Comparing larval microbiomes of the eastern oyster (*Crassostra virginica*) raised in different hatcheries

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Abstract

An ongoing goal of the aquaculture industry is the improvement of oyster larval growth and production. One component of oyster hatcheries that may contribute to the health of oyster larvae is the early establishment and subsequent development of the oyster larval microbiome. The main objectives of this study were to investigate the effects of environmental conditions (hatchery types and spawning seasons) and oyster phenotypes (larval developmental stages) on the composition and diversity of the larval microbiome. In addition, the members of core larval microbiomes were identified and quantified. Microbiome composition and diversity of larval and corresponding hatchery water samples were examined using high throughput sequencing of 16S rRNA gene amplicons and represented three development stages from two different spawning events at four different hatcheries located in Chesapeake Bay tributaries. Larval and water samples from each spawning cohort were taken at 24-hours 'D' shape (D-stage), 1-week veliger (V-stage), and 2-weeks pediveliger (PV-stage). Larval microbiomes were significantly different from water microbiomes and were significantly influenced by hatchery and spawning, with hatchery having the greatest effect on microbiome composition. While development stage did not show a significant effect on the larval microbiomes, there was a decrease in species richness from the initial D-stage larvae through the final PV-stage and nMDS clustering patterns showed some separation between early (D- and V-) and late stages (PV-) of development , suggesting a shift towards a more selective microbiome as the larvae developed. A total of 25 members (OTU level) were identified as the core larval microbiome (core OTUs), comprising approximately one quarter of the total relative abundance of the larval microbiome. Core OTUs belonging to genera *Alteronomonas* and *Roseobacter* have been shown to offer bivalve larvae some protection against pathogens, while those belonging to family *Cryomorphaceae* are

commonly isolated from microalgal species and most likely indicate an association with oyster larval feeding. These findings underscore the importance of environmental conditions on microbiome development of oyster larvae associated with hatchery success of larval cultivation. Further studies are needed to determine the contribution of the core larval microbiome to oyster health and disease resistance.

1. Introduction

Aquaculture of the eastern oyster (*Crassostrea virginica*) is a rapidly expanding and economically important industry in the Chesapeake Bay. Based on the latest Virginia survey, a reported 32.1 million aquaculture oysters were sold in 2018 and totaled $13.1 million(Hudson 2019). To meet increasing demands, oyster hatcheries are continually striving to improve oyster seed and larval health and optimize rearing conditions. The importance of the microbiome to the health and growth of oyster larvae has received increased attention from the scientific community. Early colonization of key bacteria in the oyster larval microbiome may provide advantages to the oyster as it transitions into an adult. Previous studies have suggested that bacterial colonization may play an important role not only in the development of a bivalve's gastrointestinal tract but may also reduce or prevent detrimental microorganisms from proliferating and causing disease by creating competition for nutrients, reducing space for settlement, or producing antimicrobials (Harris, 1993; Gomez-Gil et al., 2000; Castro et al., 2002; Schulze et al., 2006; Prado et al., 2010; Kesarcodi-Watson, 2012). Probiotics, for example, include beneficial bacteria that improve health or reduce disease, and when administered to bivalve larvae at early stages of development have been shown to increase the survival of oysters, possibly through inhibition of pathogenic bacteria, such as *Vibrio* species *V. alginolyticus*, *V. tubiashii*, *V. anguillarum*, and *V. splendidus* (Riquelme et al., 1996; Ruiz-Ponte et al., 1999; Schulze et al., 2006; Prado et al., 2009; Prado et al., 2010; Sohn et al., 2016; Stevick et al., 2019).

Larval microbiomes may be established during different stages of development as larvae are exposed to variable seawater quality and differing food sources. From the initial onset of oyster

68 larvae development through its later stages of organ and shell development, the oyster's exterior
69 shell surface and interior tissues are continuously exposed to bacteria in the surrounding
70 seawater. During exposure, exogenous bacteria from the hatchery environment rapidly colonize
71 larvae oyster surfaces and tissues to become resident bacteria (Brown, 1973; Kueh and Chan,
72 1985). Factors such as temperature, salinity, nutrients, and oxygen content of seawater are all
73 likely to influence the microbiomes of oyster larvae (Powell et al., 2013), and thus may affect
74 larval microbiome development.

75 Culture-based studies that began in the 1960s primarily investigated bacterial isolates related to
76 larval bivalve disease and survival, including species of *Vibrio* and *Pseudomonas* (Murchelano et
77 al., 1969; Garland et al., 1993; Nicolas et al., 1996; Sainz-Hernández and Maeda-Martínez,
78 2005). Apart from these studies, very little is known about the composition of oyster larval
79 microbiomes. Asmani et al. (2016) examined the composition of the early oyster larval
80 microbiome of Pacific oysters using non-culture based techniques. 16S rRNA gene-based
81 metagenomic analyses were used to compare the bacterial communities of 7- and 15-day-old *C.*
82 *gigas* larvae from different rearing systems including a recycling aquaculture system and a flow-
83 through system. Larval bacterial communities were found to be primarily composed of *Alpha-*
84 and *Gammaproteobacteria*. Additionally, the Asmani et al. (2016) study also demonstrated that
85 the composition of oyster larval microbiomes was highly similar regardless of treatments to the
86 rearing systems, with most variation occurring as a function of larval age. A recent study by
87 Stevick et al. (2019) examined the effect of probiotic treatments on larval microbiomes of the
88 eastern oyster *C. virginica* based on 16S rRNA gene sequences. Decreases in relative
89 abundances of potential pathogenic *Vibrio* spp. were observed in the probiotic treatments. This
90 study also found larval microbiome compositions to be significantly different from the water

91 microbiomes in rearing tanks. While both Asmani et al. (2016) and Stevick et al. (2019) reported
92 the baseline composition of oyster larval microbiomes, all experiments were conducted within
93 the same hatchery and used the same brood stock and algae feed, limiting more general
94 conclusions about the effect of variables such as brood stock or hatchery type on the
95 microbiome.

96 While different hatcheries or even different seasonal conditions (e.g., spawning events) within a
97 hatchery are likely to affect the larval oyster microbiomes, some bacteria may be present in all
98 oyster larvae regardless of locations and rearing practices. Furthermore, some of these bacteria
99 may also remain present in the oyster larvae as they transition through different developmental
100 stages. For example, Trabal et al. (2012) found that bacteria assigned to the genus of
101 *Burkholderia* identified in post-larvae gastrointestinal tracts of *C. corteziensis* and *C. gigas*
102 remained within the oysters from the post larvae stage through adulthood at different cultivation
103 sites. The bacterial taxa that are present throughout the different development stages of the oyster
104 larvae and different hatcheries may represent the 'core microbiome'. Core microbiomes may
105 comprise common or rare bacteria that have been hypothesized to be selectively adapted to
106 specialized niches provided by their host (Roeselers et al., 2011; Schmitt et al., 2012; Schauer et
107 al., 2014) and are likely to be linked to functions critical to homeostasis, development, and
108 biological functions (McFall-Ngai et al., 2013). The core microbiome in larval oysters likely
109 plays similar roles, and if so may be fundamental to larval health and production in the
110 aquaculture industry.

111 To investigate the variation and complexities of the *C. virginica* larval microbiome and examine
112 the effects of hatchery, spawn, and development stage on the larval microbiome, we conducted

high throughput sequencing of 16S rRNA gene amplicons to examine the microbiomes of *C. virginica* larvae and hatchery seawater at three development stages from two different spawns in a summer at four hatcheries. The primary objectives of this study were to (1) to compare the diversity and composition of the oyster larval microbiomes from a variety of hatcheries over a spawning season, (2) determine the changes to the larval microbiome among the different larval development stages, and (3) identify the set of bacteria that were shared among all larvae, defined here as the oyster larval core microbiome, that may serve as a potential microbial indicator of larval health.

2. Materials and methods

2.1 Sample Collection and Hatchery Descriptions

Samples of live oyster larvae were collected from four different hatcheries at three developmental stage time points: (1) D-stage (D, 48-hours), (2) Veliger (V, 1-week), and (3) Pediveliger (PV, 2-weeks), for two separate, consecutive spawning events in June and July 2015. Hatcheries were selected for larval sampling based on location proximate to the Virginia Institute of Marine Science (VIMS), where analyses were conducted. Hatcheries C and D are both commercial oyster hatcheries located on Milford Haven and the Ware River, respectively, in the Virginia part of the Chesapeake Bay tributary system. Hatcheries A and B are both research oyster hatcheries operated by the VIMS Aquaculture Genetics and Breeding Technology Center (ABC) and located on the York River and Locklies Creek, Virginia, respectively. Water treatment at hatcheries A and B is similar and includes sequential treatments with sand filtration, diatomaceous earth, and UV light treatment. At hatchery C, larvae are raised in flow through

culture using water from a single pass multimedia filter. Water at hatchery D is initially passed through bag filters of decreasing pore sizes, followed by sand filtration with fluidized charcoal, followed by a final bag filter. No antibiotics or probiotics are used to treat the water or oyster larvae at any of the facilities. At all four hatcheries, a variety of cultured algal species are grown for feeding oyster larvae. Oyster larvae at hatcheries A, B, and D are batch fed, while at hatchery C, oyster larvae are allowed to feed continuously. Several different breeding lines derived from the VIMS ABC breeding program were used at the hatcheries during the course of the experiment. Hatchery water samples were collected from larval tanks at the same time as the larval samples for each of the three larval stage time points for the first two spawning events. Dissolved inorganic nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) were measured in hatchery water samples by filtering 25 mL of hatchery water through Whatman GF/F filters (25 mm diameter, 0.7 μm nominal pore size). Filtrate was then analyzed for nutrients using a Lachat Quick-Chem 8000 automated ion analyzer (Lachat Instruments, Milwaukee, WI, USA). An additional 300 mL of hatchery site water was filtered through a 0.22-μm pore size Millipore Sterivex filter for DNA extraction. Oyster larvae were initially washed and resuspended with 20 mL of sterile seawater before being collected on a 0.22-μm pore size Millipore Sterivex (Merck KGaA, Darmstadt, Germany) filter. Both larvae and water filters were stored at -80°C until DNA extraction. Time points for hatchery B spawn 2 were excluded from this analysis as a result of spawning failure. All other spawns were considered successful based on subsequent settlement and viability of larval oysters following the PV-stage.

