the same sampling date were
234 combined. No significant differences were observed between tanks at each week according to the
235 pairwise comparisons of the diversity indices using the Kruskal-Wallis test. The biofilm dataset was
236 composed of the blank samples (three non-immersed pieces of concrete, named B0) and the samples
237 from the tanks during 16 sampling weeks (Fig. 4). The blank B0 was mostly represented by specific
238 phyla and families that were not identified in the other samples. The Actinobacteria, Firmicutes and
239 Cyanobacteria phyla and the Burkholderiaceae family were only detected in the B0 samples, and no
240 Planctomycetes were identified. At week 0, after 2 h immersion, the 0B biofilm sample already
241 harbored more Flavobacteriaceae (20.38%), Vibrionaceae (14.84%), Rhodobacteraceae (7.02%),

242 Colwelliaceae (2.74%) and Saprospiraceae (4.30%) than the B0 sample. These relative abundances
243 observed were quite similar to the proportions observed in 0W, i.e. in the tank water samples from the
244 same sampling week. Proteobacteria, Bacteroidetes and Verrucomicrobia were represented in the
245 biofilm dataset, as in the water dataset. Results also brought to light the presence of Planctomycetes,
246 Chloroflexi and Thaumarchaeota. Among these phyla, Flavobacteriaceae, Rhodobacteraceae,
247 Saprospiraceae, Rubritelaceae, Pirellulaceae, Vibrionaceae, Haliaceae and Rubinisphaeraceae were
248 the most common families observed across time in biofilm samples. Three other families,
249 Nitrosomonadaceae (from less than 0.01% in 2B to 1.71% in 30B), Nitrosopumilaceae (from less than
250 0.01% in 2B to 8.14% in 30B) and Nitrospiraceae (from less than 0.01% in 2B to 1.20% in 30B) were
251 increasingly present over time at the expense of the Flavobacteriaceae (from 37.89 % in 2B to 23.23 %
252 in 30B). All together, the phyla, Planctomycetes, Chloroflexi and Thaumarchaeota appeared to be
253 specific to biofilm samples and their relative abundance increased over time.

254 **3.3. Analyses of alpha-and beta-diversity**

255 *3.3.1. Comparison of alpha-diversity indices*

256 Significant differences were always observed when comparing mean values of alpha-diversity indices
257 between datasets. The biofilm dataset had significantly the greatest diversity than the water dataset
258 both with the richness indicator Chao1 ($H=13.87$, $p\text{-value} < 0.001$) and the richness/evenness indicator
259 Shannon ($H=70.32$, $p\text{-value} < 0.001$).

260 More specifically, there were no differences in richness between the bacterial communities of tank
261 water and biofilm samples, whereas the latter were significantly richer than samples from either water
262 inlet ($p\text{-value} < 0.001$, Fig. 5A). The Chao1 values revealed that tank water samples harbored as many
263 bacterial communities as biofilm samples, but both compartments had more bacterial communities
264 than either water inlet. Shannon's index values were significantly different between biofilm, water
265 inlet and tank water samples ($p\text{-value} < 0.001$, Fig. 5B), this latter compartment being also
266 significantly different from cold-water inlet samples ($p\text{-value} < 0.05$, Fig. 5B). Hot-water inlet samples
267 did not show differences with tank water samples or cold-water inlet samples. Shannon's index, based
268 on richness and evenness, estimated that the microbiome was richer in biofilm samples than in other

269 compartments, meaning that there were more bacterial communities and their presence was more
270 evenly distributed. The bacterial communities were also richer and more evenly distributed in the tank
271 water samples than in the cold-water inlet samples.

272 Some significant differences were observed when comparing the mean values of Shannon's index
273 between sampling weeks within tank water samples or biofilm samples. Concerning tank water
274 samples (Fig. 5C, p-value < 0.05), at weeks 16, 19 and 26 (16W, 19W, 26W Fig. 3), Shannon's index
275 values showed that the bacterial communities in tank water samples were more diverse and evenly
276 distributed than at weeks 2 and 28 (2W, 28W, Fig. 3). Regarding the biofilm samples (Fig. 5D, p-
277 value < 0.05), except for week 2 (2B, Fig. 4), none of the bacterial communities were significantly
278 different regarding their richness and evenness.

