Diversity and stability of microbiota are key factors associated to healthy and diseased *Crassostrea gigas* oysters

Running title: **Links between oyster fitness and microbiota composition**

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27   **Abstract**

28   Associations between micro- and macro-organisms (forming holobionts) are common and

29   mostly specific in nature. Although microbes might contribute to the holobiont homeostasis,

30   their role in the adaptive capacities of the holobiont remains unclear in most of the living

31   species, and studies testing the links between host fitness and microbiota characteristics are

32   still lacking.

33   To tackle this issue, we analyzed the microbiota of full-sib progenies from five biparental

34   oyster families in a controlled condition (hatchery) and after transplantation during non-

35   stressful or stressful periods in two geographic regions (Atlantic Ocean and Mediterranean

36   Sea). We used 16S rRNA gene-metabarcoding to study taxonomic compositions of microbial

37   communities. First, comparisons of microbial assemblages showed that environmental

38   conditions highly influenced oyster microbiota. However, different ecological niches existed

39   between oyster tissues and seawater, and even between the five families. Secondly, because

40   two oyster families displayed high mortalities in both stressful periods, we compared their

41   microbiota characteristics to the three other families. All oyster microbiota were highly

42   modified after transplantation and no clear microbiota dysbiosis was observed for the two

43   susceptible families. Moreover, despite similar levels of mortalities in both geographic

44   regions, we identified many differences of microbial assemblages between resistant and

45   susceptible families in the Atlantic Ocean, but few in the Mediterranean Sea. Particularly,

46   susceptible families showed lower diversity of their microbiota than the resistant ones in

47   stressful periods. Secondly, we highlighted that microbiota of susceptible oyster families had

48   higher variability than the resistant ones at the scale of bacterial orders, but also less

49   variability at the scale of OTUs. Lastly, while resistant families had increased relative

50   abundances of cyanobacteria (Subsection III, family I) during the stressful period, susceptible

51   oysters had low stability of Rhodospirillales, Rhodobacterales, Flavobacteriaceae and

52   Erythrobacteraceae.

53   This study highlighted links between microbiota diversity and low variability of microbial

54   assemblages at higher taxonomic ranks with oyster mortalities. We also identified bacterial

55   taxa related to oyster fitness.

Introduction

56

57   Interactions between micro- and macroorganisms are ubiquitous on Earth, from host-pathogen

58   relationships [1] to the widely distributed commensalim or mutualism [2,3]. Previous

59   observations suggested that composition of host-associated microbial communities (hereafter

60   named microbiota) are not random, but might be linked to host taxonomy and physiology.

61   Indeed, specific associations were found for microbiota of phylogenetically distant species of

62   mice, flies, mosquitoes, wasps and hominids [4], but also for microbiota of closely related

63   species of corals [5], hydra [6], or oysters [7]. Moreover, many studies showed that these

64   interactions were not stable when hosts were in stressful conditions or in different

65   physiological conditions. For example, microbiota compositions were found to be very

66   different between healthy and diseased corals [8], or between oysters exposed to different

67   temperatures [9].

68   Microbial associates might provide benefits to their hosts for survival, but also homeostasis

69   and development [10]. First, a stable microbiota can play a role of physical barriers against

70   pathogens. For example, healthy mice developed *Salmonella* infection after antibiotic

71   treatments [11]. Other studies highlighted protective effects of (i) secondary endosymbiont of

72   aphids against parasitoid wasps [12], (ii) toxic alkaloids produced by endophytic fungi of

73   grasses against herbivores [13], or (iii) symbiotic bacteria of frogs against pathogenic fungi

74   [14]. Secondly, because microbiota composition can change rapidly, they might represent

75   mechanisms for rapid acclimation of the holobiont to changing environments [15]. In

76   particular, fine-scale modifications of *Nematostella vectensis* microbiota were observed with

77   environmental variations [16], suggesting that microbiota might participate to the

78   maintenance of holobiont homeostasis. Hence, structure of microbiota might be multilayered

79   with at least two populations of microbes: a stable host-adapted core microbiota and a more

80   variable microbial pool [17]. However, the balance between stability and variability is still

81   poorly studied. In particular, lack of knowledge concerns links between microbiota

82   characteristics and fitness of holobionts facing environmental changes.

83   To tackle this issue, the farmed oyster *Crassostrea gigas* is a relevant model, as it filters

84   seawater to feed. As a consequence, all oysters are in contact with the same environmental

85   microbial communities, and microbiota variations are not biased towards different diets [18–

86   20]. In addition, oysters are affected by recurrent mortality syndroms affecting mainly

87   juveniles [21,22]. These mortalities are multifactorial processes due to water temperature and

3

88   quality, but also development of viral and bacterial pathogens [23–27]. Particularly, different
89   susceptibilities were observed according to oyster physiology and/or genetic backgrounds
90   [28–30], in association with microbiota dysbiosis [9]. Although oyster genetics and
91   microbiota dysbiosis were associated to mortalities, no studies tried to disantangle the links
92   between oyster fitness and microbiota characteristics.

93   Thus we produced full-sib progenies from five biparental oyster families (see Methods for
94   more details) using genitors from different origins. In particular, males and female genitors
95   were isolated from different geographic regions in France (Atlantic Ocean and Mediterranean
96   Sea), and in different areas (farming areas or sites with lower densities of oysters, and
97   potentially lower concentrations of pathogens). Then we transplanted progenies of each
98   biparental family at two time periods in the Atlantic Ocean or in the Mediterranean Sea.
99   These two time periods were selected according to seawater temperatures in order to study
100   oysters in non-stressful and stressful periods. Particularly, it was previously observed that
101   16°C was a relevant threshold to define periods of stress, because high mortality rates were
102   observed above this temperature [24,25]. After five days of transplantation, oysters were
103   sampled, seawater was filtered, and we analyzed oyster-associated and seawater bacterial
104   communities using 16S rRNA gene-metabarcoding.

105   This study aimed at (i) testing environmental effects on oyster microbiota, (ii) comparing
106   oyster microbiota between resistant and susceptible phenotypes for periods of stress, and (iii)
107   testing the balance between stability and variability of microbiota in changing environment.

108   The identification of such bacterial candidates might help to develop new production
109   techniques for oyster farming in the future.