2.2 DNA isolation and 16S rRNA gene amplification

DNA extractions for both oyster larvae and water samples were carried out using MoBio PowerSoil DNA isolation kit (Qiagen, Carlsbad, CA). For oyster larval samples, oyster larvae were carefully removed from the Sterivex filters, which were removed from cartridge housing, and then resuspended in approximately 3 mL of the MoBio bead beater solution. Approximately 10,000, 4,000 and 500 oyster larvae were added to glass bead tubes for stages D-, V-, and PV-stages, respectively. For water DNA extractions, one half of the Sterivex filter from the water samples was used after carefully extruding the filter from the cartridge housing. The remaining extraction steps were conducted following the manufacturer's protocol. Larval DNA (3 ng) and 2 water DNA (2 ng) were used for PCR. PCR amplification of the hypervariable V4 region of the 16S rRNA gene was conducted on the extracted DNA using forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al. 2011) with the Ion Torrent Personal Genome Machine (PGM). The basic manufacturer's PCR protocol was used with SuperTaq DNA Polymerase (Invitrogen, Carlsbad, CA) to create a PCR master mix with the following modification: a 1 mM dNTP mixture was used in place of a 10 mM mixture for a final concentration of 0.02 mM dNTP. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 54°C for 1 min, 68°C for 2 min. A final elongation step of 68°C for 10 min was added to ensure complete amplification. These conditions were optimized using larval DNA samples to ensure amplification of 16S rRNA genes in all samples. The amplified products were cleaned using the UltraClean GelSpin DNA Purification Kit (Mo-Bio, Carlsbad, CA). The resulting amplicon libraries were then used as templates for sequencing with the Ion PGM platform following the manufacture's instruction (Thermo Fisher Scientific, Waltham, MA). Sequences for this study are deposited at the National

178 Center for Biotechnology Information (NCBI) in the Short Read Archive (SRA) and under
179 accession ID PRJNA610764.

180 2.3 Sequence processing and OTU assignment

181 Removal of barcodes and primers from raw sequences was conducted using the Ribosomal
182 Database Project (RDP) pipeline initial process (Cole et al., 2014; http://rdp.cme.msu.edu) with a
183 minimum quality score of 20. Following initial trimming, sequences were denoised with Acacia
184 (Bragg et al., 2012) using a minimum quality score of 25. Mothur v1.35.1 (Schloss et al., 2009)
185 was used to further trim sequences against the SILVA v138 (Yilmaz et al., 2014) alignment
186 template to a length of 253 bp, precluster (diffs=1), and screen for chimeric sequences using the
187 chimera.vsearch command (Rognes et al., 2016). Unknown taxon, mitochondria, chloroplast,
188 archaea, and eukaryotic sequences were removed from analysis using SILVA v123 reference
189 taxonomy and the Wang classification method (Wang et al., 2007) with an 80% minimum
190 identity. Archaea made up < 1.2% of total sequences, and were therefore excluded from further
191 analysis. Sequences were clustered into operational taxonomic units (OTUs) based on a 97%
192 identity using the vsearch abundance-based greedy clustering (AGC) algorithm and assigned
193 consensus taxonomy using the classify.otu command in Mothur.

194 2.4 Bacterial diversity and taxonomy

195 Diversity metrics on OTUs including coverage, OTU numbers, Chao1, and Shannon diversity
196 were conducted with subsampled larvae and water sequencing reads (n=5,277) in Mothur using
197 the summary.single command. To reduce potential sequencing artifacts, OTUs with <1 sequence
198 in 1.0% of samples were removed and non-metric dimensional multidimensional scaling (nMDS)

was performed on Hellinger transformed OTU counts using Bray-Curtis resemblance matrices in the R-package Vegan (Oksanen et. al., 2019) to visualize differences between water and larvae community composition. Classifications of microbiomes and larval core microbiomes at the taxonomic class level were based on the mean relative abundance of OTUs for each microbiome sample type (larvae or water) or microbiomes within each hatchery (A, B, C, or D) using SILVA v123 reference taxonomy.

2.5 Larval core microbiome

Core OTUs were analyzed using the R package Phyloseq (McMurdie and Holmes, 2013). Sequencing reads prior to subsampling were used to prevent reduction in coverage of samples. The larval core microbiome was defined as the collection of OTUs present in at least 90% of the larvae samples being examined. A conservative 90% cutoff was selected to account for the possibility of errors associated with sampling or sequencing efforts.

2.6 Statistical analyses

Differences in Chao richness and Shannon diversity between the larval microbiomes and water microbiomes were tested using two-tailed paired t-tests. Differences relating to the main effects of larval development stage, hatchery, and spawn among the water and larval microbiomes were determined using a three factor ANOVA with post-hoc Tukey's HSD tests in R. Spearman rank correlation tests were used to determine whether Chao richness and Shannon diversity indices in larvae were correlated with water. Only samples that had both matching water and larval samples were used for this analysis (each n=18). Additional spearman rank correlation tests were conducted between Chao richness, Shannon diversity indices, and nutrients measured in the

water samples. PERMANOVA was performed on Bray-Curtis resemblance matrix derived from Hellinger transformed OTU counts to test for the effect of sample type on the combined larval and oyster microbiome using the adonis2 function and the strata function (strata=spawn). PERMDISP was conducted to determine whether multivariate dispersion had an effect on sample type. To reduce complexity of the model and further test the effects of hatchery, development stage, and spawn on larval and water microbiomes, additional PERMANOVAs and PERMDISP tests were conducted on separate larval and water microbiomes. Pairwise comparison tests between hatcheries were conducted using the pairwise.adonis2 function and p-values were corrected for FDR. PERMANOVA, pairwise tests, and PERMDISP analyses were conducted in the vegan R-package. DESeq2 (Love et al., 2014) using a Wald's significance test and local fit was performed on raw sequencing counts to test for differentially abundant OTUs between larval and water microbiomes. Pairwise comparisons were made using the contrast option in DESeq2, and Benjamini and Hochberg's p-adjusted values correcting for FDR were used to test for significance. Comparisons between the relative abundances of core larval OTUs found in the larval microbiomes and the water microbiomes were conducted using a one-tailed Wilcoxin signed rank test. All tests were based on a significance of $p < 0.05$ and error bars represent ± 1 standard error.

3. Results

3.1 Hatchery water parameters

NO_3^-, NH_4^+, and PO_4^{3-} were measured in each of the water samples for all larval development stages and spawning events (Table 1). In general, nutrients among the hatcheries, spawns, and

241 larval stages were highly variable. Concentrations of NO_3^- ranged from 0.43 μM at hatchery D
242 from spawn 2 during stage V to 154.64 μM at hatchery B from spawn 1 during stage PV. With
243 the exception of hatchery D water during spawn 2, concentrations of NO_3^- within each hatchery
244 were generally lowest at the D-stage, ranging from 4.42 μM at hatchery C from spawn 1 to 15.51
245 μM at hatchery B from spawn 1. Similar to NO_3^-, concentrations of NH_4^+ were highly variable
246 and ranged from 0.36 at hatchery A from spawn 1 during stage D to 12.56 μM at hatchery C
247 from spawn 2 during stage PV. The lowest NH_4^+ concentrations, however, were consistently
248 found at hatchery A, with an average concentration of 0.53 ± 0.20 μM. Levels of PO_4^{3-} ranged
249 from 0.26 μM at hatchery A from spawn 1 during stage D to 8.55 μM at hatchery C from spawn
250 2 during stage PV.