279 Alpha-diversity results indicated that the bacterial communities were more numerous and evenly
280 distributed within the biofilm samples than in the tank water samples. Both communities from biofilm
281 and tank water samples were more numerous and evenly distributed than communities from either
282 water inlet.

283 3.3.2. Comparison of beta-diversity

284 We used a principal coordinate analysis (PCoA) to compare each microbiome sample with every other
285 sample in each dataset (water and biofilm) and each compartment (hot- and cold-water inlets, tank
286 water and biofilm). The qualitative comparison of microbiomes (Fig. 6A) based on presence or
287 absence of considered taxa showed that two major axes explained almost 15% of the beta-diversity of
288 all samples. Along these axes, samples from both water inlets could be grouped together, as well as
289 biofilm samples on one end and tank water samples on the other end. The weighted UniFrac distance
290 greatly increased the ability to discriminate samples by compartment (Fig. 6B). Moreover, the two
291 main axes accounted for almost 50% of the variance. PERMANOVA analyses for both beta-diversity
292 UniFrac distances tested if the hypothesis that one compartment was different from another was
293 correct. Thus, compartments were statistically different between another by both the unweighted ($R^2 =$
294 0.14, $p < 0.001$) and the weighted UniFrac ($R^2=0.46$, $p < 0.001$) distances.

295 The comparison between both UniFrac distances on the same data showed that the relative abundance
296 of taxa in our samples was more compartment-discriminating than the presence/absence of different
297 considered taxa, whereas presence/absence better dissociated the samples from each other.

298 **3.4. Detection and identification of the Vibrionaceae family**

299 The study of the distribution of the Vibrionaceae family was performed on sequences identified by
300 genus within this taxonomic level. Almost 15% of all sequences were identified as Vibrionaceae,
301 namely 2,575,816 sequences (Table S4), 90% of which (2,343,502 sequences) came from the water
302 dataset. Six different already identified genera and two other groups defined as uncultured and
303 unassigned were determined within the Vibrionaceae family (Table 1). *Vibrio* spp. (1,796,843
304 sequences), *Photobacterium* spp. (404,774 sequences), *Aliivibrio* spp. (328,413 sequences) and
305 *Enterovibrio* spp. (29,895 sequences) were the most identified ASVs. Moreover, Shannon's index was
306 applied to Vibrionaceae within the tank water samples (Fig. 7). Significant differences were observed
307 for 19W and 20W samples (both in August) compared with samples from 0W to 10W (except 6W)
308 and from 26W to 30W. During these two weeks in August, the mean temperature was 20.5°C (+/-
309 0.19°C) and pH ranged from 7.24 to 7.40 (Table S1).

310 **4. Discussion**

311 Here, we conducted a seasonal survey in the North of France, from April to October 2018 in a seabass
312 farm that undergoes repeated summer outbreaks of vibriosis. Although no acute vibriosis outbreak
313 occurred during this survey, the characterization of the microbiomes from the marine aquaculture
314 environment was of paramount importance to evaluate and monitor the bacterial communities and
315 their potential interactions over time. Specifically, the characterization of the Vibrionaceae population
316 dynamics in the various aquaculture compartments shed light on which compartments (water inlets,
317 water tank and biofilm) may be a source or a reservoir of Vibrionaceae. Using Illumina MiSeq
318 technology targeting the V4-V5 variable region of the 16S rRNA gene, we detected and identified the
319 bacterial communities through a weekly sampling campaign. The presence of Vibrionaceae was
320 ubiquitous and detected in all samples (Table 1). Despite a common microbiome shared between all

321 samples, alpha-diversity indices were significantly different between sample compartments (Fig. 5A,
322 5B).

