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The Influence of Oyster Farming on Sediment Bacterial Communities

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Abstract Aquaculture currently provides half of all fish for human consumption, and this proportion is expected to increase to meet the growing global demand for protein. As aquaculture, including oyster farming, expands, it is increasingly important to understand effects on coastal ecosystems. The broad-scale ecological effects of oyster aquaculture are well documented; however, less is known regarding the influence of oyster aquaculture on sediment bacterial communities. To better understand this relationship, we compared three different oyster farming practices that varied in oyster biomass and proximity of oysters to the sediment. We used high-throughput sequencing and quantitative polymerase chain reaction to examine the effect of oyster farming on sediment bacterial communities. We examined the entire bacterial community and looked specifically at bacteria that support essential estuarine ecosystem services (denitrifiers), as well as bacteria that can be detrimental to human health (members of the *Vibrio* genus). We found that oyster biomass increased *Vibrio* richness and sediment carbon content, which influenced bacterial community composition. When compared to reference sites, the overall abundance of bacteria was increased by the bottom planting method, but the associated increases in denitrifiers and *Vibrio* were not significant. We were unable to detect *V. parahaemolyticus*, *V. vulnificus*, or *V. cholera*, the three most common *Vibrio* pathogens, in any sample, suggesting that oyster farming did not enhance these potential human pathogens in sediments at the time of sampling. These results highlight how differences in oyster farming practice can affect sediment bacterial communities, and the ecosystem services they provide.

Keywords Eastern oyster · *Crassostrea virginica* · Oyster aquaculture · Sediment bacterial communities · *Vibrio* · High throughput sequencing · 16S rRNA gene · *nirS* gene · Quantitative PCR

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Introduction

Globally, the aquaculture industry produced a record-setting 66.6 million metric tons of product in 2012 and provided nearly half of all fish for human consumption (FAO 2014). Mollusks represented a large portion of this industry, accounting for nearly 23% of global aquaculture biomass and 12% of global aquaculture revenue (FAO 2014). Since aquaculture is projected to continue to expand to meet the growing global demand for protein (World Bank 2013), it is critical to understand the role of aquaculture and mollusks in maintaining sustainable coastal ecosystems.

Oyster farming has a large influence on estuarine ecosystems (Coen et al. 2007). Oyster reefs provide a physical habitat in otherwise sandy-bottomed estuaries and can promote a diverse and abundant invertebrate community that serves as a

food source for juvenile fish (Peterson et al. 2003; Kellogg et al. 2013). Oyster reefs also provide a physical habitat for commercially important fish, thus having a dual market effect (Coen et al. 1999). Oysters filter water, which reduces suspended sediments and phytoplankton in the water column, increasing water clarity (Grizzle et al. 2006; Cerco and Noel 2007). Oyster water filtration also reduces nutrient loads by incorporating nutrients into oyster shells and biomass and by directing nutrients to the sediments through biodeposition (Newell et al. 2005; Higgins et al. 2011). Oyster biodeposits contain 2–3 times more carbon and nitrogen than do unaggregated particles (Jordan 1987); thus, oysters also alter sediment chemistry.

Oyster farming has the potential to influence sediment oxygen, nitrogen, and carbon availability (Newell et al. 2005), which can affect sediment bacterial communities and their function (Joye and Anderson 2008). Microorganisms are the main drivers of Earth's biogeochemical cycles (Falkowski et al. 2008); thus, changes in their community structure and function can influence the critical ecosystem services they provide. The influence of oyster farming on sediment chemistry may be magnified by practices that require higher oyster densities, as these farming practices are likely to have a larger effect on sediment chemistry. Therefore, the extent to which different oyster farming practices alter the benthic environment, the bacteria that inhabit benthic sediments, and the ecosystem services provided by those bacteria requires further study.

Oyster reefs can affect estuarine ecosystem services by enhancing bacterially mediated denitrification (Piehler and Smyth 2011; Kellogg et al. 2013; Smyth et al. 2013; Caffrey et al. 2016). Denitrifying bacteria convert bioavailable nitrogen to a gaseous form, ultimately allowing it to leave the system. This ecosystem service is critically important as coastal nitrogen loads are increasing, presenting serious threats worldwide (Howarth 2008). While there is clear evidence linking oyster reefs to increased potential denitrification rates (Piehler and Smyth 2011; Kellogg et al. 2013; Hoellein and Zarnoch 2014; Hoellein et al. 2015), few studies have measured how oysters alter the bacterial denitrifier community and these studies primarily focus on restored and natural reefs (Lindemann et al. 2016). Denitrification can be limited by the availability of organic carbon, nitrate, and favorable redox conditions, all of which are predicted to change with increasing oyster biodeposits (Hoellein and Zarnoch 2014). Therefore, it is important to understand how the denitrifying bacterial community changes under high oyster densities, such as those associated with oyster aquaculture.

Biodeposits associated with oyster farming may enhance denitrifying bacterial communities but could do so at a cost. Oyster farming, much like conventional agricultural systems, can be considered a monoculture, and monoculture crops can promote disease outbreaks. In oyster systems, outbreaks of parasitic microorganisms such as *Perkinsus marinus* and *Haplosporidium nelson* can disrupt oyster health by causing diseases such as Dermo and MSX (Mackin and Collier 1950; Haskin et al. 1966). Oyster farming could also promote the prevalence of human pathogens including some members of the *Vibrio* genus (Austin 2010), and it is possible that these disease agents might increase with increases in oyster stocking density. While the majority of *Vibrio* spp. are non-pathogenic (Thompson et al. 2004a, b), potentially pathogenic species, such as *V. parahaemolyticus*, *V. vulnificus*, and *V. cholera*, are a growing concern for the oyster industry as the number of seafood-associated illnesses attributed to consumption of raw or undercooked mollusks is currently on the rise (Iwamoto et al. 2010). The incidence of vibriosis, illness caused by bacteria from the *Vibrio* genus, has increased in the USA over the past decade (Newton et al. 2012), with an estimated 84,000 people suffering from some form of vibriosis annually (Scallan et al. 2011). The most common symptoms of vibriosis are gastroenteritis, diarrhea, and vomiting, but in certain cases, *Vibrio* infections can lead to septicemia and death. Given the gravity of *Vibrio* infections, and the potential for filter feeding to concentrate bacterial loads, an improved understanding of how different oyster farming practices facilitate potentially pathogenic *Vibrio* strains is critically important when considering the expansion of this industry.

In this study, we characterized changes in the sediment bacterial community associated with oyster farming, paying particular attention to denitrifying and *Vibrio* populations. We hypothesized that differences in oyster stocking density and whether the oysters were in direct contact with the sediment could alter the oyster-sediment-bacteria relationship. To test this hypothesis, we collected sediment from two different active oyster farms that use three different farming practices within Duxbury Bay, Massachusetts, and compared them to sediment from areas without oyster farming. We hypothesized that oyster biodeposits would alter the bacterial community by increasing both denitrifiers and *Vibrio* spp., and that these effects would be stronger in farming practices with higher oyster stocking density and where oysters directly interact with the sediment.

Methods

Study Area

We collected sediment samples from Duxbury Bay, Duxbury, MA (42°00′N 70°39′W) in June and July 2014. Duxbury Bay is a shallow system with an average water depth of 3 m at high tide and several exposed mudflats at low tide. The bay is approximately 5 km long and 3 km wide. The system exchanges 70% of its water volume twice daily due to tidal fluctuations of approximately 3 m (Lawson et al. 2011).