251 ## 3.2 Microbiome diversity comparisons among larvae and hatchery water

252 A total of 854,695 clean, trimmed bacterial 16S rRNA gene sequences were obtained from 19
253 oyster larval and 20 water samples. (SI Table 1) Average number of sequences was 21,195 ±
254 1,953 with an average coverage of 98.2 ± 0.2% (Table S1). Overall, richness was significantly
255 higher in the water microbiomes than the larval microbiomes (paired t-test, $t = 2.312$, $p = 0.034$),
256 while no significant difference was detected in diversity (paired t-test, $t = -1.19$, $p = 0.251$).
257 Testing for main effects only (stage, hatchery, and spawn) in the larval samples, larval
258 development stage had a significant effect on richness ($F_{2,18} = 4.272$, $p = 0.0306$), with D-stage
259 being significantly higher in richness than PV-stage (Tukey's $p = 0.0263$) (Fig. 1). No
260 developmental stage effect was found in the water samples. No significant effect of spawning
261 events was detected in either the larval or water samples. To test whether richness or diversity in
262 larvae stages was correlated with richness or diversity in water samples, Spearman rank tests

were performed on Chao and Shannon indices for each larval stage and the corresponding water sample. The rank tests showed no significant positive correlations between larvae and water microbiomes for either Chao or Shannon indices (SI Table 2). Spearman rank tests were also conducted between Chao and Shannon indices and nutrient measurements in the water and larvae microbiomes to determine whether nutrients correlated with richness and diversity of water microbiomes (Table 2). For the larval microbiomes, no significant correlations were found between nutrients and Chao or Shannon indices. For the water microbiomes, NO_3^-, NH_4^+, and PO_4^{3-} were significantly and negatively correlated with both richness and diversity.

3.3 Microbiome composition comparisons of larvae and water

Differences in β-diversity between sample types were visualized using nMDS and dispersion plots (Fig. 2) and analyzed using PERMANOVA and PERMDISP, respectively (SI Table 3). There was a clear and significant separation between the larval and oyster microbiomes based on sample type ($F_{1,39} = 3.913$, $p < 0.001$) with water showing significantly higher dispersion than larvae ($F_{1,39} = 10.679$), $p = 0.002$). To reduce complexity, larval and water microbiomes were analyzed separately for the effects of hatchery, development stage, and spawn on their respective communities. The largest, significant effect with the most variation explained in both microbiomes was due to hatchery origin with a clear separation between samples from different hatcheries (Fig. 3, SI Table 4 and 5). In the larval microbiome, the effect of developmental stage was also found to be significant ($F_{2,18} = 1.3236$, $p < 0.05$) and explained a moderate level of variation in the model ($R^2 = 0.1181$) with some separation seen between D/V- and PV-stages, but no separation seen between D- and V-stages in the nNMDS plot (SI Fig. 1A). Following FDR correction of pairwise comparisons, however, no significant differences between development

285 stages were detected. No similar separation or significance of water microbiomes were detected
286 as a function of the larval developmental stages (SI Table 5, SI Fig. 1B). In both the larval and
287 water microbiomes, the effect of the spawning events on microbiome β-diversity was significant,
288 although variation explained was low ($R^2 < 0.076$; SI Table 4A, 5A).

289 The overall compositions of both larval and water microbiomes comprised primarily
290 Alphaproteobacteria, Gammaproteobacteria, and Flavobacteriia, with Flavobacteriia having an
291 overall higher abundance in the water microbiomes and Gammaproteobacteria having an overall
292 higher relative abundance in the larval microbiome (Fig. 4). Combined, these classes represented
293 on average 58.2 ± 2.7% of total sequences for all larval and water microbiomes, regardless of
294 hatchery site. Among the larval microbiomes, the greatest differences in taxonomy at the class
295 level among sites were primarily due to a high relative abundance of Sphingobacteriia (family
296 *Saprospiraceae*) at hatchery A, Betaproteobacteria (family *Methylophilacea*e) at hatchery B,
297 Mollicutes (*family Mycoplasma*) at hatchery C, and Bacilli (family *Lactobacillus*) and
298 Planctomycetacia (family *Planctomycetaceae*) at hatchery D. In the water microbiomes, most of
299 the differences among hatchery sites were a result of few lower relative abundance classes that
300 varied among the hatcheries, including Betaproteobacteria (families *Methylophilaceae* amd
301 *Comamonadaceae*) at hatcheries B and D, Bacilli (Family XII) at hatchery C, and Actinobacteria
302 (family *Microbacteriaceae*) at hatcheries A and D.

303 Comparing the larval and water microbiomes using differential abundance testing, 5 OTUs were
304 found to be significantly higher in the oyster larval microbiomes than in the water microbiomes
305 (Table 3). Three of the OTUs, genera *Marinicella*, *Simiduia*, and an unclassified *Colwelliaceae*,

306 belonged to class Gammaproteobacteria. The other 2 OTUs, *Mycoplasma* and *Fluviicola*,
307 belonged to classes Mollicutes and Flavobacteriia, respectively.

308 3.4 Core microbiome identification in larvae

309 A total of 25 OTUs, designated as the core microbiome, were present in 90% of the larval
310 samples and made up 43.3 ± 3.8% of the larval microbiome (SI Table 6). The most abundant
311 OTUs making up the core microbiome (Fig 5) belonged to families *Rhodobacteraceae* (12
312 OTUs, 22.8 ± 2.4%), *Flavobacteriaceae* (4 OTUs, 9.5 ± 2.6%), *Alteromonadaceae* (2 OTUs, 2.5
313 ± 0.9%), *Marinicella* from an unclassified family of Gammaproteobacteria (1 OTU, 1.7 ± 0.5%),
314 *Erythrobacteraceae* (1 OTU, 2.0 ± 0.5%), and *Pseudomonadaceae* (1 OTU, 1.1 ± 0.8%). The
315 remaining 4 OTUs belonged to families *Phyllobacteriaceae*, *Oleiphilaceae*,
316 *Pseudoaltermonadaceae*, and *Cryomorphace*a and made up an average relative abundance of
317 <1%. Overall, the relative abundance of the core microbiome was higher in hatcheries A (52.9 ±
318 1.3%) and B (45.6 ± 1.7%) than in C (33.5 ± 0.6%) and D (39.1 ± 0.8%).

319 The 25 OTUs identified in the larval core were compared with the water microbiomes. In the
320 water microbiome, the larval core OTUs made up 30.2 ± 6.3% of the water microbiome, which
321 was significantly lower in relative abundance than that of the larval microbiome (z=-1.8073,
322 p=0.04). Core OTUs belonging to genera *Marinicella* and *Fluviicola* were identified as
323 differentially abundant between total larval microbiomes and water microbiome (Table 3).

324

4. Discussion

4.1 Effects of hatchery, spawn, and development stage on larval and water microbiomes

Of the three principal factors – hatchery, spawn, and development stage – examined in this study, hatchery was found to have the greatest effect on the composition and structure of the oyster larval and water microbiomes (Fig 3, SI Table 4 and 5). Variations among the hatcheries in this study included differences in location, water filtration methods, and feeding methods, which all contributed to the uniqueness of individual operations. While oyster larval and hatchery water microbiomes were both highly influenced by the effect of hatchery, there was a clear distinction between the oyster larvae and their corresponding water microbiomes (Fig 2, SI Table 3), indicating that various components of the hatchery operation may affect the oyster larvae or water microbiomes differently. For example, nutrients in water were significantly and negatively correlated with richness in the water microbiome, and had no correlation with the oyster larvae microbiome (Table 2), suggesting that different water treatment methods or other hatchery practices may have a significant impact on the water microbiome, but may not be as important to the composition of the oyster larvae microbiome.

Other oyster hatchery studies have also found distinctions between the oyster larvae microbiome and water microbiomes the larvae are raised in. Asmani et al. (2016) found a difference between *C. gigas* larval microbiomes and seawater microbiomes when examining larvae raised under different oyster rearing systems, including a flow-through and recycling system. Stevick et al. (2019) also reported *C. virginica* larval microbiome was significantly different from the

346 microbiomes of biofilm and water in a rearing tank. Both studies, like ours, suggest that the
347 oyster microbiomes are not merely reflections of the surrounding hatchery seawater, but may be
348 selectively colonized by distinct bacterial taxa. Furthermore, in our work, this distinction
349 between oyster associated bacteria at each of the hatcheries and bacteria in the seawater is further
350 evidenced by the lack of correlation between richness and diversity of the oyster larvae
351 microbiomes and their corresponding water microbiomes (SI Table2).

352 The effect of spawn season (June or July) was significant in the larval and water microbiomes,
353 however, its contribution to the overall variation in the community was low compared to the
354 hatchery effect (SI Table 4A, 5B). Environmental conditions such as temperature and substrate
355 availability, which exhibit clear seasonal trends, have been shown to strongly influence bacterial
356 communities (Shiah and Ducklow, 1994; Schultz et al., 2003), particularly those in the water
357 community. Furthermore, in the larvae samples for each of the spawning seasons different pairs
358 or groups of oysters were used to conduct the spawns, introducing a component of genetic
359 variation into the oyster larval microbiome. Genotypes have been shown to play a role in
360 shaping of oyster gill bacterial communities (Wegner et al., 2013), which may have further
361 contributed to variation due to spawning in the larvae microbiomes.