323 **4.1. Common microbiome composition**

324 A common microbiome was detected in samples from all compartments. This commonality is likely
325 due to the source of the water used to supply the tanks in water and in which the pieces of concrete
326 were immersed. Taking all the results together, the Proteobacteria (54.52 %) and Bacteroidetes (26.85
327 %) phyla formed the main components of the microbiomes from the water and biofilm compartments
328 (Fig. 1). These phyla have already been detected in a seabass farm (Duarte et al., 2019) and were also
329 predominant in the seabass skin microbiome (Chiarello et al., 2015, Pimentel et al., 2017, Rosado et
330 al., 2019). Moreover, Gamma-, Alphaproteobacteria and Bacteroides appear to be the first classes to
331 form a biofilm in a RAS (Wietz et al., 2009).

332 A core microbiome analysis (data not shown) revealed that Vibrionaceae (*Vibrio* genus, Table 1),
333 Rhodobacteraceae (with the *Loklanella*, *Sulfitobacter*, *Planktotalea* genera), Rhizobiaceae,
334 Flavobacteriaceae and Saprospiraceae were the families (belonging to the Proteobacteria and
335 Bacteroidetes phyla) shared among all samples. These results were also consistent with those of Wang
336 et al. (2018), who found that genera from the Saprospiraceae, Flavobacteriaceae and
337 Rhodobacteraceae families were shared between RAS tank water and the RAS biofilter. Moreover,
338 Flavobacteriaceae and Rhodobacteraceae have also been reported in association with zooplankton,
339 notably because of their respective capacities to participate in the carbon cycle by degrading high-
340 molecular-weight organic matter, and to contribute to biofilm formation (De Corte et al., 2018).

341 **4.2. Overall microbiota differences between sampling compartments**

342 The alpha-and beta-diversity indices used in this study (Chao1, Shannon, unweighted and weighted
343 UniFrac distances) highlighted differences in the bacterial composition between the compartments and
344 between samples within each compartment (Fig. 5, Fig. 6). The biofilm samples and tank water
345 samples harbored the most diverse bacterial populations compared with the water inlet samples (Fig.
346 5A, 5B). These observations could be partly explained by the presence of seabass in the tanks. The
347 Colwelliaceae family was more abundant in the tank water samples than in the water inlet samples

348 (Fig. 2, Fig. 3). Pimentel et al. (2017) highlighted that the accumulation of uneaten feed in the tank
349 generate anoxic areas, where the genus *Colwellia* could be found and contribute to anaerobic
350 processes. Otherwise, previous studies have shown that biofilm communities are distinct from other
351 microbial communities, for example in raw water (Luo et al., 2013). The increasing presence of the
352 Nitrosomonadaceae, Nitrospiraceae and Nitrosopumilaceae families appeared to be specific to the
353 biofilm samples (Fig. 4). In fact, these three families are involved in nitrification processes and are
354 known as ammonium-oxidizing bacteria and ammonium-oxidizing archaea (Rurangwa and Verdegem,
355 2015). Luo et al. (2013) revealed that the presence of Firmicutes and *Nitrospiraes* spp. in microbial
356 biofilms improves the quality of the raw water by dissolving oxygen and modifying nitrogen levels.
357 Planctomycetes, such as Pirellulaceae (Fig. 4), also participate in ammonium oxidation in anaerobic
358 conditions (Schreier et al., 2010). The presence of these bacterial populations in a biofilm is crucial
359 because they oxidize ammonia and nitrite into less toxic compounds, such as nitrates (Rurangwa and
360 Verdegem, 2015).

361 Moreover, our results showed that bacterial populations composing biofilm established between the 2
362 hours immersion and the second sampling week. Bacterial communities on the non-immersed blank
363 disappeared after just 2 h of immersion, as manifested by the Actinobacteria, Cyanobacteria and
364 Firmicutes families. Firmicutes have been found in bacterial communities from RAS biofilters or in
365 biofilms in raw water distribution systems (Luo et al., 2013, Schreier et al., 2010). These families have
366 often been observed in various inhabited areas, such as utensils or on stones (Ng et al., 2016). The
367 populations unique to biofilms changed rapidly, despite the presence and constancy of families
368 common to all compartments. Our results showed that Planctomycetes, Chloroflexi and
369 Thaumarchaeota phyla as being specific to biofilm samples.