Duxbury Bay is the largest producer of oysters in the state of Massachusetts although only 1.3% of the bay's total area, 0.3 km^2, is used for aquaculture (Massachusetts Department of Marine Fisheries 2014). Oyster farming has long been established in Duxbury Bay, and there are currently 29 lease holders who primarily grow *C. virginica*. Given the history of oyster aquaculture and the variety of growth methods employed within Duxbury Bay, this is an ideal location for an observational study of the effects of oyster aquaculture on sediment bacterial communities.

We collected samples from within working oyster farms to assess the sediment bacterial community associated with different oyster farming practices. However, because we were working within active oyster farms, certain combinations of oyster age class and stocking density were unavailable for collection. Our sampling scheme therefore incorporates all age classes and stocking densities available from these farms in order to provide a dataset as robust as possible. To sample as many farming practices as possible, we collected samples from two different locations in Duxbury Bay (Fig. 1): DU1, an intertidal portion of the bay, and DU2, a subtidal portion of the bay.

The DU1 location included an oyster lease that used both the tray method and off-bottom growth method for culturing *C. virginica* (Fig. 1). The tray method is a high stocking density method, approximately 4800 oysters per m^2, where oysters are collected in trays and grown directly on the sediment surface. Off-bottom growth is an intermediate stocking density method, approximately 1500 oysters per m^2. In this system, large baskets of oysters are suspended in the water column approximately 0.3 m from the sediment surface and therefore have no direct contact with the benthos. At DU1, we sampled four sites: areas with 1-year-old oysters grown using the tray system, areas with 1-year-old oysters grown off-bottom, areas with 2-year-old oysters grown off-bottom, and a reference site with no oysters, which was located approximately 100 m from the farmed area (Table 1). These four sites encompassed all age classes of oysters grown at this location as well as a reference site with similar sediments, for comparison.

The subtidal DU2 location in the bay contained an oyster lease that used the bottom-planting method. Bottom-planting is a low stocking density method, approximately 250 oysters per m^2, where oysters are grown directly on the sediment surface. From this location, we collected sediment samples from areas with 2-year-old oysters, areas with 3-year-old oysters, and a reference site with no oysters, located approximately 600 m from the area where oysters were farmed (Table 1). This encompasses all sampling combinations available at this location.

Estimation of Oyster Biomass

Many factors varied across our sampling sites (oyster farming practice, oyster stocking density, and oyster age class); therefore, we decided to collapse two of these variables, oyster stocking density and age class, into a single variable, oyster biomass, to provide more direct comparisons between different farming practices. Since there were no direct biomass measurements available for oysters grown in Duxbury Bay, we used estimates from the literature. Oyster biomass can differ with food availability and quality, water temperature, and salinity (Shumway 1996); therefore, we opted to use only data collected from Massachusetts-cultivated oysters in our estimation. Based on data from oysters farmed in Massachusetts (Shaw 1963), we identified the following

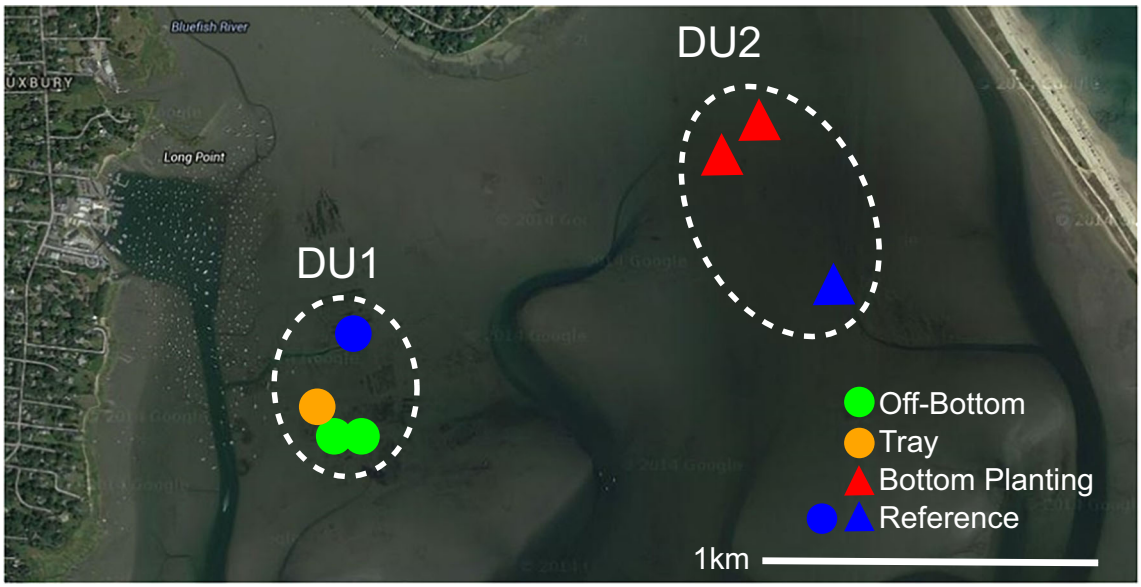

Fig. 1 Aerial image of sediment sampling locations in Duxbury Bay, MA (42°00′N 70°39′W). DU1 is intertidal, DU2 is subtidal. Reference sites have no oyster growth. Note the effects of oyster aquaculture on the benthic environment are visible at DU1

Table 1 Summary of farming method, oyster stocking density, oyster age class, and estimated oyster biomass for each sampling site

Location	Method	Density: Oysters/m^2	Oyster age class	Estimated oyster biomass (kg/m^2)
DU1	Tray	4800	1 year old	5.28
	Off-Bottom	1500	1 year old	1.65
	Off-Bottom	1500	2 year old	9.75
	Reference	0	–	0
DU2	Bottom Planting	250	2 year old	1.63
	Bottom Planting	250	3 year old	2.78
	Reference	0	–	0

relationship $B = 0.0372*SL^{1.99}$, where B is biomass in grams of oyster meat and SL is the shell length (dorsal to ventral measurement) in mm. We used a power function to describe this relationship since this function was previously defined for oyster biomass estimations (Mann et al. 2009; Southworth et al. 2010). We have confidence in this estimate as the scaling exponent falls within the previously defined range for oysters (Powell et al. 2015). We applied the identified shell height to biomass relationship to oyster shell height data from Shaw (1962) using measurements from oysters farmed in Mills Creek, West Chatham, Massachusetts, which included the three age classes of oyster observed in our current study. This allowed us to account for differences in oyster age class and to compare across different stocking densities using a single metric. Throughout, the phrase "oyster stocking density" is used when we consider the number of oysters in a location, whereas "oyster biomass" is used when the age and number of the oysters are both taken into account.

Sample Collection and Sediment Carbon and Nitrogen Content

At each sampling site, we collected five sediment cores using a 9.5-cm-diameter polyvinyl chloride sediment corer. To sample the top 1–2 cm of sediment, we used a 5 ml syringe with the tip cut off and took a series of four to five sediment plugs. We homogenized the sediment plugs from each core and aliquoted them into sterile cryovials for subsequent extraction of bacterial DNA. All DNA samples were frozen immediately using liquid nitrogen and transferred to −80 °C for storage. In addition to sediments for DNA analysis, we filled a 50-ml Falcon Tube with sediments from the top 1–2 cm of each of the abovementioned cores for analysis of sediment carbon and nitrogen content. These sediment samples were stored on ice until we were able to transfer them to −20 °C.