362 In both the larval microbiome sample, development stage as an oyster phenotype was found to
363 have a significant effect and explained a moderate level of variability (SI Fig 1A, SI Table 4A).
364 While nMDS showed a distinction between early D-stage and PV-stage, pairwise testing
365 revealed no significant difference in β-diversity between the stages. Further testing with larger
366 sample sizes may be necessary to identify significant compositional differences between the
367 larval stages. One measurable difference among the larval development stages, however, was an

368 overall decrease in species richness, with a significant difference between D-stage and PV-stage
369 (Fig 1). Chao richness indices for PV-stage larvae determined in this study (301.1 ± 31.9) were
370 consistent with those found in different post larvae *Crassostrea* oysters from Trabal Fernández et
371 al. (2014), suggesting that by the PV-stage, oyster larval richness stabilizes as it enters post larval
372 growth. This same decrease in species richness or effect of developmental stage on the
373 microbiome was not found in the water microbiomes, signifying a different selection pressure on
374 the oyster microbiome compared to the water microbiome. Early D-stage larvae may be more
375 likely to be rapidly colonized by several transient bacterial taxa from the surrounding water
376 column, while later PV-stage larvae microbiomes may prevent bacterial colonization and instead
377 host permanent, resident bacteria. Alternatively, bacteria that have a competitive advantage may
378 begin to outcompete and replace other bacteria by the PV-stage. Several bacteria species are
379 known to colonize surfaces and produce polymers, inhibitory compounds, and antimicrobials
380 that prevent competitors from succeeding (Bruhn et al., 2005; Rao et al., 2005; Xavier and
381 Foster, 2207).

382 ## 4.2 Larval core microbiome and its implications

383 Evidence of the establishment of a set of resident bacteria associated with oyster larvae is
384 demonstrated by examining the larval core microbiome. Of the 9,261 OTUs found in oyster
385 larvae, a total of 25 OTUs were present across 90% of the oyster larvae regardless of
386 development stage, hatchery, or spawning event (SI Table 6). While the number of larval core
387 OTUs was small, the combined core OTUs averaged more than 43% of the larval microbiomes
388 indicating a strong presence of a core microbiome in larval oysters. In comparison, adult oyster

389 gut core microbiomes have been shown to have a higher number of shared OTU, but represent a
390 lower percentage of the total microbiome (Pierce and Ward, 2019).

391 The majority (12 OTUs) and most abundant core OTUs belonged to family *Rhodobacteraceae*
392 from the class Alphaproteobacteria (Fig 5). Rhodobacter are rapid primary surface colonizers
393 (Dang et al., 2008) and have been shown to be abundant in phytoplankton cultures used in
394 bivalve larvae feed (Nicolas et al., 2004), and thus may explain the dominance of
395 *Rhodobacteraceae* as a dominant family in early core larval microbiomes. Some
396 *Rhodobacteraceae* bacteria, specifically *Phaeobacter* [*Roseobacter*] *gallaciensis,* have been
397 shown to benefit mollusc larvae (Ruiz-Ponte et al., 1999) and provide protection against
398 pathogens (Kesarcodi-Watson et al., 2012), while others have been shown to contribute to
399 diseases like Juvenile oyster disease (Boettcher et al., 2000). Additionally, *Phaeobacter* has been
400 shown to be a core genus present in the gut microbiomes of adult mussels and oysters across
401 seasons (Pierce and Ward, 2019), indicating a potential relationship with the bivalve hosts.

402 The second and third most abundant families shared by the larvae were *Flavobacteriaceae* from
403 class Flavobacteriia (4 OTUs) and *Alteromonadaceae* (2 OTUs) from class
404 Gammaproteobacteria. OTU #00010 from family *Flavobacteriaceae* most closely identified
405 with genus *Tenacibaculum*, which has been identified in juvenile and adult oysters (Fernandez-
406 Piquer et al., 2012; Lee et al., 2009; Trabal Fernández et al., 2014) as well as in other marine
407 animals and macroalgae (Suzuki et al., 2001; Heindl et al, 2008; Wang et al., 2008). Of the two
408 OTUs (OTU #00015) from family *Alteromonadaceae*, one closely identified with *Alteromonas*
409 *macleodii. A. macleodii* has been isolated from microalgal cultures in an aquaculture hatchery
410 (Schulze et al., 2006) and found in larval cultures of flat oysters (Farto et al., 2006). In mollusc

411 larvae, *A. macleodii* has been demonstrated to offer some protection against oyster larvae
412 pathogens *V. coralliilyticus* and *V. pectenicida* (Kesarcodi-Watson et al., 2012).

413 Of the OTUs identified in the larval core microbiome, only core OTUs from family
414 *Cryomorphaceae* and *Marinicella* from an unidentified Gammaproteobacteria family were found
415 to be differentially abundant from the water samples (Table 3). *Cryomorphaceae* has not been
416 previously linked to the oyster core microbiome, however, it has been found to be abundant in
417 algal cultures (Asmania et al., 2016). This may indicate that higher abundances of
418 *Cryomophaceae* in the oyster larval core may be due to algae during feeding in the later larval
419 stages of development. While still part of the core microbiome by definition, these bacteria may
420 be an important component of the oyster larval diet rather than permanent resident bacteria. In
421 comparison to *Cryomorphaceae*, *Marinicella* has been found to be part of the oyster core
422 microbiome in *Crassostrea sikameae*, remaining consistently present during probiotic treatments
423 and depuration (García Bernal et al., 2017). It is unclear as to the role of *Marinicella* in oyster
424 larvae, however, its higher abundance in oyster larvae compared to the surrounding water
425 suggest that it may be an important component of larval health or is preferentially consumed
426 during feeding. Overall, the presence of 25 core OTUs in successfully reared larvae provides the
427 first insight into the development of potentially important microbial indicators to evaluate and
428 predict the success of larvae rearing practices at hatcheries.

429 Oyster larvae showed a wide range of variation in their microbiomes primarily due to the
430 hatchery in which they were raised. These significant effects of hatchery on the larval
431 microbiome may have implications in the selection of hatchery operation and rearing methods.
432 However, our study was limited in the number of samples and our findings may not sufficiently

capture or represent the full range of variation present in oyster microbiomes at each hatchery, season or time point. In our study, hatcheries varied according to water treatment, feeding methods, and location. Isolation and testing of each of these different methods on larvae microbiome is necessary to identify which specific hatchery practices have the most impact on the oyster larval microbiome. While development stage was not determined to have a significant effect in our study, there were a few distinct differences between the larval stages of development including a significant decrease in species richness between the early and late stages of oyster development and greater variability in class composition than corresponding D-stage larval and water microbiomes. Together, our data suggests a shift towards a more selective larval microbiome as the oyster develops. The transition in microbiomes from D-stage to PV-stage may be a critical time period in larval development to ensure the oyster larvae are exposed to beneficial bacteria, including probiotics. Additionally, the 25 core OTUs identified here in successfully settled larval oysters provides insight into core microbiomes that may be essential to oyster growth and development. Future studies comparing larval core OTUs in this study to larval core OTUs from other geographical locations in the U.S. and Canada will help further determine the presence and potential importance of these OTUs in developing oysters. The investigation of changes that occur to the oyster larval microbiome as it develops as well as identification of the larval core provides an important step in unravelling the complexity associated with the oyster larval microbiome aid in the development diagnostic tools to monitor hatchery practices.

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References

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., and Nicolas, J-L., 2016. Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea Gigas*. Aquaculture. 464, 434–44. https://doi.org/10.1016/j.aquaculture.2016.07.020.

Boettcher, K.J., Barber, B.J., Singer, J.T., 2000. Additional evidence that juvenile oyster disease is caused by a member of the *Roseobacter* group and colonization of nonaffected animals by *Stappia stellulata*-like strains. Environ. Microbiol. 66(9), 3924-30. https://doi.org/10.1128/AEM.66.9.3924-3930.2000.

Bragg, L., Stong, G., Imelfort, M., Hugenholtz, P., Tyson, G.W., 2012. Fast, accurate error-correction of amplicon pyrosequences using Acacia. Nat. Methods. 9, 425-26. https://doi.org/10.1038/nmeth.1990.

Brown, C., 1973. The effects of some selected bacteria on embryos and larvae of the American oyster, *Crassostrea virginica*. J. Invertebr. Pathol. 21, 215-23. https://doi.org/10.1016/0022-2011(73)90206-1.

Bruhn, J.B., Nielsen, K.F., Hjelm, M., Hansen, M., Bresciania, J., Schulz, S., and Gram, L., 2005. Ecology, inhibitory activitym and morphogenesis of a marine antagonistic bacteium belonging to the *Roseobacter* clade. Environ. Microbiol. 71(11), 7263-70. https://doi.org/10.1128/AEM.71.11.7263-7270.2005.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyon, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of

480 sequences per sample. PNAS. 108, 4516. https://doi.org/10.1073/pnas.1000080107.

481 Castro, D., Pujalte, M.J., Garay, E., and Borrego, J.J., 2002. Vibrios isolated from the cultured
482 manila clam (*Ruditapes Phillippinarum*): numerical taxonomy and antibacterial activities. J.
483 Appl. Microbiol. 93, 438-447. https://doi.org/10.1046/j.1365-2672.2002.01709.x

484 Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro,
485 A., Kuske, C.R., Tiedje, J.T., 2014. Ribosomal database project: data and tools for high
486 throughput rRNA analysis. Nucleic Acids Res. 42, D633–42.
487 https://doi.org/10.1093/nar/gkt1244.