370 During this seasonal campaign, the cold-water inlet underwent the most temperature changes (from
371 9.08°C to 21.1°C), due to warmer waters in the summer, but no major differences in bacterial
372 communities were observed in this compartment. The water temperature in the tanks was maintained
373 approximately constant by mixing in variable proportions the hot- and cold-water inlets (from 17.97°C
374 to 21.17°C). Nevertheless, the bacterial communities did not change with the slightly higher
375 temperatures during summer (Fig. 5C, 5D). Moreover, a Spearman correlation test was performed

376 between all physical parameters (temperature, pH, oxygen, salinity and conductivity) measured in the
377 tanks, the diversity indicators (Chao1 and Shannon from tank water and biofilm samples) and the
378 relative abundance of Vibrionaceae (from the tank water and biofilm samples). No significant
379 association was revealed between evenness and richness variations from the tank water and biofilm
380 samples and temperature, oxygen, salinity or conductivity considered independently. A negative
381 correlation was revealed only between pH and both diversity indicators in biofilm samples. Bacterial
382 communities of biofilm samples appeared more diverse when the pH was lower. The pH variation
383 ranged from 6.35 to 7.97 during the sampling campaign (Table S1). The pH decrease may be linked to
384 the nitrification process occurring in biofilm samples. Finally, the relative abundance of Vibrionaceae
385 from water tank samples correlated negatively with pH and oxygen levels in the tanks, whereas the
386 relative abundance of Vibrionaceae from biofilm samples correlated positively with the temperature in
387 the tanks. Then the temperature rising seemed promote Vibrionaceae proliferation in biofilm samples
388 and their role as reservoir in case of dysbiosis.

389 **4.3. Potential pathogens detected**

390 We then focused on the Vibrionaceae family in tank water samples, because this compartment
391 contained more Vibrionaceae than the other compartments. Our results suggested that biofilms may be
392 a reservoir of Vibrionaceae, and tank water seemed amenable to its proliferation. A lower Shannon
393 index was indeed obtained (18W, 24W and 28W, Fig. 5C) when the Vibrionaceae were most abundant
394 (18W, 24W and 28W, Fig. 3). The balance between bacterial communities was thus disturbed, because
395 one bacterial population had proliferated in particular. Among the Vibrionaceae family, four genera
396 were frequent, in order of importance: *Vibrio*, *Photobacterium*, *Aliivibrio* and *Enterovibrio*. More
397 precisely, the species level determination identified *Vibrio ponticus*, *Vibrio aestivus*, *Vibrio*
398 *sinaloensis* and *Vibrio anguillarum*, as well as *Photobacterium damsela* subsp. *damsela*,
399 *Photobacterium swingsii*, *Photobacterium gaetbulicola* Gung47, *Photobacterium rosenbergii*, and
400 *Photobacterium damsela* subsp. *piscicida*, and *Aliivibrio fischeri* and *Enterovibrio nigricans* (Table
401 1). Most pathogenic *Vibrio* species, such as *Vibrio harveyi* or *Vibrio vulnificus*, were not identified,
402 although we expected to find *V. harveyi* since it has already been identified as major pathogens in this

403 aquaculture infrastructure (Mougin et al., 2020). The Vibrionaceae population more than doubled
404 during the sampling campaign (20% at 0W and 50% at 28W, Fig. 3). The peaks of diversity were
405 reached at weeks 19 and 20 (during August), suggesting that all genera were present and in stable
406 proportions (Fig. 7). Despite this variation, the equilibrium between genera remained relatively stable
407 throughout the sampling weeks.