To analyze sediment carbon and nitrogen content, each 50 ml sediment sample was thawed and thoroughly homogenized. Approximately 10 ml of homogenized sediment was transferred to an acid-washed scintillation vial and dried for 3–14 days at 55 °C. We transferred 2–3 mg of dried sediment to a weighed tin capsule and measured the sediment carbon and nitrogen content on a Perkin Elmer 2400 Series II CHN Analyzer (Perkin Elmer, Waltham, MA, USA) following the manufacturer's instructions using acetanilide as an internal standard. Carbon and nitrogen content from different samples were then compared in two ways: using a Welch's *t* test to find significant differences in content among locations while accounting for unequal variance and using linear models to test for correlation to oyster biomass (R Core Team 2014).

Nucleic Acid Extraction and Sequencing

We used the MOBIO PowerSoil® DNA Isolation Kit on sediment samples following the manufacturer's instructions (MOBIO, Carlsbad, CA, USA). We confirmed the presence of DNA using an ethidium bromide-stained 0.8% agarose gel and amplified each DNA extract in triplicate using uniquely barcoded, bacterial specific primers. These primers targeted the V4 region of the 16S rRNA gene (515F (5′-GTGC CAGCMGCCGCGGTAA-3′) and 806R (5′-GGAC TACHVGGGTWTCTAAT-3′)) and contained adaptors for high-throughput Illumina sequencing (Caporaso et al. 2012). For each PCR reaction, we added the following: 10 μl 5-Prime Hot Master Mix (5 Prime®, Thermo Fisher Scientific, Waltham, MA, USA), 0.2 μM of each primer, 1 μl of template DNA (1:100 dilution), and 13.5 μl DEPC water. We used the Qiagen QIAquick® Gel Extraction Kit to purify all PCR products following the manufacturer's instructions (Qiagen, Valencia, CA, USA) and used a Qubit® 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) to quantify the purified PCR products. We combined the purified PCR products in equimolar amounts and used the KAPA Library Quantification Kit for Illumina Sequencing Platforms (Kapa Biosystems, Wilmington, MA, USA) to check the quantity of the pool. We loaded the samples on a V2 300 cycle kit for sequencing using an Illumina MiSeq (Illumina, San Diego, CA, USA).

Sequence Analysis

We used QIIME to perform all quality filtering and sequence analysis (Caporaso et al. 2010). We joined paired-end reads specifying at least 10 base pairs in overlap and retained sequences with a Phred score of 30 or higher (indicating 99.9% accuracy in base calling). We identified chimeric sequences using usearch61, an algorithm that performs both de novo and reference-based chimera detection, using the Genomes Online Database (GOLD) as a reference, and removed all identified chimeras (Edgar et al. 2011; Thomas et al. 2014). We assigned operational taxonomic units (OTUs) using uclust and a 97% sequence identity threshold (Edgar 2010) and assigned taxonomy using the Ribosomal Database Project (RDP) classifier

(Wang et al. 2007). We removed all OTUs identified as archaea, due to incomplete coverage of this group by the primers used. Thus, our study focuses exclusively, but comprehensively, on the bacterial domain. We also removed any sequences that were unassigned at the kingdom level, though we did not remove mitochondrial sequences since they accounted for less than 0.02% of all sequences. We rarified all samples to the lowest sequencing depth, 12,233 sequences per sample.

We used the weighted UniFrac metric to compare bacterial community composition among sites because it takes into account both abundance and phylogenetic similarity (Lozupone et al. 2011). We visualized this comparison using a principal coordinate analysis (PCoA) and used a permutational multivariate analysis of variance (PERMANOVA, Anderson 2001) to test for significant differences in the clustering of samples. We also used the primary axis of this PCoA as a proxy for bacterial community composition and evaluated potential drivers of community structure using structural equation modeling. We started by testing a full model of all hypothesized drivers using the piecewiseSEM package in R (Lefcheck 2015) and subsequently eliminated drivers for which there was little or no support ($p \geq 0.01$). We eliminated paths until we found a model where the modeled covariance matrix was not significantly different from the observed covariance matrix ($p = 0.258$) indicating good agreement between our model and the data. To establish support for our model, we allowed carbon and nitrogen to covary, as they do in natural systems. We followed this same technique to build models that included oyster biomass, oyster stocking density, and a six-level oyster biomass ranking metric that was not scaled (i.e., absent, low, medium-low, medium, medium-high, high), as the main driver of our model to determine how these different estimations might affect the outcome. All models yielded the same results in terms of supported paths, though the marginal sum of squares differed slightly. We present only our oyster biomass model as it contained the most information by including both oyster stocking density and age class.

Quantitative PCR

To quantify bacterial abundances in the sediment, we first quantified DNA extracts using a Qubit® 2.0 Fluorometer and normalized all extracts to 3 ng μl^{-1} to ensure similar amplification efficiencies from each sample regardless of DNA recovery from extraction. Next, we performed quantitative PCR (qPCR) in triplicate for each gene of interest on a Strategene Mx3005P (Agilent Technologies Inc., Santa Clara, CA, USA). All amplification products were checked for quality via gel electrophoresis, and all SYBR products (16S, *nirS*, and *Vibrio* genus) were checked for specificity by examining melt curves. For amplifications that passed quality checks, we compared sample Ct values to those obtained from samples that had known concentrations of the gene of interest (see below). We then used the calculated copy number, along with the sample dilution factor, to calculate the number of copies of each gene per gram of dry sediment. These copy numbers, normalized per gram of dry sediment, were then compared using a Welch's *t* test to account for unequal variance (R Core Team 2014).

With the exception of the multiplex qPCR, we created standards for each gene via purified and quantified PCR amplicons from environmental samples. We then serially diluted the purified product to create standards of known concentration. For the *Vibrio* multiplex standards, we generated standards as above, except that initial amplicons were generated from known isolates of each species of interest (*V. parahaemolyticus*: ATTC 17802, *V. vulnificus*: ATTC 27562, *V. cholera*: ATTC 39315).

To assess total bacterial abundance, we quantified the 16S rRNA gene. Each 25 μl reaction contained the following: 10 μl 5-Prime Hot Master Mix, 1.125 μl of 20 μM primers 357F and 519R, 1 μl of 25 mM $MgCl_2$, 1 μl of 25× SYBR Green (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), 0.3 μl of 1/500× ROX as reference dye (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), and 6 ng of template DNA (Biddle et al. 2008). Samples were amplified using the following conditions: 95 °C for 3 m, followed by 40 cycles of 95 °C for 20 s, 60 °C for 45 s, and 72 °C for 30 s. qPCR efficiency for this reaction ranged from 90 to 101%.

The *nirS* gene is often used as a proxy for the denitrifier community in estuarine systems because it represents the first step in the denitrification pathway where nitrogen is converted to a gaseous intermediate that can then leave the system (Zumft 1997). We quantified *nirS* following the methods of Jayakumar et al. (2009). For each 25 μl reaction, we added 10 μl 5-Prime Hot Master Mix, 0.3 μl of each 100 μM primer, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 25× SYBR Green, 0.3 μl of 1/500× ROX as reference dye, and 6 ng of template DNA. Samples were amplified using the following conditions: 95 °C for 3 m, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. *nirS* qPCR efficiency ranged from 90 to 95%.