488 Dang, H., Li, T., Chen, M., Huang, G. 2008. Cross-ocean distribution of *Rhodobacterales*
489 bacteria as primary surface colonizers in temperate coastal marine waters. Appl. Environ.
490 Microbiol. 74(1), 52–60. https://doi.org/10.1128/AEM.01400-07.

491 Farto, R., Guisande, J.A., Armada, S.P., Prado, S., Nieto, T.P., 2006. An improved and rapid
492 biochemical identification of indigenous aerobic culturable bacteria associated with
493 Galician oyster production. J. Shellfish. Res. 25(3), 1059-65. https://doi.org/10.2983/0730-
494 8000(2006)25[1059:AIARBI]2.0.CO;2.

495 Fernandez-Piquer, J., Bowman, J.P., Ross, T., and Tamplin, M.L., 2012. Molecular analysis of
496 the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of
497 postharvest temperature on its structure. J. Appl. Microbiol. 112(6), 1134–43.
498 https://doi.org/10.1111/j.1365-2672.2012.05287.x.

499 García Bernal, M., Trabal Fernandez, N., Saucedo Lastra, P.E., Medina Marrero R., Mazon-

500 Suastegui, J.M., 2017. *Streptomyces* effect on the bacterial microbiota associated to
501 *Crassostrea sikamea* oyster. J. Appl. Microbiol. 122, 601-614.
502 http://doi.org/10.1111/jam.13382.

503 Garland, C.D., Nash, G.V., Summer, C.E., McMeekin, T.A., 1983. Bacterial pathogens of oyster
504 larvae (*Crassostrea gigas*) in a Tasmanian hatchery. Mar. Freshwater Res. 34, 483-87.
505 https://doi.org/10.1071/MF9830483.

506 Gomez-Gil, B., Roque, A., Turnball, J.F., 2000. The use and selection of probiotic bacteria for
507 use in the culture of larval aquatic organisms. Aquaculture 191, 259-270.
508 https://doi.org/10.1016/S0044-8486(00)00431-2.

509 Harris, J.M., 1993. The presense, nature, and role of gut microflora in aquatic invertebrates: a
510 synthesis. Microb. Ecol. 25, 195-231. https://doi.org/10.1007/BF00171889.

511 Heindl, H., Wiese, J., Imhoff, J.F. 2008. Tenacibaculum adriaticum sp. nov., from a bryozoan in
512 the Adriatic Sea. Int. J. Syst. Evol. Micr. 58, 542-47. https://doi.org/10.1099/ijs.0.65383-0.

513 His, E., Maurer, D., 1988. Shell growth and gross biochemical composition of oyster larvae
514 (*Crassostrea gigas*) in the field. Aquaculture 69, 185-94. https://doi.org/10.1016/0044-
515 8486(88)90195-0.

516 Hudson, K. (2019) Virginia shellfish aquaculture situation and outlook report: Results of the
517 2018 Virginia Shellfish Aquaculture Crop Reporting Survey May 2019 (VIMS Resource
518 Report No. 2019-8).

519 Kesarcodi-Watson, A., Miner, P., Nicolas, J.-L., and Robert, R., 2012. Protective effect of four

potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). Aquaculture 344-349, 29-34. https://doi.org/10.1016/j.aquaculture.2012.02.029.

Kniprath, E., 1981. Ontogeny of the molluscan shell field: a review. Zool. Scr. 10, 61-79. https://doi.org/10.1111/j.1463-6409.1981.tb00485.x.

Kueh, C.S., Chan, K.Y., 1985. Bacteria in bivalve shellfish with special reference to the oyster. J Appl. Bacteriol. 59, 41-47. https://doi.org/10.1111/j.1365-2672.1985.tb01773.x.

Lee, Y.S., Baik, K.S., Park, S.Y., Kim, E.M., Lee, D-H., Kahng, H-Y., Jeon, C.O., Jung, J.S., 2009. *Tenacibaculum crassostrea* sp. nov., isolated from the Pacific oyster, *Crassostrea gigas*. Int. J. Syst. Evol. Micr. 59, 1609-14. https://doi.org/10.1099/ijs.0.006866-0.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15(12), 550. https://doi.org/10.1186/s13059-014-0550-8.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Duilier, N., et al., 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. USA 110-3229–36. https://doi.org/10.1073/pnas.1218525110.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 8, e61217. https://doi.org/10.1371/journal.pone.0061217.

Miyazaki, Y., Nishida, T., Aoki, H., Samata, T., 2010. Expression of genes responsible for biomineralization of *Pinctada fucata* during development. Comp. Biochem. Phys. B. 155, 241-48. https://doi.org/10.1016/j.cbpb.2009.11.009.

Murchelano, R.A., Bishop, J.L., 1969. Bacteriological study of laboratory-reared juvenile American oysters (*Crassostrea virgnica*). J. Invertebr. Pathol. 14, 321-27. https://doi.org/10.1016/0022-2011(69)90158-X.

Nicolas, J.L., Corre, S., Gauthier, G., Robert, R., Ansquer, D., 1996. Bacterial problems associated with scallop *Pecten maximus* larval culture. Dis. Aquat. Org. 27, 67-76. https://doi.org/10.3354/dao027067.

Nicolas, J.L., Corre, S., Cochard, J.C., 2004. Bacterial population association with phytoplankton cultured in a bivalve hatchery. Microb. Ecol. 48(3), 400–13. https://doi.org/10.1007/s00248-003-2031-6.

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. Vegan: Community Ecology Package from https://cran.r-project.org.

Pierce, M.L., Ward, J.E., 2019. Gut microbiomes of the eastern oyster (Crassostrea virginica) and the blue mussel (Mytilus edulis): temporal variation and the influence of marine aggregate-associated microbial communities. mSphere 4:e00730-19. https://doi.org/10.1128/mSphere.00730-19.

Powell, S.M., Chapman, C.C., Bermudes, M., Tamplin, M.L., 2013. Dynamics of seawater

560 bacterial communities in a shellfish hatchery. Microb. Ecol. 66, 245-56.
561 https://doi.org/10.1007/s00248-013-0183-6.

562 Prado, S., Montes, J., Romalde, J.L., and Barja, J.L., 2009. Inhibitory activity of *Phaeobacter*
563 strains against aquaculture of pathogenic bacteria. Int. Microbiol. 12, 107-14.

564 Prado, S., Romalde, J.L., Barja, J.L., 2010. Review of probiotics for use in bivalve hatcheries.
565 Vet. Microbiol. 145, 187-97. https://doi.org/10.1016/j.vetmic.2010.08.021.

566 Rao, D., Webb, J.S., Kjelleberg, S., 2005. Competitive interaction in mixed-species biofilms
567 containing the marine bacterium *Pseudoalteromonas tunicata*. Appl. Enivron. Microb.
568 71(4), 1729-36. https://doi.org/10.1128/AEM.71.4.1729-1736.2005.

569 Riquelme, C., Toranzo, A.E., Barja, J.L., Vergara, N., and Araya, R., 1996. Association of
570 *Aeromonas hydrophila* and *Vibrio alginolyticus* with larval mortalities of Scallop
571 (*Argopecten purpuratus*). J. Invertebr. Pathol. 67, 213-28.
572 https://doi.org/10.1006/jipa.1996.0035.

573 Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K.,
574 Rawls, J.F., 2011. Evidence for a core gut microbiota in the zebrafish. ISME J. 5-1595–
575 1608. https://doi.org/10.1038/ismej.2011.38.

576 Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open
577 source tool for metagenomics. PeerJ. 4, e2584. https://doi.org/10.7717/peerj.2584.

578 Ruiz-Ponte, C., Samain, J.F., Sánchez, J.L., Nicolas, J.L., 1999. The benefit of *Roseabacter*
579 species on the survival of scallop larvae. Mar. Biotechnol. 1, 52-99.

https://doi.org/10.1007/PL00011751.

Sainz-Hernández, J.C., Maeda-Martinez, A.N., 2005. Sources of *Vibrio* bacteria in mollusc hatcheries and control methods: a case study. Aquac. Res. 36-1611-18. https://doi.org/10.1111/j.1365-2109.2005.01386.x.

Schauer, C., Thompson, C., Brune, A., 2014. Pyrotag sequencing of the gut micobiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS One. 9, e85861. https://doi.org/10.1371/journal.pone.0085861.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B, Lesniewski, R.A., et al., 2009. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Micob. 75, 7537–41. https://doi.org/10.1128/AEM.01541-09.

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., Perez, T., Rodrigo, A., Schupp, P., Vacelet, J., Webster, N., Hentschel, U., Taylor, M.W., 2012. Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. ISME J. 6, 564–576. https://doi.org/10.1038/ismej.2011.116.

Schultz, G.E., White, E.D., Ducklow, H.W., 2003. Bacterioplankton dynamics in the York River estuary: primary influence of temperature and freshwater inputs. Aquat. Microb. Ecol. 30, 135-48. https://doi.org/10.3354/ame030135.