<div style="text-align:center">Methods</div>

Genitors from different geographic origins

Biparental *C. gigas* oysters families were produced using a recently developed methodology that allowed the production of pathogen-free juveniles [25,27,31]. Four oysters families (F09, F15, F32 and F44) were produced from wild genitor sampled in farming and non-farming area in two geographic regions, the Mediterranean Sea and the Atlantic Ocean (Fig. S1). In addition, family F21 was produced using genitors from a massal breeding program aiming at increasing the resistance of *C. gigas* oysters against OsHV1-virus. It was performed by breeding disease survivors throughout four generations of selection [32]. Each family (cohort) thus corresponded to the offspring of a biparental reproduction (full-sib progenies). Maturation induction, reproduction and larval breeding were performed as described previously [25,33]. In the larval and post-larval stages, the oysters were fed with the same diet as the genitors at a concentration between 1500-2000 μm^3 μl^{-1} [34]. Before experiments, all oyster families were maintained in controlled condition at the laboratory (Argenton, France) using seawater treated with UV, filtered through 1 μm mesh, and enriched with a bi-specific phytoplankton diet made of *Tisochrysis lutea* (CCAP 927/14) and *Chaetoceros muelleri* (CCAP 1010/3) (in equal biomass proportion) at a ratio equivalent to 6% of the oyster dry mass [34]. Finally, all oysters remained free of any abnormal mortality.

Experimental design

About 140 individuals of each oyster family were either kept in the controlled condition or placed in one out of four natural environments for five days. The four natural environments corresponded to two geographic site (Atlantic Ocean and Mediterranean Sea, see Fig. 1), and two time periods. These two time periods coincided to non-stressful and stressful periods according to seawater temperatures (below and above 16°C, respectively) (Table S1), and were confirmed by the observed mortality rates. After five days of transplantation, no mortality occurred and about 15 individuals of each family were flash frozen in liquid nitrogen and stored at -80°C. In addition, 125 oysters per family were also placed in controlled condition to monitor mortality rates. The number of dead oysters was estimated at day 13 (*i.e.*, eight days after the end of transplantation). Moreover, serial filtrations of 5 liters of seawater were performed for the five conditions using porosity of 10 µm (Whatman[TM]

<div style="text-align:center">5</div>

141 111115 Nucleopore^{TM} Track-Etch Polycarbonate Membrane filter, 47 mm diameter), 5 µm

142 (Whatman^{TM} 111113 Nucleopore^{TM} Track-Etch Polycarbonate Membrane filter, 47 mm

143 diameter), 0.8 µm (Whatman^{TM} 111109 Nucleopore^{TM} Track-Etch Polycarbonate Membrane

144 filter, 47 mm diameter), and 0.2 µm (Whatman^{TM} 111106 Nucleopore^{TM} Track-Etch

145 Polycarbonate Membrane filter, 47 mm diameter).

146

147 **DNA extraction, PCR and sequencing**

148 Frozen oysters were ground in liquid nitrogen in 50 ml stainless steel bowls using 20 mm

149 diameter grinding balls (Retsch MM400 mill). The powders were stored at -80°C, and were

150 then used for DNA extractions using the DNA from tissue Macherey-Nagel kit (reference

151 740952.250) according to the manufacturer's protocol. In order to improve DNA extractions,

152 we nevertheless added a crushing step, that consisted in an additional 12 minutes mechanical

153 lysis using zirconium beads before the 90 min enzymatic lysis in the presence of proteinase

154 K. The same lysis and DNA extraction protocol was used for the different filters. DNA

155 concentration and quality were checked with Epoch microplate spectrophotometer (BioTek

156 Instruments, Inc.).

157 Then, the 16S rRNA gene of bacterial communities was amplified and sequenced using the

158 variable V3V4 loops (341F: 5'-CCTACGGGNGGCWGCAG-3'; 805R: 5'-

159 GACTACHVGGGTATCTAATCC-3') [35]. Paired-end sequencing (250 bp read length)

160 was performed at the McGill University (Génome Québec Innovation Centre, Montréal,

161 Canada) on the MiSeq system (Illumina) using the v2 chemistry according to the

162 manufacturer's protocol. Raw sequence data are available in the SRA database (BioProject ID

163 PRJNA419907).

164

165 **Sequence analyses**

166 The FROGS pipeline (Find Rapidly OTU with Galaxy Solution) implemented into a galaxy

167 instance (http://sigenae-workbench.toulouse.inra.fr/galaxy/) was used to define Operational

168 Taxonomic Units (OTU), and computed taxonomic affiliations [36]. Briefly, paired reads

169 were merged using FLASH [37]. After denoising and primer/adapters removal with cutadapt

170 [38], *de novo* clustering was performed using SWARM that uses a local clustering threshold,

6

171 with aggregation distance d=3 [39]. Chimera were removed using VSEARCH (*de novo*

172 chimera detection) [40]. Particularly, this method divided each sequence into four fragments,

173 and then looked for similarity with putative parents in the whole set of OTUs. We filtered the

174 dataset for singletons and we annotated OTUs using Blast+ against the Silva database (release

175 123, September 2015) to produce an OTU and affiliation table in standard BIOM format.

176 Rarefaction curves of species richness were produced using the {phyloseq} R package, and

177 the rarefy_even_depth and ggrare functions [41]. The alpha diversity metrics (Chao1,

178 Simpson and Shannon) were estimated at the OTU level with the estimate_richness function.

179 Moreover, Pielou's measure of species evenness was computed using the diversity function in

180 {vegan}. We also used phyloseq to obtain abundances at differents taxonomic ranks (from

181 genus to phylum) (tax_glom function). Multi-affiliation and unknown taxa were removed for

182 the different taxonomic ranks if they corresponded to the addition of multiple OTUs having

183 the same annotation. Because samples had different sequencing depths, we used relative

184 abundances of OTUs and the different taxonomic ranks for subsequent analyses. We

185 computed Bray-Curtis dissimilarities to study beta diversity, *i.e.*, distances between samples

186 for OTU compositions (vegdist function, {vegan}).