408 Our results revealed that the water from the tanks contained Vibrionaceae and may be sufficiently rich
409 in bacteria to harbor pathogens and their antagonistic members. Some studies have indeed reported
410 that *Roseobacter* spp. (family Rhodobacteraceae) may establish antagonistic activity against
411 Vibrionaceae and particularly against *V. anguillarum* (Balcázar et al., 2010, Hjelm et al., 2004).
412 Among the Flavobacteriaceae, *Polaribacter* is an opportunist common fish farm bacteria colonizing an
413 already weakened fish and has already been detected in the core microbiome of seabass and seabream
414 (Rosado et al., 2019). Our results showed that increases in Rhodobacteraceae and Flavobacteriaceae
415 were associated with decreases in Vibrionaceae. The absence of acute vibriosis outbreaks during the
416 campaign suggests that there is an equilibrium between pathogenic Vibrionaceae and the other
417 antagonistic bacterial communities. It would be relevant to study the putative antagonistic activity
418 between sampled Vibrionaceae, Rhodobacteraceae and Flavobacteriaceae. Simultaneously,
419 identification and detection of biofilm microbiomes now makes it possible to investigate the capacity
420 of horizontal gene transfer of the different species and to investigate antibiotic resistance. Despite the
421 presence of potential pathogens, there were no disease outbreaks; therefore, the vaccination campaign
422 appears to have worked. In the present study, no sampling was carried out directly on fish firstly for
423 ethical reasons and secondly because the main objective was to consider the aquaculture
424 compartments. Nevertheless, future studies should compare our results with fish microbiomes to
425 confirm the efficiency of vaccination. Finally, our results demonstrated the importance of
426 simultaneously studying all bacterial communities.

427 **5. Conclusion**

428 In conclusion, this is the first study to compare bacterial communities from several water
429 compartments and from biofilms in an aquaculture farm over time with a seven-month weekly

430 sampling campaign. Proteobacteria and Bacteroidetes, which are common phyla in water ecosystems,
431 were abundant. The diversity of bacterial communities differed within compartments, although there
432 were no significant correlations with water quality parameters. Biofilm samples contained higher
433 bacterial diversity than water samples and tank water samples were richer than samples from water
434 inlets. The proportion of Vibrionaceae was high in tank water samples. All six genera composing the
435 Vibrionaceae family were identified. The natural environment seemed to be a source of Vibrionaceae,
436 which found a favorable environment to develop and then persist in biofilms in the aquaculture
437 system. In the future, we plan to investigate the antagonistic effects between Vibrionaceae and other
438 already present bacteria to design solutions such as probiotics use to monitor bacterial populations and
439 promote sustainable aquaculture. Moreover, the aquaculture environment can be monitored using a
440 metatranscriptomic analysis. The bacterial populations detected can be linked to the primary and
441 secondary metabolic functions observed, such as virulence.

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447 **Declaration of competing interest**

448 The authors declare that they have no known competing financial interests or personal relationships
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602

603

604 **Figure Legends**

605 Figure 1: The six most abundant phyla (from all samples, over all sampling times), representing
606 94.38% of all sequences. The number above each bar indicates the percentage of each phylum. The
607 percentage of each class of Proteobacteria is shown in the blue donut chart.

608 Figure 2: Bar plots of relative abundance (%) of bacterial families throughout the 30 sampling weeks
609 composing A) the cold-water inlet samples, B) the hot-water inlet samples. Bacterial families are
610 grouped by phylum with the second color scale on the top. Families unassigned or that did not reach a
611 minimum of 3% in at least one sample were grouped in the “Others” category.

612 Figure 3: Bar plots of relative abundance (%) of bacterial families composing the tank water samples
613 throughout the 30 sampling weeks. Bacterial families are grouped by phylum with the second color
614 scale on the top. Families unassigned or that did not reach a minimum of 3% in at least one sample
615 were grouped in the “Others” category.

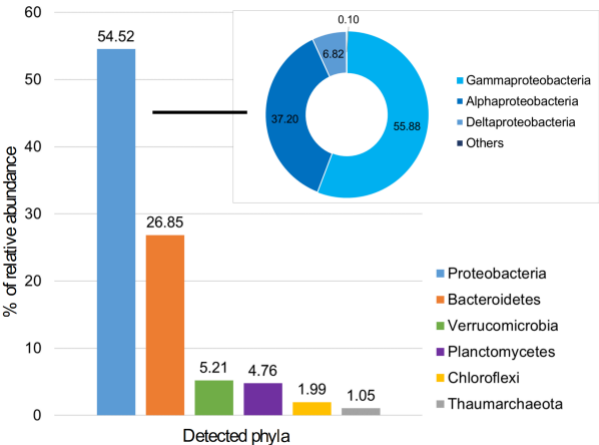
616 Figure 4: Bar plots of relative abundance (%) of bacterial families across biofilm samples across
617 sampling weeks. Bacterial families are grouped by phylum and by family (color scale on the right).
618 Unassigned families or those that did not reach a minimum of 1% in at least one sample were grouped
619 in the “Others” category.