Schulze, A.D., Alabi, A.O., Tattersall-Sheldrake, A.R., Miller, K.M., 2006. Bacterial diversity in

a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. Aquaculture 256, 50-73. https://doi.org/10.1016/j.aquaculture.2006.02.008.

Shiah, F-K., Ducklow, H.W., 1994. Temperature and substrate regulation of bacterial abundance, production and specific growth rate in Chesapeake Bay, USA. Mar. Ecol. Prog. Ser. 103, 297-308. https://doi.org/10.3354/meps104297.

Sohn, S., Lundgren, K.M., Tammi, K., Karim, M., Smolowitz, R., Nelson, D.R., Rowley, D.C., Gómez-Chiarri, M., 2016. Probioitic strains for disease management in hatchery larviculture of the eastern oyster *Crassostrea virginica*. J. Shellfish Res. 35, 307-317. https://doi.org/10.2983/035.035.0205.

Suzuki, M., Nakagawa, Y., Harayama, S., Yamamoto, S., 2001. Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bactria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amyloticum* sp. nov. Int. J. Syst. Evol. Micr. 51, 1639-52.

Stevick, R.J., Sohn, S., Modak, T.H., Nelson, D.R., Rowley, D.C., Tammi, K., Smolowitz, R., Markey Lundgren, K., Post, A.F., Gómez-Chiarri, M., 2019. Bacterial Community Dynamics in an Oyster Hatchery in Response to Probiotic Treatment. Front. Microbiol. https://doi.org/10.3389/fmicb.2019.01060.

Trabal, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Ascencio-Valle, F., Morales-Bojórquez, E., and Romero, J., 2012. Molecular analysis of bacterial microbiota associated with oysters

(*Crassostrea gigas* and *Crassostrea cortieziensis*) in different growth phases at two cultivation sites. Microb. Ecol. 64, 555-69. https://doi.org/10.1007/s00248-012-0039-5.

Trabal Fernández, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Ascencio-Valle, F., Romero, J., 2014. Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. FEMS Microbiol. Ecol. 88, 69–83. https://doi.org/10.1111/1574-6941.12270.

Wang, J-T., Chou, Y-J., Chou, J-H., Chen, CA., Chen, W-M., 2008. *Tenacibaculum aiptasiae* sp. nov., isolated from a sea anemone Aiptasia pulchella. Int. J. Syst. Evol. Micr. 58, 761-66. https://doi.org/10.1099/ijs.0.65437-0.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Micob. 73, 5261–67. https://doi.org/10.1128/AEM.00062-07.

Wegner, K.M., Volkenborn, N., Peter, H., Eiler, A., 2013. Disturbance induced decoupling between host genetics and composition of the associated microbiome. BMC Microbiol. 13, 252. https://doi.org/10.1186/1471-2180-13-252.

Xavier, J.B., Foster, K.R., 2007. Cooperation and conflict in microbial biofilms. Proc. Natl. Acad. Sci. USA. 104(3), 876-81. https://doi.org/10.1073/pnas.0607651104.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Ludwig, W., Pruesse, E., Quast, C., Schweer, T., Glo, F.O., 2014. The SILVA "and all-species living tree project (LTP)" taxonomic

640 frameworks. Nucleic Acids Res. 42, D643–48. https://doi.org/10.1093/nar/gkt1209.

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642 Figure legends

643 Figure 1. Chao richness and Shannon diversity in oyster larval and water microbiomes by
644 developmental stage. Mean Chao richness in (A) oyster larvae (B) and water and mean Shannon
645 diversity in (C) oyster larvae and (D) water for larval stages D-, V-, and PV (oyster, n = 18;
646 water, n = 18). Significance differences between larvae stages are denoted with different letters
647 ($p<0.5$). Error bars represent ± SE.

648 Figure 2. Beta-diversity among different sample types in the larval and water microbiomes. Non-
649 metric multidimensional scaling plots based on a Bray-Curtis resemblance matrix (A) and
650 dispersion plots (B) depicting β-diversity between sample types: larvae (n = 19) and water (n =
651 20). Ellipses represent ± SE and significance ($p < 0.05$) indicated by (*).

652 Figure 3. Beta-diversity among different hatcheries in the larval and water microbiomes. Non-
653 metric multidimensional scaling (nMDS) plot based on Bray-Curtis resemblance matrices
654 depicting differences in hatcheries between (A) larval and (B) water microbiomes. Ellipses
655 represent ± SE.

656 Figure 4. Taxonomic composition of oyster larvae and water microbiomes. Mean relative
657 abundances of bacterial classes by hatchery and sample type (oyster, n = 19; water, n = 20). Only
658 classes with > 1% relative abundance are shown.

659 Figure 5. Taxonomic composition of larval core microbiome. Mean relative abundances by
660 hatchery of bacterial families in the larval core microbiome (oyster, n = 19). Core microbiome is
661 defined as OTUs found in 90% of oyster larvae samples.

SI Figure 1. Beta-diversity among different developmental stages in the larval and water microbiomes. Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis resemblance matrices depicting differences in developmental stages between (A) larval and (B) water microbiomes. Ellipses represent ± SE.

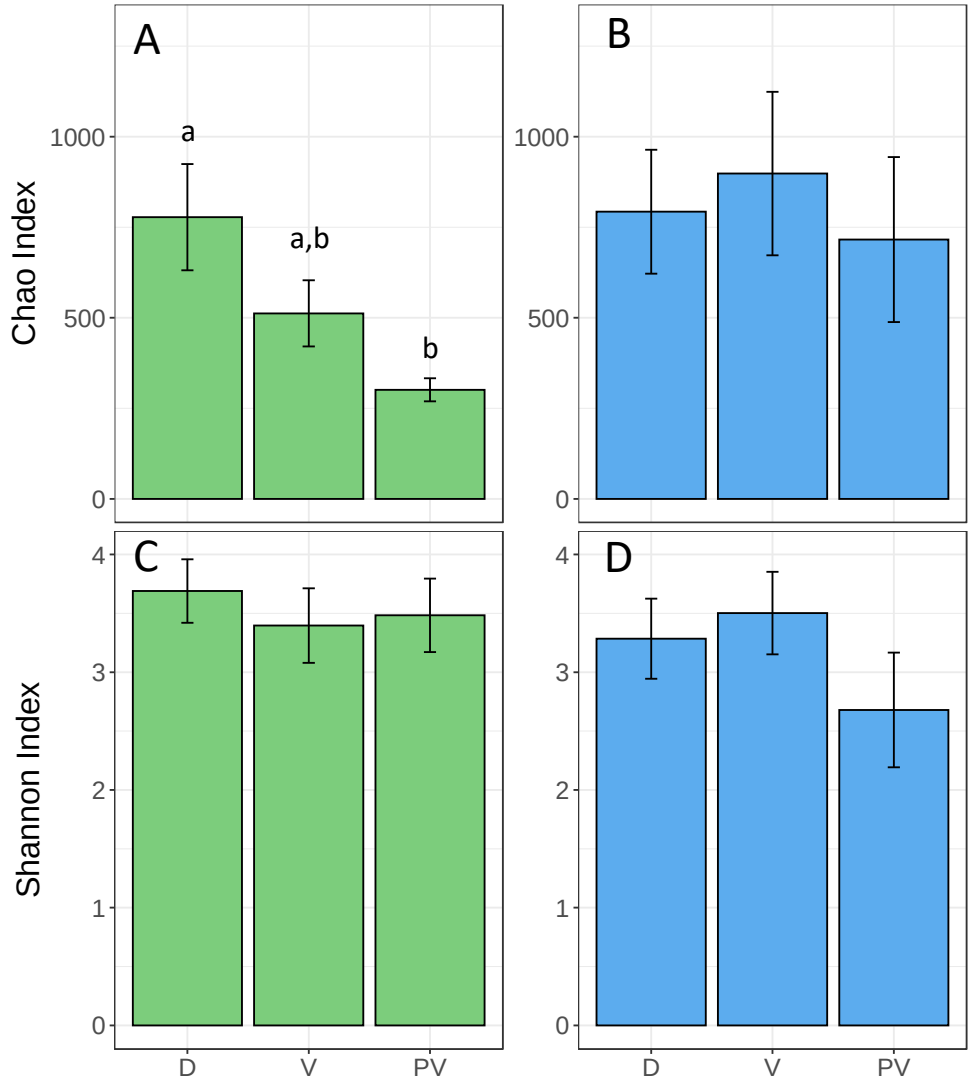
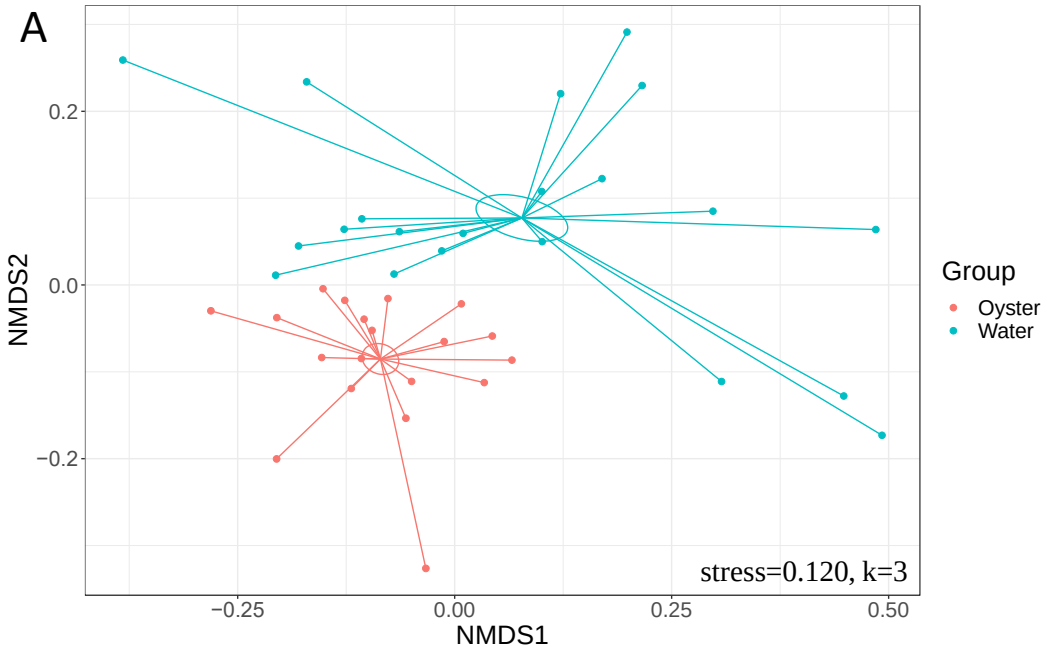