187

Statistical and multivariate analyses

189 All statistical analyses were done using R v3.3.1 (R: a language and environment for

190 statistical computing, 2008; R Development Core Team, R Foundation for Statistical

191 Computing, Vienna, Austria [http://www.R-project.org]).

192 Clustering and ordination methods were used to describe and compare composition of

193 microbial communities between samples. Hierarchical clustering (average linkage (hclust

194 {stats})) and principal coordinate analyses (hereafter named PCoA) of microbial communities

195 were computed using Bray-Curtis dissimilarities (vegdist {vegan}). Factors associated to each

196 sample were plotted face to clustering using the heatmap.2 function and the {gplots} package.

197 Variability of oyster microbiota for stressful and non-stressful periods was estimated using

198 PCoA and distances to centroid using betadisper {vegan}.

199 In order to explain modifications of microbial assemblages, we used redundancy analysis [42]

200 which investigate the variations of the different OTUs under the constraint of differents

201 factors. OTUs were Hellinger-transformed [43] before testing their links with the following

7

202   variables: family, period (stressful or non-stressful) and site (Atlantic Ocean or Mediterranean

203   Sea) of transplantation. In particular, we estimated the influence of these variables on

204   microbial assemblages using rda and anova.cca functions ({vegan}).

205   We performed Student's t-test (t.test {stats}) or non-parametric Wilcoxon test (wilcox.test

206   {stats}) (when normality was rejected with the Shapiro-Wilk test, (shapiro.test {stats})) to

207   compare oyster families for (i) alpha diversity metrics (Chao1, Simpson, evenness and

208   Shannon), and (ii) distances to centroid.

209   Wilcoxon tests were used to identify candidates (OTUs or orders) related to (i) seawater or

210   oysters, and to (ii) stressful or non-stressful periods.

211   Finally, we computed enrichment analyses of the significant OTUs to identify over-

212   represented higher taxonomic ranks using Fisher's exact tests at each taxonomic rank.

213   For all analyses, the threshold significance level was set at 0.05. P-values were corrected for

214   multiple comparisons using Benjamini and Hochberg's method [44] (p.adjust, {stats}).

215

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Results

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Environmental conditions highly influenced oyster microbiota

Oyster families were produced using wild genitors sampled from sites of high and low density of oyster populations. Full-sib progenies of five biparental families (F09, F15, F21, F32 and F44) were produced and maintained in a controlled condition (laboratory of Argenton, France). In order to study microbiota compositions in changing environments, individuals of each oyster family were then transplanted from the controlled condition into two natural environments at two time periods (non-stressful or stressful) for five days (Fig. 1). For the five conditions of this study, microbial communities were sequenced using the 16S rRNA gene from about 14 individuals of each oyster families (347 oyster-associated microbiota), and from seawater (19 filters). In average, each sample contained ~17,550 sequences representing ~1,140 OTUs (Fig. S2-S4, Table S2 and S3).

First, oyster microbiota were highly variable between the different conditions (Fig. 2 and Fig. S5). Particularly, we used redundancy analyses to identify factors (oyster family, period (non-stressful or stressful) and site (Atlantic Ocean and the Mediterranean Sea) of transplantation) that signficantly explained microbiota changes in the Atlantic Ocean and the Mediterranean Sea. We found that the period and site of transplantation were the most important factors (p=0.001) (Fig. 3A and Fig. S5). Hence, the environment highly influenced the composition of oyster microbiota.

Oyster tissues nevertheless exhibited different ecological niches from seawater

Although environmental conditions highly influenced oyster microbiota, we found that oyster tissues constituted different ecological niches from seawater.

First, redundancy analysis also highlighted the effect of oyster family on oyster microbiota (p=0.001) (Fig. 3A). In particular, this variable significantly constrained bacterial assemblages within the five conditions (Fig. 3B). Secondly, analyses of oyster-associated and seawater bacterial communities revealed that both types clustered separately within the five conditions (Fig. 2). Notably, the most abundant OTUs for seawater and oysters belonged to bacterial families Rhodobacteraceae (~16% of total sequences) and Mycoplasmataceae (~11.4% of total sequences), respectively. To identify significant differences between

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246   seawater and oysters for OTUs, we computed non-parametric Wilcoxon tests. While we found

247   OTUs with significantly higher relative abundances in seawater compared to oysters (Table

248   S4 and Fig. S6), we did not observed OTUs significantly associated with oysters. This lack of

249   significance might result from our stringent analysis procedure. Indeed, the combination of

250   Wilcoxon tests and p-values corrections for multiple comparisons only kept highly significant

251   OTUs. However, it suggested that most oyster OTUs came from the environment, although

252   oyster tissues constrained their growth. Among them, 48 OTUs from the Proteobacteria,

253   Bacteroidetes, Actinobacteria and Chloroflexi phyla were significantly higher in seawater for

254   the four natural environments. Then we performed enrichment analyses of significant OTUs

255   in each condition to identify taxonomic ranks that were over-represented in seawater. We

256   found in particular that Proteobacteria from Rhodobacteraceae, Litoricolaceae, SAR86 and

257   SAR116 families were enriched in seawater of natural environments.

258   Overall, these results suggested that oyster tissues constrained bacterial assemblages

259   differently (i) from seawater, and even (ii) between oyster families having differents genetic

260   backgrounds.

261

262   Oyster families derived from farming area showed low mortality rates

263   Then we particularly focused our attention on oyster microbiota and mortality rates in

264   response to period of stress.

265   While no mortalities were observed for control and non-stressful periods in both the Atlantic

266   Ocean and the Mediterranean Sea, many oysters died during stressful periods (in May and

267   July for the Mediterranean and Atlantic sites, respectively). In particular, two oyster families

268   (F15 and F32) displayed high mortality rates (hereafter named HM) (from 87 to 100%, n=125

269   oysters per family) in both geographic regions, whereas the three others (F09, F21 and F44)

270   showed lower mortality rates (hereafter named LM) (from 0 to 52%, n=125 oysters per

271   family) (Fig. 4). Notably, these two clusters of phenotypes were not linked to geographic

272   origin of genitors (Atlantic Ocean or Mediterranean Sea), but rather to the fact that they

273   originated or not from farming areas. This suggested that selective pressures by pathogens

274   were higher in farming areas.

