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Microbial interactions with oysters from the Great Bay Estuary: Characterization of endemic Vibrio choleraeand oyster metagenetics

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MICROBIAL INTERACTIONS WITH OYSTERS FROM THE GREAT BAY ESTUARY: CHARACTERIZATION OF ENDEMIC *Vibrio cholerae* AND OYSTER METAGENETICS

BY

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ABSTRACT

BACTERIAL ECOLOGY OF THE GREAT BAY ESTUARY: THE DISCOVERY OF ENDEMIC *Vibrio cholerae* AND DEVELOPING A METAGENETICS APPROACH TO EXPLORE THE OYSTER MICROBIOTA

By

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University of New Hampshire. December, 2010

Within the last two decades the number of gastroenteritis seafood-borne outbreaks due to *Vibrio* species has increased. These infections are usually caused by the consumption of raw or undercooked shellfish. I took two approaches to describe the ecology of Vibrios in the Great Bay Estuary, Durham, New Hampshire. First, I performed a phenotypic and multi-locus sequence analysis of 31 *V. cholerae* strains isolated from oysters, water and sediment in 2008 and 2009. I concluded that the *V. cholerae* population is endemic, diverse and highly recombinatory, but none of the isolates contain the virulence markers indicative of clinical strains. Next, I performed a metagenetic microbial census of 39 individual oysters and the overlying water from the Nannie Island and Oyster River oyster beds. I concluded that the microbial populations in the oyster are differential from the overlying water. Furthermore, the microbial populations structure in *V. parahaemolyticus* contaminated oysters contain unique signatures.

CHAPTER I

INTRODUCTION

Over at least the past thirty years, outbreaks of *Vibrio* infections have increased sharply, especially in industrialized nations where disease incidence typically stabilizes or diminishes. The consumption of raw shellfish, particularly oysters, is a common vector for gastroenteritis infections caused by Vibrios. Changes in dietary habits, changes in land and resource usage, and especially seasonal weather changes which are possibly indicative of global climate change have raised concerns about emerging pathogens in environments atypical for these outbreaks, including Northern coastal United States (DePaola *et al.*, 2000; McLaughlin *et al.*, 2005; Constantin de Magny *et al.*, 2009; Lipp *et al.*, 2003). These new public health trends challenge researchers to explore ecological dynamics that are driving these outbreaks of shellfish-borne *Vibrio* infections.

Little is known about natural populations of *Vibrio parahaemolyticus* and *Vibrio cholerae* in cold temperate regions where disease is rare but is recently emerging. Environmental Vibrios do not have equal potential to cause disease, and multiple biovars with different levels of virulence often coexist (Deepanjali *et al.*, 2005; DePaola *et al.*, 2000; Louis *et al.*, 2003). In many cases, infections caused by these organisms in temperate regions are simply attributed to travelers contracting the disease elsewhere, but other incidents are not as clear (MacRae *et al.*, 1983; McLaughlin *et al.*, 2005). In cases where the pathogens emerge from these temperate environments, it is not known in what

context of the natural microbial population these arise. For example, different strains or species, either non-pathogenic or pathogenic, could displace one other as environmental conditions influence changes in the local population structure. Another potential is gene flow between strains with different potentials to cause disease, and different potentials to survive in their particular environment, in which new hybrid strains can arise. Few studies have explored the dynamics of these pathogens and non-pathogens as they relate to each other and as they relate to the total microbial population in the environment.

Another question is how changes in environmental conditions affect the population dynamics of Vibrios, especially in northern environments which experience extremely cold winters and warm summers. Some studies have been performed that demonstrate increasing temperature of surface waters and other environmental conditions increase the total *V. cholerae* population (Constantin de Magny *et al.*, 2009; Lipp *et al.*, 2002), but have not yet explored how this dynamic influences interactions between different biovars of Vibrios including recombination. Recombination in natural populations is particularly important to understand because most *Vibrio* pathogenicity islands and toxins are transferred horizontally (Garg *et al.*, 2003). A particular concern in northern regions is that non-pathogenic but robust cold adapted strains could exchange genes with transient pathogenic strains that likely cannot persist in the cold environment.

The Great Bay Estuary (GBE) in New Hampshire is a unique location to study these dynamics because changes in environmental conditions will probably approach conditions similar to endemic outbreak regions (New England Regional Assessment, 2001; Sokolov et al., 2009). Incidences of these organisms that typically reside in warmer conditions have already been reported (O'Neill *et al.*, 1992), but little is known about

how these endemic strains interact with each other, and their surrounding microbial inhabitants. Due to extreme changes in environmental conditions over relatively small spatial scales, the GBE provides an excellent opportunity to study the dynamics of the microbial populations residing there. Additionally, different oyster beds in the GBE are influenced by different factors. The Oyster River (OR) oyster bed site is classified as prohibited for shellfish harvesting because of close proximity to a wastewater treatment facility, which causes low dissolved oxygen and high dissolver organic carbon levels (Fig 1.1). Comparably, the Nannie Island (NI) oyster bed is more distant from the facility and is thought to be less influenced by waste water effluent. NI is generally accepted as a cleaner site, so that recreational oyster harvesting is approved there (Fig. 1.1).