Figure 1. Chao richness and Shannon diversity in oyster larval and water microbiomes by developmental stage. Mean Chao richness in (A) oyster larvae (B) and water and mean Shannon diversity in (C) oyster larvae and (D) water for larval stages D-, V-, and PV (oyster, n = 18; water, n =18). Significance differences between larvae stages are denoted with different letters ($p<0.5$). Error bars represent ± SE.



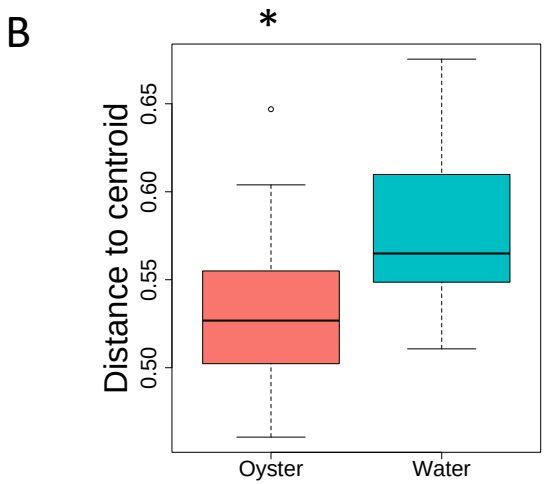


Figure 2. Beta-diversity among different sample types in the larval and water microbiomes. Non-metric multidimensional scaling plots based on a Bray-Curtis resemblance matrix (A) and dispersion plots (B) depicting β-diversity between sample types: larvae (n=19) and water (n=20). Ellipses represent ± SE and significance ($p < 0.05$) indicated by (*).

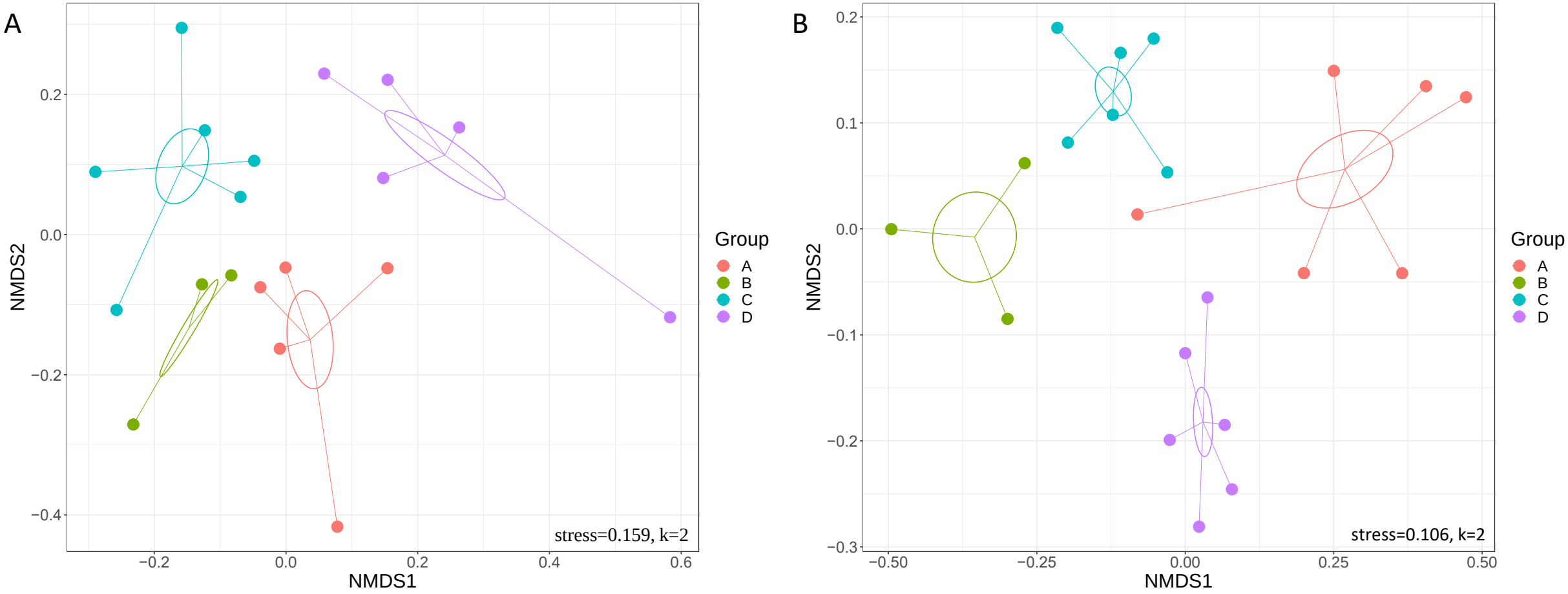


Figure 3. Beta-diversity among different hatcheries in the larval and water microbiomes. Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis resemblance matrices depicting differences in hatcheries between (A) larval and (B) water microbiomes**.** Ellipses represent ± SE.

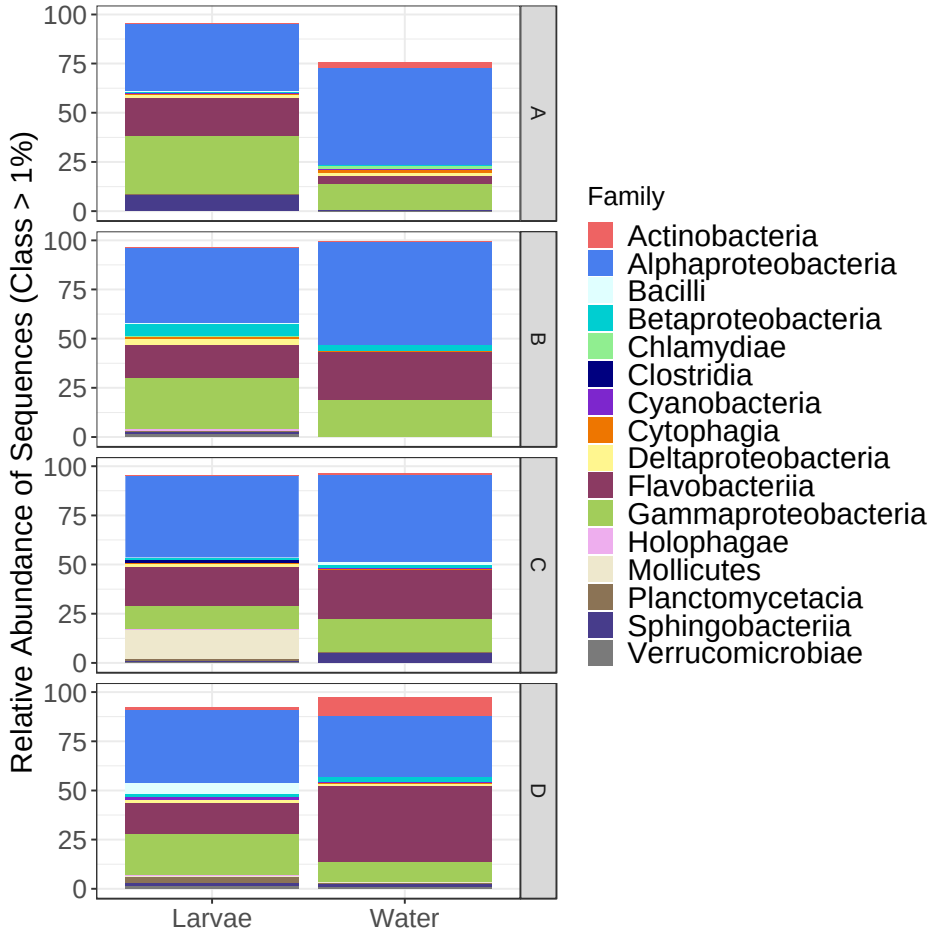


Figure 4. Taxonomic composition of oyster larvae and water microbiomes. Mean relative abundances of bacterial classes by hatchery and sample type (oyster, n =19; water, n =20). Only classes with > 1% relative abundance are shown.