We used two different methods to quantify *Vibrio* populations. First, to quantify bacteria from the *Vibrio* genus, we used 16S rRNA gene primers 567F and 680R that are specific to this genus (Thompson et al. 2004b). We used the following chemistry: 10 μl 5-Prime Hot Master Mix, 1 μl of each 10 μM primer, 1 μl of 25 mM $MgCl_2$, 0.25 μl of BSA, 1 μl of 25× SYBR Green, 0.3 μl of 1/500× ROX as reference dye, 6 ng of template DNA, and 8.45 μl of DEPC water (Mansergh and Zehr 2014). We amplified the samples using the following

conditions: 95 °C for 10 m, followed by 40 cycles of 95 °C for 15 s and 64 °C for 1 m. qPCR efficiency for this reaction ranged from 92 to 110%.

We also performed multiplex qPCR to quantify the abundance of the *tlh*, *vvhA*, and *ompW* genes from *V. parahaemolyticus*, *V. vulnificus*, and *V. cholera*, respectively. We adapted the TaqMan multiplex qPCR approach outlined in Garrido-Maestu et al. (2014), which simultaneously amplifies these three genes. We used 10 μl Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies Inc., Santa Clara, CA, USA) and 0.25 μl of each primer and probe in the following concentrations: *vvhA*F 10 μM, *vvhA*R 10 μM, *vvhA*P 10 μM, *tlh*F 7.5 μM, *tlh*R 7.5 μM, *tlh*P 10 μM, *ompW*F 7.5 μM, *ompW*R 7.5 μM, *ompW*P 15 μM, 0.3 μl of 1/500× ROX as reference dye; 6 ng of template DNA; and 5.96 μl of DEPC water. Samples were amplified using the following conditions: 95 °C for 3 m, followed by 40 cycles of 95°for 15 s and 58 °C for 30 s. qPCR efficiency for all three genes ranged from 83 to 90%. The limit of detection in the multiplex assay was *V. parahaemolyticus* = 39 copies, *V. vulnificus* = 83 copies, and *V. cholera* = 370 copies.

Oligotype Analysis

We used oligotype analysis to examine ecological patterns within the *Vibrio* genus. Oligotyping is a supervised bioinformatics tool that allows for discrimination between closely related sequences based on identifying information-rich nucleotide positions (Eren et al. 2013). We assigned taxonomy using the uclust classification tool and Greengenes 13.5 as a reference database and selected all sequences assigned to the family *Vibrionacea* and the genus *Vibrio* (DeSantis et al. 2006; Edgar 2010). After preparing the sequences by padding gaps at the 5′ end, we followed the oligotyping pipeline (Eren et al. 2013). We started with a single entropy component; decomposed the oligotypes; added an additional, manually chosen, component that best explained the remaining entropy across all oligotypes; and re-ran the analysis. We repeated this procedure until all oligotypes were decomposed. At each step, we removed any oligotypes that were present in fewer than five samples, did not represent at least 0.5% of a single sample, or whose most abundant unique sequence was present less than three times. Once the oligotypes were decomposed, we used the NCBI Basic Local Alignment Tool (BLAST) to find highly similar sequences using the non-redundant database. We compared the number of oligotypes recovered from no or low stocking density farming practices (reference and bottom planting) and high stocking density farming practices (tray and off-bottom) using a Welch's *t* test, and also tested for correlation between the richness of oligotypes and oyster biomass (R Core Team 2014).

Results

Sediment Carbon and Nitrogen Content

Average sediment carbon and nitrogen content for all sampling locations were 0.73 ± 0.30% (Fig. 2a) and 0.10 ± 0.03% (Fig. 2b), respectively, at the time of sampling. Carbon content was significantly higher in DU1 samples (intertidal, 0.90 ± 0.27%) compared to DU2 samples (subtidal, 0.51 ± 0.14%) when all samples were included in the analysis (Welch's *t* test, $t = 4.00$, $df = 7$, $p = 0.005$) and when comparing reference sites ($t = 3.22$, $df = 8$, $p = 0.01$). Therefore, we only compared oyster-associated sediments with their location-specific reference samples. Percent of carbon in sediments was significantly higher in all areas where oysters were

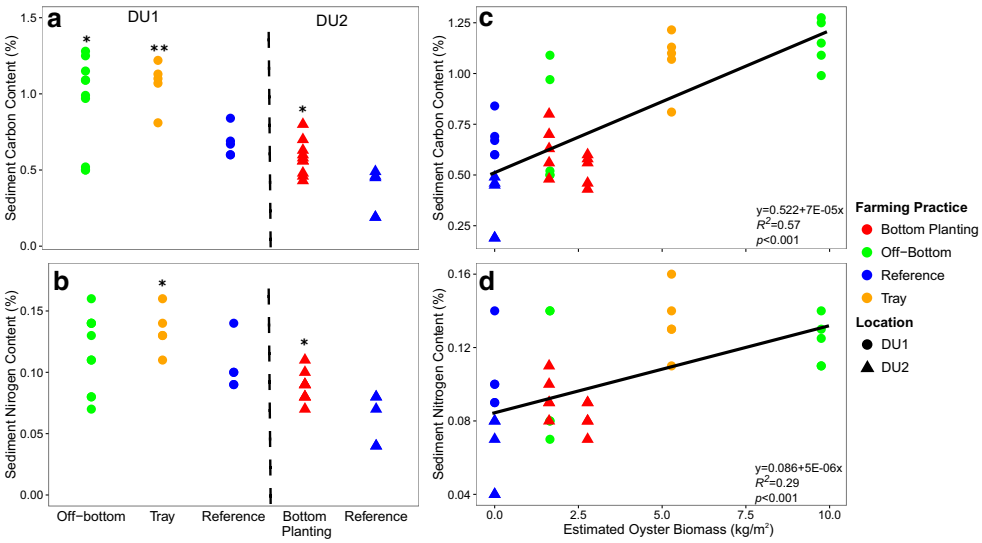

Fig. 2 Percent of carbon (**a**) and nitrogen (**b**) in sediment samples taken from oyster farms and reference sites in Duxbury Bay, and their relationship to estimated oyster biomass (**c**, **d**). Colors represent different oyster farming practices, and shapes represent different sampling locations. Samples from different farming practices were compared to their corresponding reference site based on sampling location using a Welch's *t* test, * $p < 0.05$; ** $p < 0.005$

grown (Fig. 2a, off-bottom: $t = 2.36$, $df = 12$, $p = 0.036$; tray: $t = 4.73$, $df = 7$, $p = 0.002$; bottom planting: $t = 2.92$, $df = 7$, $p = 0.025$), compared to their associated reference sites. Percent of nitrogen was significantly higher at two of the three oyster sampling sites (Fig. 2b, off-bottom: $t = 0.39$, $df = 12$, $p = 0.890$; tray: $t = 2.43$, $df = 7$, $p = 0.042$; bottom planting: $t = 2.64$, $df = 7$, $p = 0.044$).

Variation in carbon and nitrogen content can be explained, in part, by location within the bay (carbon ANOVA, $F = 25.41$, $R^2 = 0.42$, $p < 0.001$; nitrogen $F = 21.61$, $R^2 = 0.38$, $p < 0.001$). However, some of this variation can also be attributed to oyster biomass. Differences in oyster age class in the off-bottom- and bottom-planting farming practices resulted in variable oyster biomass (Table 1). Sediment carbon and nitrogen content were both positively correlated to oyster biomass (Fig. 2c, carbon, $F = 43.76$, $R^2 = 0.57$, $p < 0.001$ and Fig. 2d, nitrogen, $F = 13.19$, $R^2 = 0.29$, $p < 0.001$), suggesting that in addition to the influence of location within the bay, the number and age of oysters being farmed also likely influence sediment carbon and nitrogen content.