275

Microbiota of LM oysters were more diverse and stable in stressful periods

276

277 Although high mortality rates affected HM oyster families, no clear pattern of microbiota
278 dysbiosis was observed during stressful periods (Fig. 2). Oysters were placed for five days in
279 the field, because previous observations showed that this time period was long enough for
280 disease development, but not for mortality [27]. As a consequence, disease possibly just
281 started after five days in our experiment, and we expected to find fine-scale changes of
282 microbial communities rather than complete dysbiosis. We thus studied more precisely
283 microbiota of LM and HM using alpha (Chao1, Simpson, evenness and Shannon) and beta
284 (Bray-Curtis dissimilarities) diversity indices.

285 First, analyses of alpha diversity (Table S5) showed that LM had higher values of Simpson,
286 evenness, and Shannon indices than HM in control, and in the stressful period for the Atlantic
287 Ocean (Table 1). Moreover, LM had also higher values of evenness in the Mediterranean Sea,
288 but we did not identify significant differences for Simpson and Shannon.

289 Secondly, we used Bray-Curtis dissimilarities and PCoA to estimate the variability of
290 bacterial assemblages for LM and HM at different taxonomic ranks using distances to
291 centroid as a proxy. We found that oyster-associated microbiota were less variable for LM
292 than HM in controlled condition for many taxonomic ranks ($p<0.05$, Wilcoxon test; except for
293 family, class and phylum) (Table 2). Furthermore, while no significant differences were
294 observed between LM and HM during the stressful period in the Mediterranean Sea, LM
295 microbiota were also less variable than HM for the stressful period in the Atlantic Ocean
296 considering higher taxonomic ranks (from order to class; $p<0.01$, Wilcoxon test), but the
297 opposite trend was observed for OTUs in the same region ($p=0.02$, Wilcoxon test).

298 Hence, all these results suggested that although no clear pattern of microbiota dysbiosis was
299 observed during stressful periods, microbiota of HM families had lower diversity than LM in
300 the Atlantic Ocean. In addition, HM microbiota assemblages were more variable than LM at
301 higher taxonomic ranks, and less variable for OTUs within this region. In contrast, few
302 significant differences were observed in the Mediterranean Sea for microbiota diversity and
303 assemblage variability. It suggested that disease development did not start before oysters were
304 sampled after five days of transplantation in this region.

305

306

11

307 **Microbiota of LM oysters had significant increase of cyanobacteria**

308 Our results revealed that oyster microbiota of LM showed higher variability and stability than
309 HM in the Atlantic Ocean for OTU and orders, respectively. As a consequence, we used
310 Wilcoxon tests to compare taxa of non-stressful and stressful periods within this region. In
311 particular, we tried to identify parallel changes between oyster families of both LM and HM.

312 First, LM shared increased abundances of 16 OTUs in stressful period (Fig. 5A and Table
313 S4), and HM had similar decreases for 29 OTUs (Table S4). In particular among them, five
314 cyanobacteria and three Flavobacteriaceae were significant for LM, and 13 Flavobacteriaceae
315 and five Erythrobacteraceae significantly decreased for HM. Then enrichment analyses of the
316 significant OTUs for each oyster family highlighted that variability mostly concerned
317 cyanobacteria (Subsection III, Family I) for all LM. In contrast, decreased OTUs over-
318 represented Flavobacteriaceae and Erythrobacteraceae families for all HM. Furthermore, we
319 found that although LM had similar increases for 3 bacterial orders in stressful period
320 (Acidobacteria Subgroup 3, Holophagae Subgroup 10, Phycisphaerales), 4 other orders were
321 stable compared to HM (Fig. 5B). Particularly, the relative abundances of Rhodospirillales
322 increased in stressful period for HM, and the fraction of Rhodobacterales, Sphingomonadales,
323 and Cytophagia (Order II) significantly decreased.

12

324 **Discussion**

325 **Oyster microbiota were influenced by the environmental conditions**

326 We found that the environment highly constrained oyster microbiota. This was not surprising

327 because oysters are sessile organisms that filter seawater to feed. Hence, because they face

328 continuous environmental changes, it is expected that microbiota reaches different stable

329 states [45]. Moreover, stable interactions across environments may not be favored in oysters,

330 because they do not have specialized anatomical structures hosting microbiota [17], such as

331 nodules of legume plant [46], or light organ of bobtail squid [47]. As a consequence and

332 according to our observations that oyster microbiota is highly variable, we hypothesize that

333 oyster bacterial assemblages have high diversity, high horizontal transmission of facultative

334 symbionts, high efficiency to purge deleterious mutations, and that they may facilitate rapid

335 acclimation to environmental changes [17].

336

337 **Mortality rates were linked to the origin of genitors**

338 Oysters were transplanted for five days in the field. According to previous observations [27],

339 this time period was considered sufficient for disease development during stressful periods,

340 and no mortalities were observed before oysters were sampled. As a consequence, we could

341 analyze microbiota modifications linked to perturbations (*i.e.*, environmental changes), and

342 not to moribund individuals.

343 To date, most oyster diseases were associated to *Vibrio* strains and *herpesvirus* (OsHV-1

344 μvar) [27,48,49], although other studies highlighted the role of *Roseovarius crassostreae*

345 [50,51], *Tenacibaculum soleae* [52], and possibly Chlamydiales [53] and *Arcobacter* sp.

346 [9,52]. Here we did not analyze the causal origin of diseases, but we observed similar

347 mortality rates in both geographic regions. In particular, the five oyster families formed two

348 clusters of low and high mortality rates during stressful periods in both the Atlantic Ocean

349 and the Mediterranean Sea. Moreover, this clustering was highly associated to the origin of

350 genitors from farming areas or sites with lower oyster densities. Thus these results highlighted

351 that resistance to mortalities was (i) as previously observed influenced by oyster genetics [29],

352 (ii) replicable between two French coastal regions, and (iii) higher for oyster families derived

353 from genitors sampled in farming areas. Because aquaculture uses very high oyster densities,

354 and is affected by many mortality syndroms [21,54], it is likely that selective pressures from

13

355   pathogens are higher in these areas. The low mortalities observed in this study for oyster

356   progenies derived from genitors of farming areas thus suggested that past events might

357   influence their current immune responses. In particular, these different mortality levels might

358   be linked to transgenerational immune priming [55], and/or to natural selection of genetic

359   variants.