620 Figure 5: Mean values and standard deviations of Chao1 (A) and Shannon (B, C, D) alpha-diversity
621 indices plotted by compartment (A, B) or by sampling week for tank water samples (C) and for
622 biofilm samples (D). All purple and gray box plots respectively represent tank water samples and
623 biofilm samples from the three breeding tanks. Blue and red box plots correspond to cold- and hot-
624 water inlet samples, respectively. Kruskal-Wallis tests for pairwise comparisons were carried out and
625 different letters indicate significant differences.

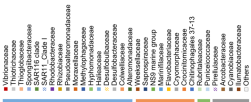
626 Figure 6: Beta-diversity of the 159 samples. (A) Unweighted UniFrac and (B) weighted UniFrac
627 distances were analyzed and plotted using principal coordinates analysis (PCoA). Each point
628 represents a microbiome sample (composed of 29,725 sequences corresponding to the rarefaction
629 depth). Rings and stars distinguish the water dataset from the biofilm dataset, respectively. Blue and
630 red colors correspond to cold- and hot-water inlet samples, respectively, whereas the three shades of
631 purple indicate the water or biofilm samples from the three breeding tanks. The brown star indicates
632 the B0 sample.

633 Figure 7: Mean values and standard deviations of Shannon alpha-diversity index of the Vibrionaceae
634 family, plotted by sampling week for tank water samples. Kruskal-Wallis tests for pairwise
635 comparisons were carried out and different letters indicate significant differences.