Bacterial Community Composition

We assessed how oyster farming practices influenced the sediment bacterial community by analyzing over 1.5 million 16S rRNA gene sequences clustered at 97% sequence identity. Initial analysis of bacterial community composition revealed that differences between samples were minor at broad taxonomic scales (Fig. S1), but that there were oyster-associated differences at higher taxonomic levels (Fig. S2 and Table S1). We used principal coordinate analysis (PCoA) of the weighted UniFrac metric to further analyze these differences and found a separation in community similarity along the primary axis (Fig. 3, PC1). This separation could result from two different explanations; differences in community composition could be associated with location within the bay (Fig. 3, circles vs. triangles) or could be explained by differences in farming practice and associated stocking density. Low stocking density bottom-planting samples (Fig. 3, red) clustered with reference sites from both sides of the bay (Fig. 3, blue), and intermediate and high stocking density practices, off-bottom and tray, formed their own cluster (Fig. 3, green and orange). PERMANOVA results indicate that both groupings are statistically significant, though clustering based on oyster stocking density explained more of the variance in the data than location (PERMANOVA, location: $F = 14.22$, $R^2 = 0.30$, $p = 0.001$; stocking density: $F = 18.13$, $R^2 = 0.35$, $p = 0.001$). In addition to the clear separation along the primary axis (Fig. 3 PC1), there was also separation along the secondary axis (PC2) where tray samples cluster separately. While this separation is statistically significant (PERMANOVA, $F = 2.94$, $R^2 = 0.08$, $p = 0.033$), this clustering explains far less of the variance in the data.

To determine how sediment variability and oyster farming practice influenced the bacterial community, we built a causal model predicting these relationships and tested it with structural equation modeling (Fig. 4). Our hypothesized model predicted that changes in the bacterial community (as approximated by PC1 in Fig. 3) were driven directly by changes in oyster biomass and indirectly by changes in oyster biomass that altered sediment carbon and nitrogen content (Fig. 4a). We found no support for a direct relationship between oyster biomass and bacterial community composition, though oyster biomass did drive both sediment carbon and nitrogen content (Fig. 4b). The model supported an indirect link between oyster biomass and bacterial community

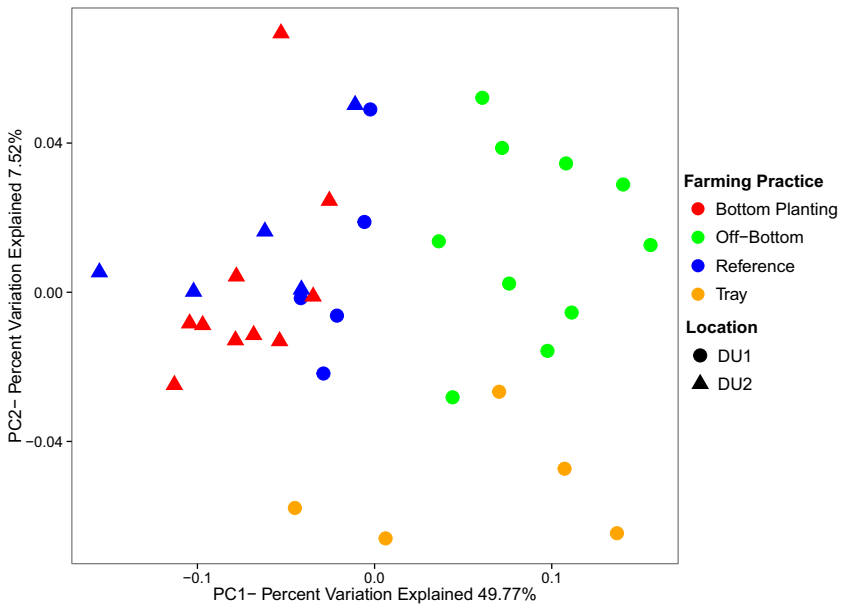

Fig. 3 Principal coordinates analysis of weighted UniFrac metric comparing bacterial community composition from sediment samples. Colors represent different oyster farming practices, and shapes represent different sampling locations

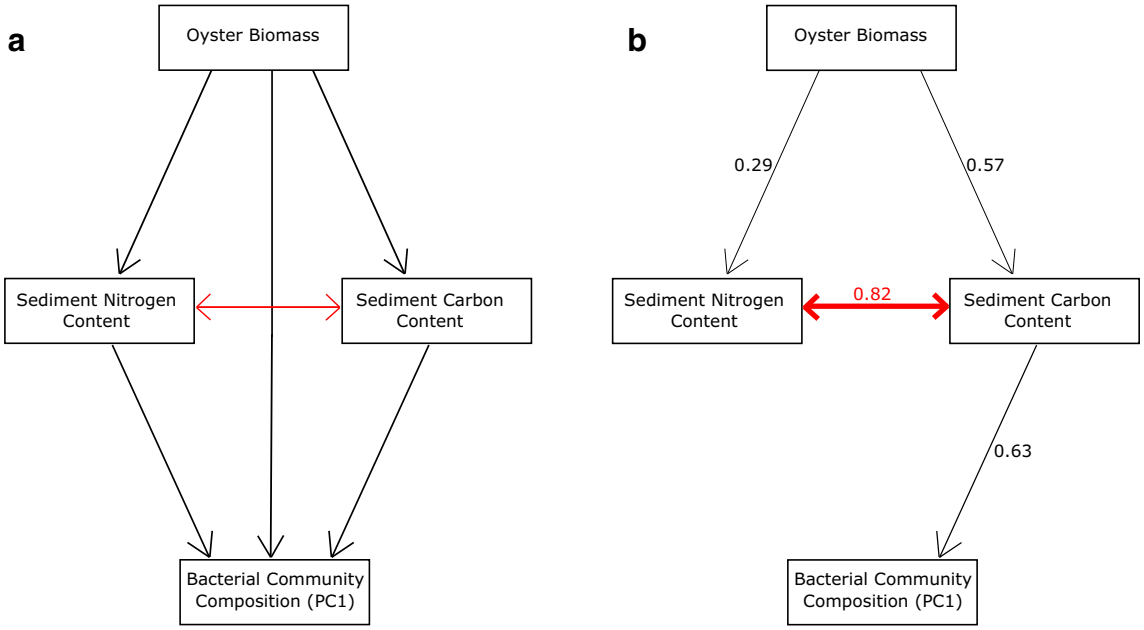

Fig. 4 Hypothesized (**a**) and supported (**b**) models explaining the drivers of sediment microbial community composition in Duxbury Bay. **a** Depicts all relationships tested and indicates their directionality. Black arrows are causal relationships; red arrows represent correlations. **b** Shows paths that were supported by structural equation modeling. The marginal sum of squares for each relationship is given alongside the supported path, and the relative support is indicated by line thickness

composition mediated through sediment carbon content, though there was no evidence of an indirect link through sediment nitrogen content.