360

Stability for bacterial orders and variability of OTUs were linked to oyster mortalities

362   No clear patterns of microbiota dysbiosis were observed in both stressful periods for HM

363   oyster families, suggesting that oysters were sampled before the massive community structure

364   disruption that occurred for moribund and dead oysters [9]. In order to identify fine-scale

365   modifications of oyster microbiota during disease development, we thus performed analyses

366   of alpha diversity indices and variability of microbial assemblages using distances to centroid

367   as a proxy.

368   Higher values of alpha diversity indices (Simpson, evenness, Shannon) was the most striking

369   parallel found for LM compared to HM for the stressful period of the Atlantic Ocean.

370   Particularly, this result highlighted that OTU abundances within LM microbiota were more

371   equally distributed than HM microbiota. For example, evenness may possibly have important

372   effects on oyster fitness, because it was already found to be positively linked to ecosystem

373   productivity [56,57], functional stability [58,59], and invasion resistance [60,61].

374   Furthermore, for the stressful period of the Atlantic Ocean, LM were more stable than HM for

375   higher taxonomic ranks, and more variable for OTUs. Similar results of stability were also

376   obtained for hydra [16], and in oyster hemolymph [9]. However, we precisely identified the

377   stable taxonomic ranks in this study (*i.e.*, from order to class).

378   Surprisingly, we did not find significant differences between HM and LM in the

379   Mediterranean Sea for variability of microbial assemblages, and only evenness and Chao1

380   were significant for alpha diversity indices. Because oysters were all sampled after five days

381   of transplantation, it is very likely that disease development was different between both

382   geographic regions, and even between oyster families. Thus first signs of microbiota

383   dysbiosis were possibly missed in this study if they occurred later in the Mediterranean Sea.

384   For instance, it was observed in a previous work that primate microbiota was altered at the

385   very last stages of a viral infection [62].

14

Rhodospirillales, Rhodobacterales, Flavobacteriaceae, Erythrobacteraceae and cyanobacteria are putative candidates associated to oyster fitness

Because stability and variability of microbiota are putative beneficial characteristics of the holobiont [11,16], we tried to identify taxa related to LM and HM in the Atlantic Ocean where differences for microbiota diversity and stability were observed. We particularly focused our attention on parallel changes between oyster families, as they could point out factors involved in adaptive processes [63–65]. Furthermore, parallels are likely to be reproducible in future studies.

Similarly to *C. virginica* oysters from Hackberry Bay [66], Mycoplasmataceae was the dominant taxa of oyster microbiota in this study. Despite this dominance, stability of the Rhodospiralles and Rhodobacterales orders, and Erythrobacteraceae and Flavobacteriaceae families might be the most important factor for oyster fitness. Indeed, HM had high significant changes for both orders between non-stressful and stressful periods. Moreover, enrichment analysis showed that Erythrobacteraceae and Flavobacteriaceae families were over-represented by OTUs that significantly decreased between both time periods. Among them, Erythrobacteraceae were already isolated from marine invertebrates [67], and Rhodospiralles were found in diseased tissues of *Platygyra carnosus* corals [68]. Conversely, presence and variability of cyanobacteria OTUs might be linked to rapid acclimation of oyster holobiont to environmental changes. Indeed, increased OTUs in stressful period for LM over-represented cyanobacteria (Subsection III, Family I). Although cyanobacteria had a negative effect on other marine invertebrates such as scleractinian corals [69], they were already observed at high relative abundances within oyster microbiota [70]. Particularly, they were abundant in the digestive gland, connective tissue, mantle, and gonad of oysters [71]. Because cyanobacteria persisted in oyster tissues without signs of alterations, a possible endosymbiotic relationship was even proposed [71]. Notably, cyanobacteria were also negatively correlated to culturable *Vibrio* abundance in free-living microbial communities [72]. Hence, cyanobacteria might play a role of barrier for competitors/pathogens, because vibrios are the main bacterial pathogens of oysters [54],. In particular, photosynthetic activity of cyanobacteria could inhibited pathogen growth through the production of reactive oxygen species, such as observed for *Plasmodium* infection in *Anopheles gambiae* [73].

15

Conclusions

418

419 Our study highlighted that oyster microbiota were highly influenced by environmental
420 conditions. However, different ecological niches existed for bacterial communities between
421 seawater and oyster tissues, and even between oyster families having different genetic
422 backgrounds. Although similar mortality rates were observed for each family in the two
423 geographic regions 12 days after transplantation, disease development during stressful periods
424 were possibly more advanced at the time of sampling in the Atlantic Ocean compared to the
425 Mediterranean Sea. Thus, we recommended that future studies control disease development in
426 order to compare microbiota of different individuals at the same stage of infection. For the
427 Atlantic Ocean, we identified many links between microbiota characteristics and oyster
428 mortalities, and particularly that LM had higher diversity, higher variability of bacterial OTUs
429 and higher stability of bacterial orders and classes compared to HM. Furthermore,
430 Rhodospiralles and Rhodobacterales orders, as well as Erythrobacteraceae, Flavobacteriaceae,
431 and cyanobacteria (Subsection III, Family I) families were putative candidates associated with
432 oyster fitness.

433 However, we did not know whether these differences were causes or consequences of the
434 observed mortalities. Hence, experimental microbiota manipulations might help in the future
435 to understand if such modifications favored oysters facing recurrent mortality syndroms. For
436 example, they might lead to develop farming procedures using bacterial probiotics.

437

Declarations

438

439 Ethics approval and consent to participate: Not applicable.

440 Consent for publication: Not applicable.

441 Availability of data and materials: The datasets generated during the current study are
442 available in the Sequence Read Archive repository under BioProject ID PRJNA419907 (to be
443 released upon publication).

444 Competing interests: The authors declare that they have no competing interests.

16

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460

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651

Legends of figures

FIG. 1. Experimental design. Five oyster full-sib families produced with genitors from different origins in terms of geography (Atlantic Ocean or Mediterranean Sea) and area (from farming area or sites where densities of oyster populations were lower) were produced in controlled condition (hatchery), and placed for five days in natural conditions in the Atlantic Ocean (latitude: 48.335263; longitude: 4.317922) or the Mediterranean Sea (latitude: 43.379087; longitude: 3.571483) at two time periods (non-stressful or stressful). Then, oysters were flash frozen, DNA were extracted, and microbiota were sequenced using 16S rRNA gene-metabarcoding.