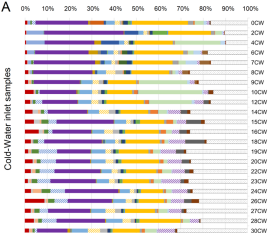
636



Proteobacteria Bacteroidetes Vernucomicrobia Other Phyla

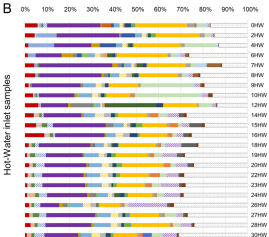


A



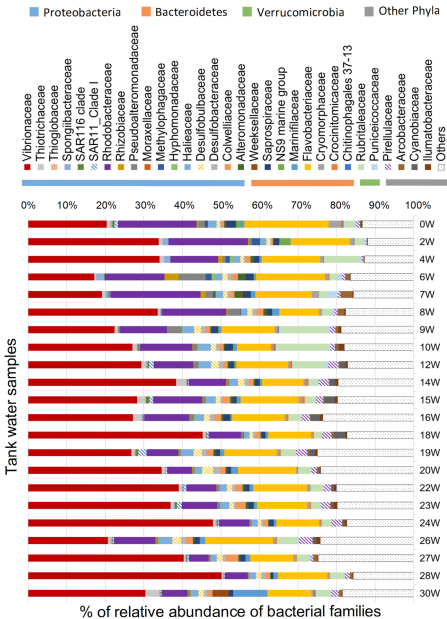
Cold-Water inlet samples

B



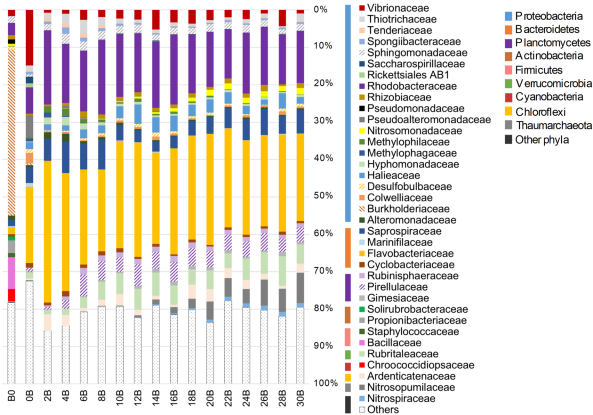
Hot-Water inlet samples

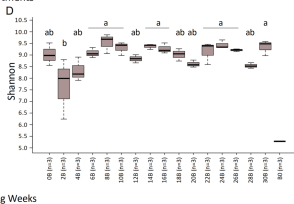
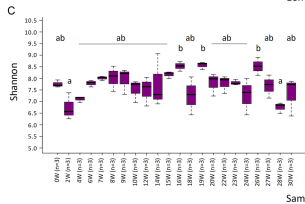
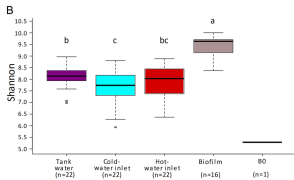
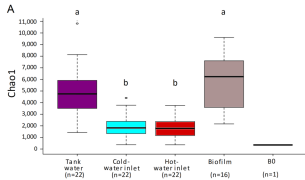
% of relative abundance of bacterial families

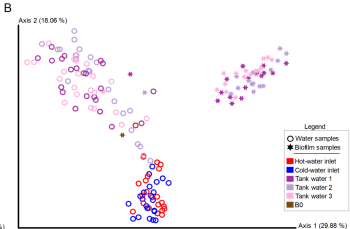
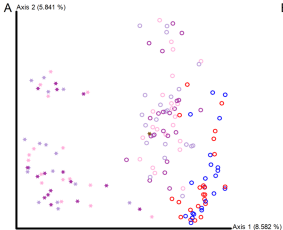


Biofilm samples

% of relative abundance of bacterial families







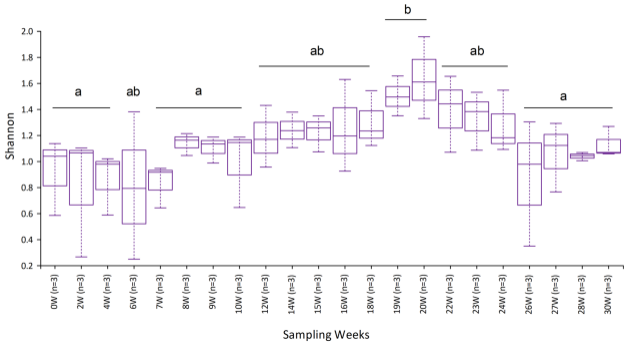


Table 1: Number of sequences identified, using the SILVA 132 database, in the Vibrionaceae family and number of samples

Taxonomy of Vibrionaceae detected						All samples		
phylum	class	order	family	genus	species	Number of sequences	Number of samples observed in	Number of total samples
Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio		1,796,843	159	159
				—		1,750,394	159	-
				<i>Vibrio ponticus</i>		24,369	68	-
				<i>Vibrio aestivus</i>		16,467	20	-
				<i>uncultured bacterium</i>		2,803	67	-
				<i>Vibrio sinaloensis</i>		2,436	6	-
				<i>Vibrio anguillarum</i>		336	28	-
				<i>uncultured Shewanella sp.</i>		29	10	-
				<i>uncultured Thalassotalea sp.</i>		9	1	-
				Photobacterium		404,774	150	159
				—		198,025	136	-
				<i>Photobacterium damsela subsp. damsela</i>		203,839	98	-
				<i>Photobacterium swingsii</i>		2,878	71	-
				<i>Photobacterium gaetbulicola Gung47</i>		14	4	-
				<i>uncultured gamma proteobacterium</i>		11	1	-
				<i>Photobacterium rosenbergii</i>		5	1	-
				<i>Photobacterium damsela subsp. piscicida</i>		2	1	-
				Aliivibrio		328,413	130	159
				—		273,651	123	-
				<i>Aliivibrio fischeri</i>		54,762	108	-
				Enterovibrio		29,895	77	159

	—	29,812	74	-
	<i>Enterovibrio nigricans</i>	42	5	-
	<i>uncultured bacterium</i>	41	5	-
Salinivibrio	—	55	9	159
Grimontia	—	29	7	159
uncultured	—	57	6	159
	—	15750	140	159