Quantification of the Bacterial Community

Our quantification results showed a large amount of variation among samples from the same farming practice (Fig. 5). The total number of bacteria in bottom-planting samples was significantly higher than in reference sites (Fig. 5a, Welch's *t* test, bottom planting: $t = 4.16$, $df = 11$, $p = 0.002$), and although the mean abundance in the tray farming practice was also higher than in the associated reference locations, this difference was not significant (tray: $t = 1.85$, $df = 4$, $p = 0.139$). Mean bacterial abundance was lower in off-bottom samples than in reference samples, though again, this difference was not statistically significant due to large sample-to-sample variability (off-bottom: $t = -0.81$, $df = 11$, $p = 0.441$). There were no significant differences in the copy number of *nirS* genes recovered from any of the sites (Fig. 5b, off-bottom: $t = -0.60$, $df = 11$, $p = 0.563$; bottom planting: $t = 0.02$, $df = 5$, $p = 0.980$; tray: $t = 2.16$, $df = 4$, $p = 0.096$). These differences were also not significant when the number of *nirS* genes was normalized for 16S rRNA gene copy number (Table S2). There were a significantly greater number of bacteria from the *Vibrio* genus recovered from the tray farming practice compared to the relevant reference site (Fig. 5c, tray: $t = 2.62$, $df = 4$, $p = 0.050$), and although the mean abundance of *Vibrio* was also higher in the bottom planting site compared to the reference site, this difference was not significant (bottom planting: $t = 1.45$, $df = 9$, $p = 0.180$). The abundance of bacteria from the *Vibrio* genus was lower in off-bottom samples than in reference samples, though again, this difference was not statistically significant (off-bottom: $t = -0.48$, $df = 9$, $p = 0.640$). When the number of *Vibrio* was normalized for 16S rRNA gene copy number, differences between samples were not significant (Table S3). We tried quantifying three potentially pathogenic *Vibrio* species: *V. parahaemolyticus*, *V. vulnificus*, and *V. cholera*; however, none of these species were present above the limit of detection in any sample. The relationship between gene abundance and oyster biomass was not statistically significant for any of the genes measured (16S: $F = 1.34$, $R^2 = 0.01$, $p = 0.256$; *nirS*: $F = 3.98$, $R^2 = 0.08$, $p = 0.06$; *Vibrio*: $F = 2.07$, $R^2 = 0.03$, $p = 0.159$).

Oligotype Analysis

Given the importance of oyster-associated *Vibrio* populations to human health, we further characterized this portion of the bacterial community using oligotype analysis (Fig. 6). This allowed us to examine the within-genus diversity of *Vibrio* across our sites. Oligotype analysis revealed 15 different oligotypes within the *Vibrio* genus. The majority of the samples were dominated by Oligotype 0, with four samples composed entirely of this oligotype. Oligotype 0 is most likely related to *V. comitans* or *V. halioticoli* (Table 2) and constituted, on average, 63% of the *Vibrio* in all samples (Fig. 6). Oligotype 3 was the second most abundant oligotype,

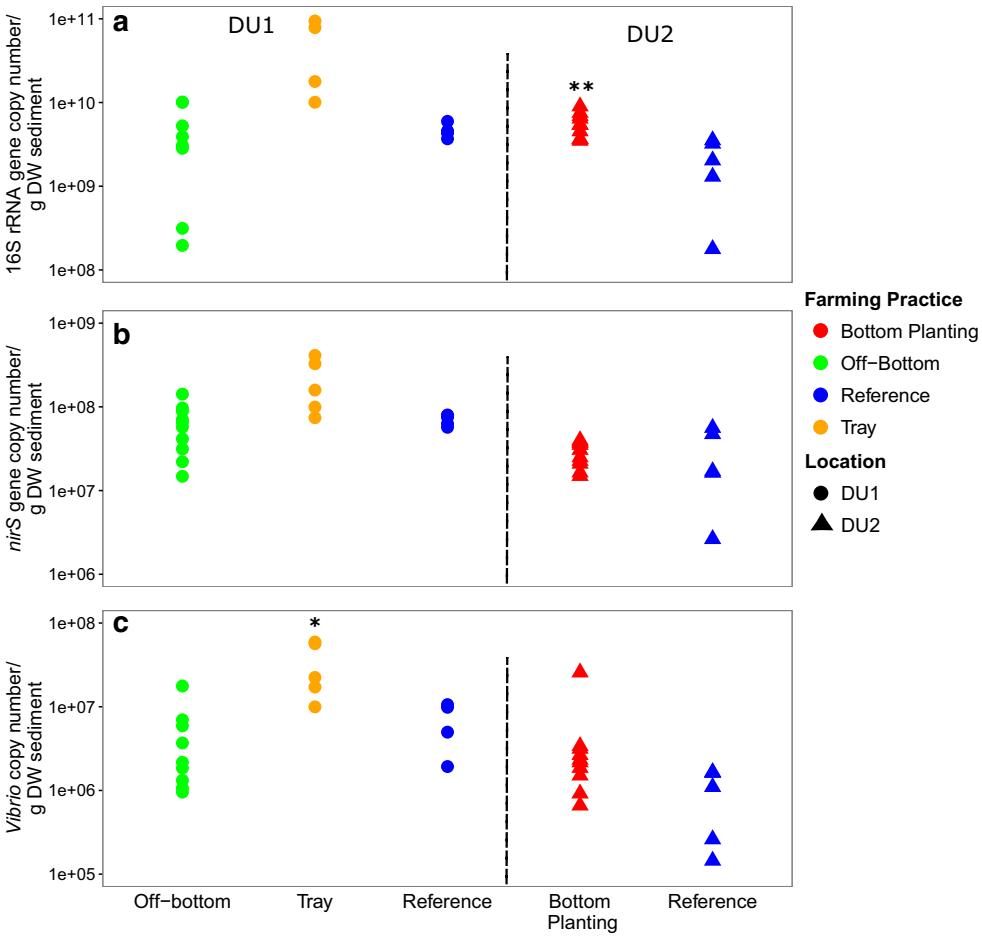

Fig. 5 Quantitative PCR results comparing the number of copies of 16S rRNA gene (**a**), *nirS* gene (**b**), and *Vibrio* spp. (**c**). All results are per 1 g dry weight sediment. Colors represent different oyster farming practices, and shapes represent different sampling locations. Samples were compared to their corresponding reference site based on sampling location using a Welch's *t* test, * $p < 0.05$; ** $p < 0.005$

constituting, on average, 9% of *Vibrio* across all samples and was the sole oligotype identified in one DU2 reference sample. Oligotypes 1, 2, 4, and 5 each constituted an average of 3–5% of the *Vibrio* recovered in this study, while the remaining oligotypes accounted for less than 2% each. There was a significant difference in the number of oligotypes recovered from different oyster stocking densities (Welch's *t* test, high stocking density vs. low stocking density: $t = 7.38$, $df = 19$, $p < 0.001$), and the richness of oligotypes was positively correlated to oyster biomass ($F = 21.88$, $R^2 = 0.39$, $p < 0.001$).

Discussion

We observed an increase in sediment carbon and nitrogen content at sites where oysters were present relative to adjacent reference sites with no oysters (Fig. 2a, b). It is possible that some of the variability in carbon content could be attributed to differences in carbonate content, but since these samples were collected from highly similar sediments that were only a few meters apart, we assumed carbonate levels to be relatively similar. More plausibly, the differences in carbon and nitrogen content were a result of oyster water filtration and biodeposition (Haven and Morales-Alamo 1966). Similar results have been reported in other studies (Piehler and Smyth 2011; Kellogg et al. 2013; Hoellein and Zarnoch 2014; Lindemann et al. 2016). Given that our sampling scheme included sites with different farming practices, we were able to build on past studies and connect the observed increases in sediment carbon and nitrogen content to changes in oyster biomass. Not surprisingly, sediment carbon and nitrogen content increased with increasing oyster biomass (Fig. 2c, d), suggesting that farming practices that promote high oyster biomass have a larger influence on sediment carbon and nitrogen content than do lower biomass practices.