FIG. 2. Clustering analyses of microbial communities in the five conditions. Dark blue, light blue, black, light green, dark green, and orange boxes correspond to F09, F15, F21, F32, F44 and seawater, respectively. Clustering were computed using Bray-Curtis dissimilarities based on relative abundances of OTUs, and the average linkage method.

FIG. 3. Redundancy analyses of oyster microbiota. Plots show cumulative variations explained by the addition of variables using redundancy analysis. Period: period of transplantation (stressful or non-stressful). Site: site of transplantation (Atlantic Ocean or Mediterranean Sea). Family: oyster families. Residuals: fraction not explained by the variables used in this study. **A.** Analyses based on the microbiota samples from Atlantic Ocean and Mediterranean Sea. **B.** Analyses of the five conditions.

FIG. 4. Mortalities rates (%) for the five oyster families recorded for the transplantation experiments realized during stressful periods. Mortalities were estimated 12 days after individuals were placed in the Atlantic Ocean or in the Mediterranean Sea sites.

FIG. 5. Variability and stability of LM microbiota compared to HM in the Atlantic Ocean. A. Significant increased OTUs for all LM and no HM. **B.** Significant changes of bacterial orders for all HM and no LM.

22

678 **Tables**

679 **Table 1. Comparison of alpha diversity indices between LM and HM.** Numbers into
680 brackets are p-values. NS: not significant. LM or HM indicate which group had significantly
681 higher values.

Index	Hatchery (control)	Med. Sea (non-stressful)	Atl. Ocean (non-stressful)	Med. Sea (stressful)	Atl. Ocean (stressful)
Chao1	NS (0.74)	LM (0.042)	NS (0.199)	HM (0.033)	NS (0.080)
Simpson	LM (0.005)	NS (0.848)	HM (0.024)	NS (0.059)	LM (<0.001)
Evenness	LM (0.003)	NS (0.722)	NS (0.918)	LM (0.039)	LM (<0.001)
Shannon	LM (0.004)	NS (0.565)	NS (0.913)	NS (0.085)	LM (<0.001)

682

683 **Table 2. Comparison of microbiota variability between LM and HM.** Numbers into
684 brackets are p-values. NS: not significant. LM and HM indicate which group had significantly
685 higher values.

Taxonomic rank	Hatchery (control)	Med. Sea (non-stressful)	Atl. Ocean (non-stressful)	Med. Sea (stressful)	Atl. Ocean (stressful)
OTU	HM (0.044)	HM (0.036)	NS (0.427)	NS (0.278)	LM (0.023)
Genus	HM (0.026)	NS (0.889)	NS (0.358)	NS (0.730)	NS (0.507)
Family	NS (0.925)	NS (0.676)	NS (0.385)	NS (0.694)	NS (0.944)
Order	HM (0.036)	NS (0.676)	NS (0.376)	NS (0.793)	HM (0.012)
Class	NS (0.828)	NS (0.464)	NS (0.239)	NS (0.909)	HM (0.001)
Phylum	NS (0.844)	NS (0.076)	LM (0.020)	NS (0.938)	NS (0.865)

686

687

23

688   **Supplementary informations**

689   **FIG. S1. Origin of the five oyster full-sib families.** F09, F21 and F44 were isolated in

690   farming area, where densities of oyster populations and pathogens are expected to be high.

691   F09: Logonna Daoulas (latitude: 48.335263; longitude: -4.317922); F15: Dellec (latitude:

692   48.353970; longitude: -4.566123); F21: Charente Maritime (latitude: 45.781741; longitude: -

693   1.121910); F32: Vidourle (latitude: 43.553906; longitude: 4.095175); F44: pond of Thau

694   (latitude: 43.418736; longitude: 3.622620).

695   **FIG. S2. Rarefaction analysis of species richness for oyster microbiota and filters in the**

696   **controlled condition (hatchery).**

697   **FIG. S3. Rarefaction analysis of species richness for oyster microbiota and filters in the**

698   **Mediterranean Sea.** SW: seawater.

699   **FIG. S4. Rarefaction analysis of species richness for oyster microbiota and filters in the**

700   **Atlantic Ocean.** SW: seawater.

701   **FIG. S5. Clustering analyses of oyster microbiota from the whole samples of natural**

702   **environments.** For the family variable, dark blue, light blue, black, light green, and dark

703   green boxes correspond to F09, F15, F21, F32, and F44, respectively. For the geographic

704   origin, dark and light blue correspond to Atlantic Ocean and Mediterranean Sea, respectively.

705   For area of origin, dark and light blue correspond to farming and non-farming areas,

706   respectively. For transplantation site, dark and light blue correspond to Atlantic Ocean and

707   Mediterranean Sea, respectively. For period of transplantation, dark and light blue correspond

708   to stressful and non-stressful periods, respectively. Mortality rates (%) and temperatures from

709   low to high are indicated by light to dark red. Clustering were computed using Bray-Curtis

710   dissimilarities based on relative abundances of OTUs, and the average linkage method.

711   **FIG. S6. Similarities for significant OTUs between the five conditions (A) and oyster**

712   **families (B).** Black numbers indicate seawater- and stressful-associated OTUs for the five

713   conditions and oyster families, respectively. Red numbers indicate oyster- and non-stressful-

714   associated OTUs for the five conditions and oyster families, respectively. This figure

715   summarizes informations given in Table S4.