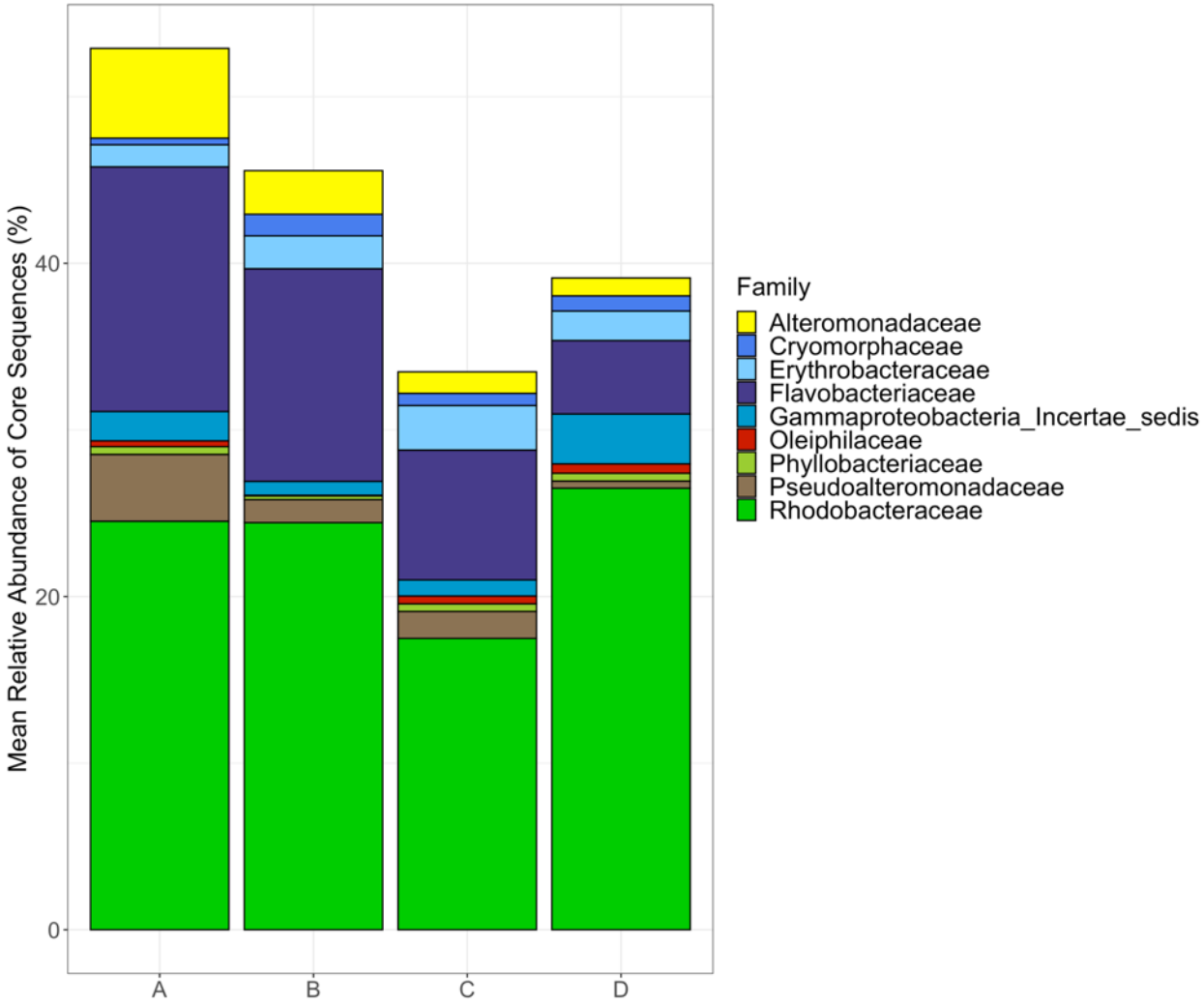


Figure 5. Taxonomic composition of larval core microbiome. Mean relative abundances by hatchery of bacterial families in the larval core microbiome (oyster, n = 19). Core microbiome is defined as OTUs found in 90% of oyster larvae samples.

Table 1. Nutrient parameters of hatchery water. Water samples were collected from each hatchery corresponding to different spawning events and larval development stages. Missing data points from sample collection are indicated by "*NA*".

Hatchery	Spawn	Stage	NO_3^- (µM)	NH_4^+ (µM)	PO_4^{3-} (µM)
A	1	D	5.43	0.36	1.16
		V	4.04	0.55	0.26
		PV	5.46	0.57	0.29
A	2	D	9.71	0.89	0.76
		V	20.75	0.48	1.14
		PV	11.90	0.31	0.72
B	1	D	15.51	0.68	0.32
		V	36.28	0.53	0.55
		PV	154.64	10.29	3.42
C	1	D	4.42	1.22	0.78
		V	12.59	2.02	1.96
		PV	17.11	7.74	3.06
C	2	D	*NA*	*NA*	*NA*
		V	48.18	2.70	6.10
		PV	63.58	12.56	8.55
D	1	D	14.52	11.71	0.72
		V	15.32	4.73	0.76
		PV	16.51	5.08	1.61
D	2	D	19.90	1.06	0.93
		V	0.43	0.69	0.18
		PV	0.45	2.21	0.29

Table 2. Correlation between nutrients and microbiome richness and diversity. Spearman rank correlations between nutrients and corresponding Chao richness and Shannon diversity in oyster larvae and water microbiomes. Significance is denoted in bold ($p<0.05$).

Sample Type	Nutrient	ρ (rho)	p-value	ρ (rho)	p-value
Oyster	NO_3^-	0.07	0.77	0.12	0.60
	NH_4^+	0.07	0.78	0.35	0.14
	PO_4^{3-}	-0.16	0.53	-0.09	0.72
Water	NO_3^-	-0.55	**0.02**	-0.24	0.34
	NH_4^+	-0.47	0.05	-0.46	0.06
	PO_4^{3-}	-0.49	**0.04**	-0.30	0.22

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Table 3. Differentially abundant OTUs between oyster larval microbiomes and water microbiomes. OTUs were identified from DESeq2 using Benjamini Hochberg's p-adjusted values corrected for FDR. Only OTUs that were found to be significant ($p<0.5$) are listed. OTUs indicated in bold are OTUs found in the core larval microbiome. Larvae and water values given are mean relative abundances of OTUs.

OTU	Phylum	Class	Order	Family	Genus	log2Fold Change	p-adusted value	Larvae*	Water*
Otu00009	*Tenericutes*	*Mollicutes*	*Mycoplasmatales*	*Mycoplasmataceae*	*Mycoplasma*	8.89	5.80E-05	4.92 ± 3.60	0
Otu00031	*Proteobacteria*	*Gammaproteobacteria*	Incertae_Sedis	Incertae_Sedis	*Marinicella*	2.87	2.80E-02	1.70 ± 0.48	0.07 ± 0.03
Otu00046	*Proteobacteria*	*Gammaproteobacteria*	*Cellvibrionales*	*Cellvibrionaceae*	*Simiduia*	5.90	2.80E-02	0.50 ± 0.25	0
Otu00060	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Cryomorphaceae*	*Fluviicola*	3.18	8.87E-03	0.76 ± 0.23	0.13 ± 0.07
Otu00086	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*	Unclassified *Colwelliaceae*	5.18	4.44E-02	0.42 ± 0.24	0

**Mean relative abundances < 0.01 are represented as 0*

Table 3. Differentially abundant OTUs between oyster larval microbiomes and water microbiomes. OTUs were identified from DESeq2 using Benjamini Hochberg's p-adjusted values corrected for FDR. Only OTUs that were found to be significant ($p<0.5$) are listed. OTUs indicated in bold are OTUs found in the core larval microbiome. Larvae and water values given are mean relative abundances of OTUs.

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Otu00031	*Proteobacteria*	*Gammaproteobacteria*	Order_Incertae_Sedis	Family_Incertae_Sedis	*Marinicella*	2.87	2.80E-02	1.70 ± 0.48	0.07 ± 0.03
Otu00046	*Proteobacteria*	*Gammaproteobacteria*	*Cellvibrionales*	*Cellvibrionaceae*	*Simiduia*	5.90	2.80E-02	0.50 ± 0.25	0
Otu00060	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Cryomorphaceae*	*Fluviicola*	3.18	8.87E-03	0.76 ± 0.23	0.13 ± 0.07
Otu00086	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*	Unclassified	5.18	4.44E-02	0.42 ± 0.24	0

*Mean relative abundances < 0.01 are represented as 0