We tested the relationship between oyster biomass and sediment carbon and nitrogen content using structural equation modeling. Our hypothesis that oyster biomass was a driver of sediment carbon and nitrogen content was well supported (Fig. 4b). This helps explain the high variance in sediment carbon and nitrogen content and different gene abundances observed in the off-bottom growth system (Figs. 2 and 5), as this system had two significantly different ranges of oyster biomass based on the different age classes present. Even though sediment carbon and nitrogen content both increased with increased oyster biomass, our model suggests that only

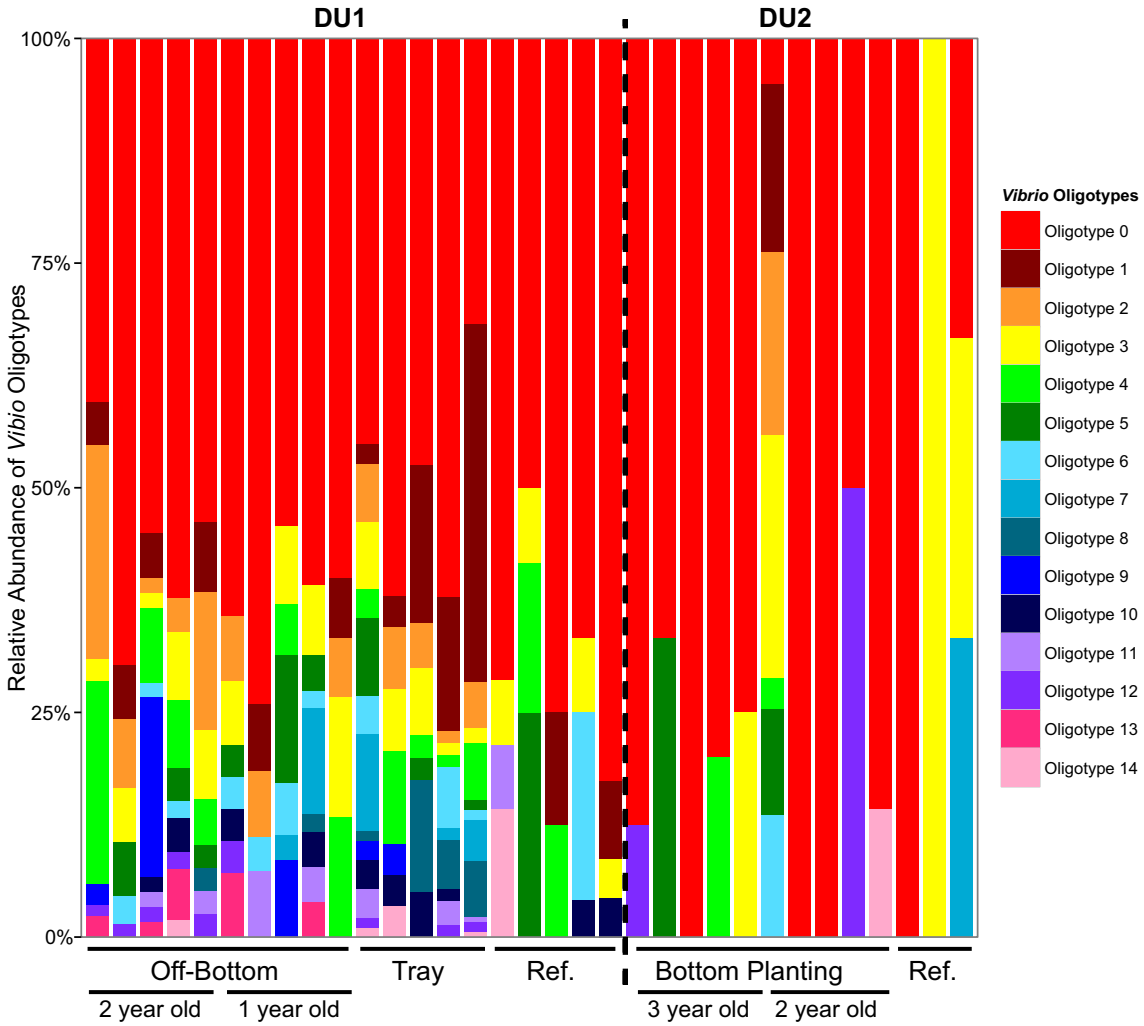

Fig. 6 Results of oligotyping analysis on the *Vibrio* genus. The relative abundance of each oligotype is shown for each sample. For farming practices where more than one age class of oyster was present (off-bottom and bottom planting), samples are grouped by age class with the oldest oysters appearing first

sediment carbon content was significant in explaining changes in sediment bacterial community composition (Fig. 4b). This suggests that either the changes in nitrogen content associated with increased oyster biomass were not significant enough to perturb the sediment bacterial community or that the bacterial community was resistant to these perturbations. Previous research has shown that sediment bacterial communities may be resistant to changes in nitrogen content (Bowen et al. 2011), although changes may be more apparent among denitrifying organisms (Bowen et al. 2014; Kearns et al. 2015).

Our structural equation model showed no support for a direct connection between changes in oyster biomass and changes in sediment bacterial community composition (Fig. 4b). This is contrary to other oyster-associated macroorganisms such as commercially important fish and invertebrates, which are often promoted in the presence of oyster reefs (Coen et al. 1999; Peterson et al. 2003), and differs from other microbial components of the benthic environment such as the microphytobenthos (Newell et al. 2002). It appears that the physical structure provided by oyster farming does not necessarily promote different populations of bacteria. Instead, the observed changes in the sediment bacterial community are primarily due to increases in sediment carbon content that occur with increased oyster biomass.

Although oyster biomass played an indirect role in structuring bacterial communities, the abundance of bacteria associated with different farming practices followed a different pattern (Fig. 5). Differences in bacterial abundance were not well correlated to oyster biomass. This could be attributed to heterogeneity in the number of RNA operons in bacterial taxa (Coenye and Vandamme 2003); however, we have no reason to believe this heterogeneity would differentially affect different sediment bacterial communities. Instead, the nature of the oyster-sediment interaction may be important in determining bacterial abundance. The bottom-planting method had

Table 2 Closest cultured representative for each oligotype based on NCBI BLAST results from the non-redundant database