716

717

718   **TABLE S1. Temperatures in the five conditions.**
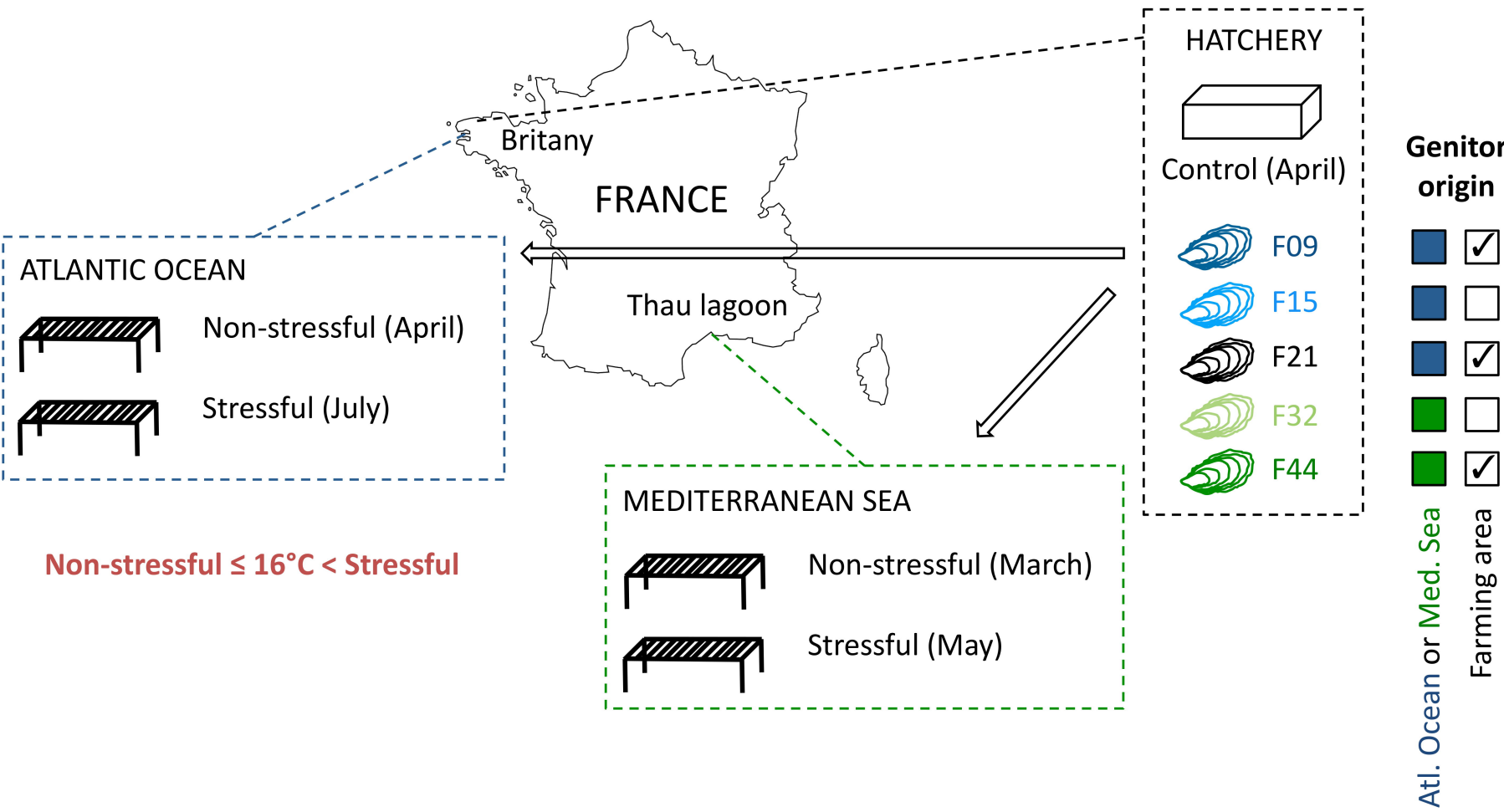
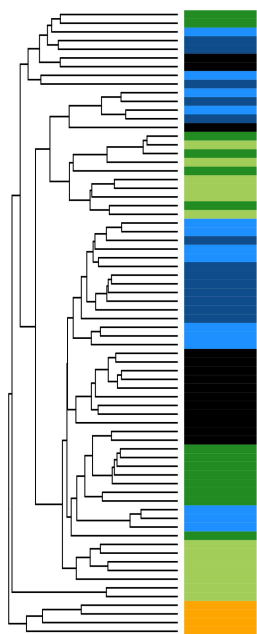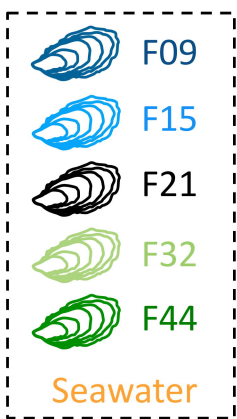719   **TABLE S2. Number of sequences and OTUs.**

720   **TABLE S3. OTU annotations and abundances in the 347 oyster microbiota and on the**
721   **19 filters of seawater microbial communities.**

722   **TABLE S4. Significant OTUs.** For columns *Seawater- or oyster-associated*, *seawater* and
723   *oyster* indicate if abundances of each OTU were higher in seawater or within oysters,
724   respectively (p<0.05, Wilcoxon test). For columns *Stressful- or non-stressful-associated*,
725   *stressful* and *non-stressful* indicate if abundances of each OTU were higher in stressful or
726   non-stressful periods for each oyster family, respectively (p<0.05, Wilcoxon test). NS: not
727   significant. NT: not tested (absent from the dataset).