Oligotype ID	% Identical	Number of BLAST hits	Top species hits
Oligotype 0	100	99	*Vibrio breoganii* (8)
			Vibrio comitans (13)
			Vibrio halioticoli (13)
Oligotype 1	100	2	Not defined at species level
Oligotype 2	100	99	*Vibrio alginolyticus* (5)[a]
			Vibrio brasiliensis (4)
			Vibrio campbellii (4)[b]
			Vibrio harveyi (6)[b]
			Vibrio parahaemolyticus (28)[a]
			Vibrio tubiashii (7)[b]
			Vibrio xuii (8)
Oligotype 3	100	1	Not defined at species level
Oligotype 4	100	42	*Vibrio cortegadensis* (4)
			Vibrio tapetis (14)
Oligotype 5	100	2	*Vibrio ponticus* (1)[b]
Oligotype 6	100	1	Not defined at species level
Oligotype 7	100	16	*Vibrio pectenicida* (3)[b]
Oligotype 8	99	77	*Vibrio litoralis* (14)
			Vibrio rumoiensis (15)
Oligotype 9	99	71	*Vibrio litoralis* (14)
			Vibrio rumoiensis (15)
Oligotype 10	99	100 (max searched)	*Vibrio breoganii* (9)
			Vibrio comitans (14)
			Vibrio ezurae (5)[b]
			Vibrio halioticoli (8)
			Vibrio inusitatus (4)
			Vibrio neonatus (4)
Oligotype 11	99	100 (max searched)	*Vibrio cortegadensis* (4)
			Vibrio mediterranei (15)
			Vibrio shilonii (10)[b]
			Vibrio tapetis (16)[b]
Oligotype 12	-	Not resolved	–
Oligotype 13	100	12	Not defined at species level
Oligotype 14	100	34	*Vibrio tapetis* (30)[b]

Numbers in parentheses indicate the number of hits associated with each species.

[a] Species that contain strains that are known human pathogens

[b] Species associated with diseases in aquatic organisms

significantly higher bacterial abundance compared to reference sites. Although not significant due to high variance, the tray system also tended to have higher bacterial abundance than reference sites. Both these farming practices involve oysters sitting directly on the sediment surface thereby more directly altering the sediment water interface, the structure of the sediment surface, and local hydrodynamics. Oysters in the bottom-planting and tray methods may promote bacterial abundance by increasing surface area and niche space, whereas in the off-bottom system, sediment-oyster interactions are minimized because oysters are suspended above the sediment surface and local currents buffer exchange between oysters and the sediments below. Previous studies have shown that the landscape context is important to oyster-mediated ecosystem services; oysters present in relatively homogeneous habitats such as mudflats showed increased potential denitrification rates compared to oysters present in complex habitat such as seagrass beds (Smyth et al. 2015). Off-bottom oysters may not be altering bacterial

abundance as they are not adding any additional heterogeneity to the sediment system, whereas bottom planting and tray oysters do increase sediment heterogeneity.

Previous research reported increased denitrification rates associated with oyster reefs (Piehler and Smyth 2011; Kellogg et al. 2013; Smyth et al. 2013). Therefore, we hypothesized there would be an increase in the number of denitrifiers, as approximated by *nirS* gene copy number, in oyster-associated sediment samples. Our data do not support this hypothesis, suggesting that denitrification rates may not be closely coupled to *nirS* gene copy number. This discrepancy has been seen in other sediment systems (Bowen et al. 2014). *nirS* and *nirK* are two functionally redundant genes that encode enzymes that reduce nitrite. In general, *nirS* is thought to be more abundant in marine systems (Bowen et al. 2014), but it is possible that including analysis of the *nirK* gene would improve the correlation between the abundance of denitrifiers and oyster biomass. In addition, quantification of *nirS* genes from DNA does not allow us to assess the level of gene expression (via mRNA), which may be a better predictor of denitrification activity in response to oyster cultivation.

Due to high recovery rates of potentially pathogenic *Vibrio* spp. from estuarine sediments (Johnson et al. 2012; Chase et al. 2015), sediments have been implicated as a reservoir for oyster-associated *Vibrio* infections (Cole et al. 2015). Our results suggest that oyster farming does not significantly enhance the abundance of *Vibrio* spp. in the sediment (Fig. 5c), or any of the *Vibrio* species most commonly associated with human illness (*V. parahaemolyticus*, *V. vulnificus*, and *V. cholera*). However, oyster farming did increase the number of *Vibrio* oligotypes recovered from the sediments (Fig. 6). Sediment samples from farming practices with high oyster stocking density had a significantly higher number of *Vibrio* oligotypes than samples from low oyster stocking density, and the number of oligotypes was positively correlated to oyster biomass. Despite this increase in oligotype richness, the oligotypes recovered in this study were largely not associated with pathogenic strains of *Vibrio*. Only Oligotype 2 was closely associated with potential human pathogens (Table 2). This oligotype was present in all sediments from high and intermediate oyster stocking density farming practices (average abundance 7.7%) but was found in only four out of 15 reference or low oyster stocking density samples (average abundance 1.8%). This suggests that high oyster stocking density may promote this potential pathogen; however, much more work is needed to confirm the pathogenicity of this oligotype.

Of the *Vibrio* sequences recovered in this study, the majority belonged to Oligotype 0 (average abundance 62.8%) and Oligotype 3 (average abundance 9.1%). Oligotype 0 was most similar to *V. comitans* or *V. halioticoli* (Table 2). These two closely related non-motile, fermentative bacteria are alginolytic, a trait that allows them to persist as abalone symbionts (Sawabe et al. 1998; Sawabe et al. 2007), though they have also been recovered from outside the host (Albakosh et al. 2015). The second most abundant *Vibrio* oligotype, Oligotype 3, did not share 100% sequence identity with any representatives in the GenBank database, although it did share 99% sequence identity with a number of *V. tapetis* isolates. *V. tapetis* is the causative agent of brown ring disease in clams (Borrego et al. 1996) and has been associated with disease in a number of other aquatic organisms.

Our observations of the sediment bacterial community associated with oyster farming are from a single time point. They only provide a snap-shot of this community and are not reflective of seasonal differences that might occur. However, other studies have shown that summer is generally peak timing for both the bacterial communities of interest: denitrifiers and *Vibrio*. Several studies measured potential denitrification rates associated with oyster reefs and report peak denitrification in the summer (Piehler and Smyth 2011; Kellogg et al. 2013; Smyth et al. 2013). Summer is therefore the best time of year to evaluate changes in denitrifiers since this is when they will have the most influence on estuarine biogeochemistry. Similarly, studies of sediment *Vibrio* from Washington, Louisiana, Mississippi, and Maryland reported peak *Vibrio* populations in the summer months (Johnson et al. 2012; Chase et al. 2015), which also coincides with increased incidence of vibriosis outbreaks (Newton et al. 2012). Analysis of seasonal changes in the bacterial community associated with different oyster farming practices would greatly add to our understanding of the dynamics of this system; however, this study, which encompasses peak seasonal activity of both communities, helps us begin to disentangle the role that oyster farming, and oyster biomass, plays in structuring sediment microbial communities.

In conclusion, we observed that oyster farming practice does differentially affect sediment carbon and nitrogen content and bacterial community composition. Sediment percent carbon and nitrogen were both positively correlated with oyster biomass, and increases in carbon altered the sediment bacterial community. High oyster biomass was also correlated with an increase in the number of largely non-pathogenic *Vibrio* oligotypes. The abundance of bacteria in the sediment was not significantly influenced by oyster biomass; instead, the proximity of oysters to the sediment appeared to influence bacterial abundance. Finally, we observed no significant increase in the abundance of *Vibrio* spp. associated with oyster farming and were unable to recover evidence of *V. parahaemolyticus*, *V. vulnificus*, or *V. cholera* in sediment samples. Changes in bacterial community composition and abundance associated with different oyster farming practices are important to consider as oyster aquaculture activities expand. As the aquaculture industry develops, oyster farmers and policy makers will need to establish best practices that facilitate farming success as well as the long-term sustainability of coastal waters, and oyster biomass should be considered in the decision-making process.

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