728   **TABLE S5. Metadata and alpha diversity indices.**

729

25

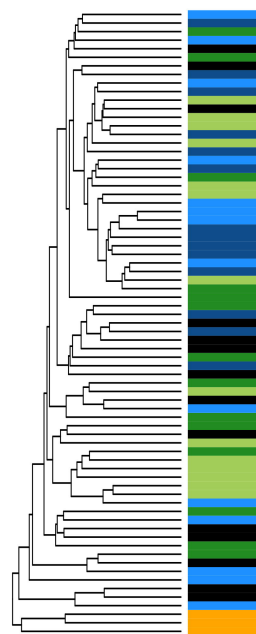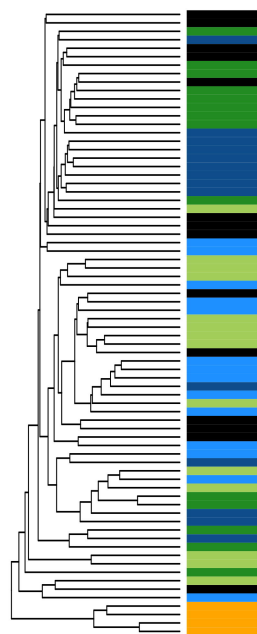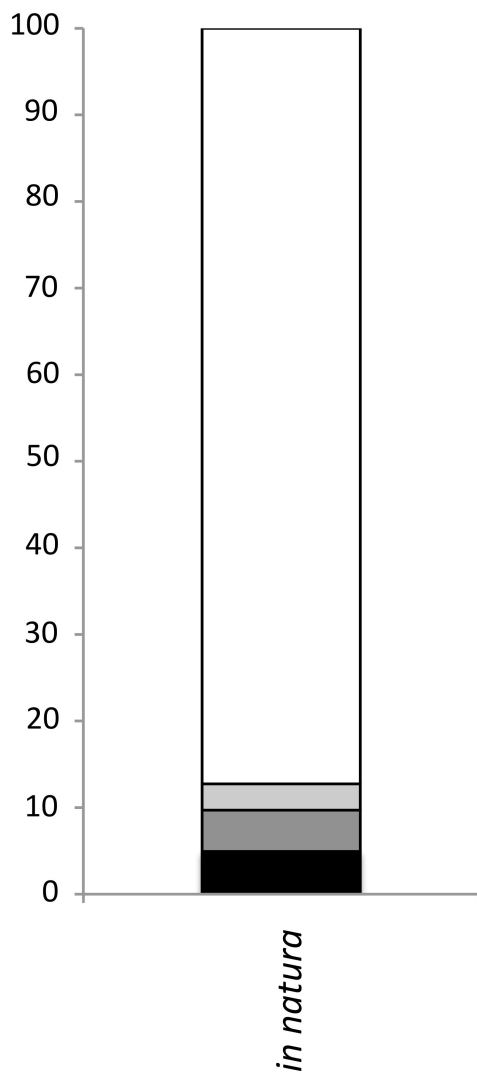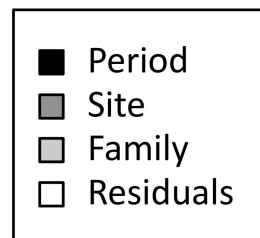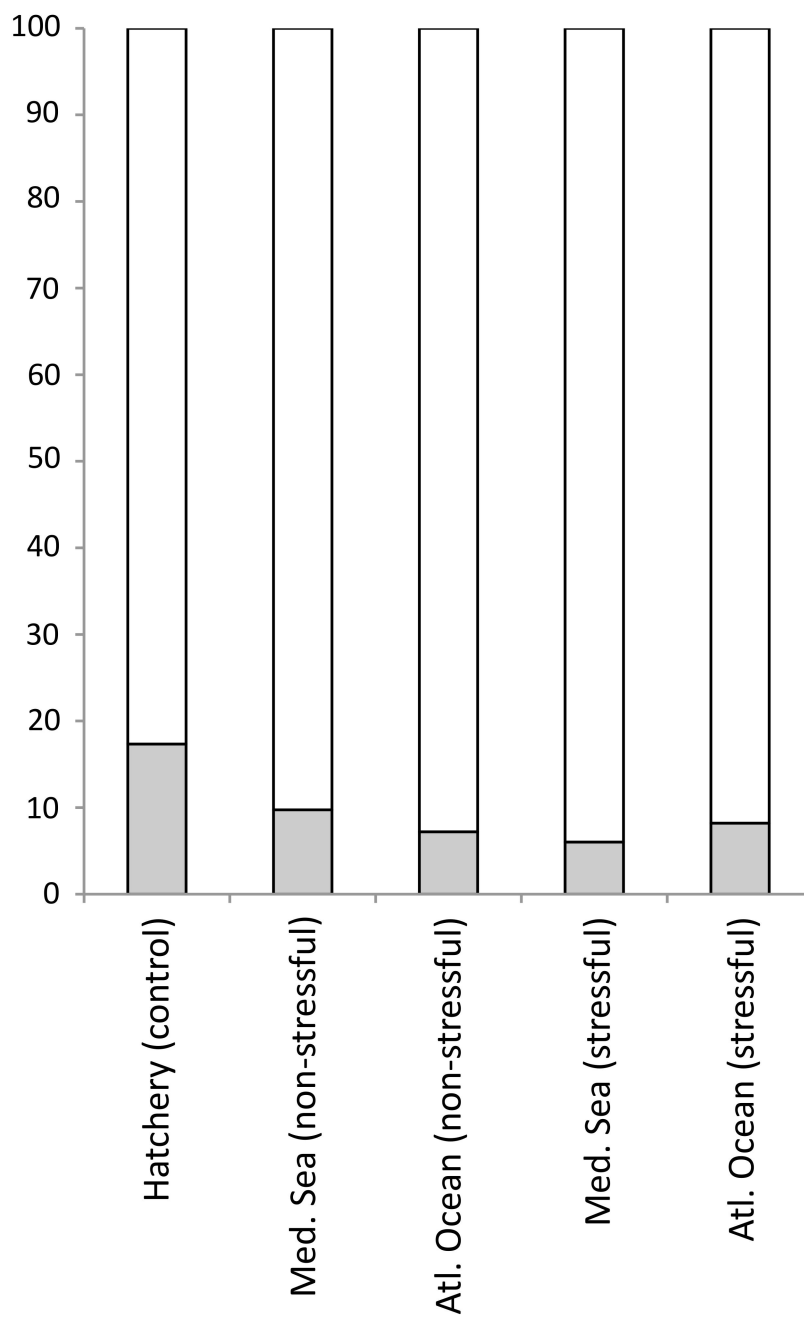|	
F09	
F15	
F21	
F32	
F44	
Seawater	

Hatchery (control) Med. Sea (non-stressful) Atl. Ocean (non-stressful) Med. Sea (stressful) Atl. Ocean (stressful)

A

Color Key
and Histogram

Frequency

B

Color Key
and Histogram

Frequency

F09 (stressful)
F21 (stressful)
F44 (stressful)

F09 (non-stressful)
F21 (non-stressful)
F44 (non-stressful)

F15 (stressful)
F32 (stressful)

F15 (non-stressful)
F32 (non-stressful)

Phormidium sp. · *Phormidium* · *Pseudahrensia sp.* · *Leptolyngbya* · *Cyanobacterium* · CA002 · *Phormidium* · Puniceicoccaceae · *Maribacter* · *Roseobacter* · *Pibocella* · DEV007 · JTB25 · Saprospiraceae · *Croceitalea sp.* · *Roseobacter*

Rhodospirillales · Rhodobacterales · Cytophagia (Order II) · Sphingomonadales