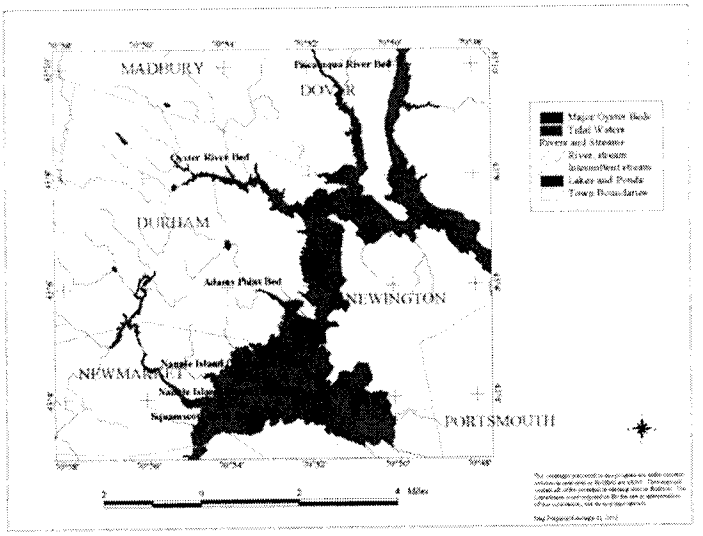

Figure 1.1: Great Bay Estuary Shellfish Classification Map. Courtesy of the NH Shellfish Program.

We took two approaches to study microbial populations associated with oysters in the GBE, one focused on a single human pathogenic species, and another focused on the mixed microbial populations associated with *Vibrio* free and *Vibrio* infested oysters. First, we will use a newly discovered endemic population of *V. cholerae* to 1) correlate

incidence and abundance with environmental conditions, 2) describe the structure of that particular population including strain relatedness to each other and with known toxigenic strains, and 3) explore any evidence of recombination between the local strains with a multi-locus sequence analysis (MLSA). In a parallel study to understand microbial populations in the GBE as they correlate and interact with a pathogen, *V. parahaemolyticus*, we will perform a microbial population survey by a 16s rDNA metagenetic approach. This survey will allow us to explore 1) differentiations between populations from various environments, including the OR or NI, and 2) identify host and water specific microbial community members. Understanding the dynamics of these populations, how they interact, how they differ, and how they are influenced by the surrounding environment will give insight into how Vibrios interact with the environment.

CHAPTER II

PHENOTYPIC AND MULTI-LOCUS SEQUENCE ANALYSIS OF *Vibrio cholerae* ISOLATED FROM THE GREAT BAY ESTUARY OF NEW HAMPSHIRE REVEALS GENETIC RECOMBINATION WITHIN THIS DIVERSE ENDEMIC POPULATION

INTRODUCTION

Vibrio cholerae, the causative agent of cholera, is responsible for deadly epidemics in developing nations (Albert *et al.*, 1993; Mekalanos *et al.*, 1997; Pascual *et al.*, 2002; Sack *et al.*, 2004). Untreated infections result in severe dehydrating diarrheal disease. Although only an average of five non-fatal incidents are reported every year in the United States (http://www.cdc.gov/cholera), fatal epidemics are ongoing in warm, subtropical climates, the most recent in Haiti which had been cholera-free for decades. Variation in disease incidence is caused in part by greater transmission between patients by fecal-contaminated drinking water supplies, a symptom of prevalent poverty in developing nations, but also by endemic populations of toxigenic cholera in warm subtropical environments (Constantin de Magny and Colwell, 2009; Colwell and Spira, 1992; Lipp *et al.*, 2002). Although *V. cholerae* is a ubiquitous waterborne bacterium comprised of over 200 serotypes, many of which can cause gastroenteritis and wound infections, only two serotypes constrained to warm waters, O1 (biotypes Classical and El Tor) and O139, are currently responsible for significant epidemic and pandemic cholera disease outbreaks (Albert *et al.*, 1993; Pascual *et al.*, 2002; Sack *et al.*, 2004).

Although strains of global epidemiological relevance are phylogenetically closely related to and derived from Asiatic strains, the emergence of new infective and/or toxigenic strains from genetically distinct endemic populations remains a concern. Endemic cholera has not been a problem in temperate regions in modern times (Lipp *et al.*, 2002; Sack *et al.*, 2004); however, given the predicted conditions associated with climate change of increasing estuarine temperatures and decreasing salinity from increased rainfall, endemic cholera may emerge as a threat in regions not previously thought at risk (Constantin de Magny and Colwell, 2009; Lipp *et al.*, 2002). Toxigenic O1 and O139 serotypes of *V. cholerae* are not regularly isolated from temperate waters in the United States, but some environmental non-O1/non-O139 populations associated with disease have been described (Colwell and Spira, 1992; MacRae 1983; Preheim 2010). Even so, the virulence potential of these endemic populations is not well understood. Despite considerable knowledge about the most clinically relevant strains, few studies have focused on resident environmental strains. An understanding of the potential reservoirs of pathogens provides a foundation for understanding how ecological factors influence their population dynamics and the potential for disease emergence.

Here we describe the genotypic and phenotypic characteristics of a newly identified population of non-O1 *V. cholerae* isolated from water, sediment, and live oysters from the Great Bay Estuary (GBE) in New Hampshire, a location where until now *V. cholerae* has not been isolated. Major rainfall events were the primary predictor for incidence, which also correlated more weakly with increased temperature and decreased salinity. Multi-locus sequence analysis identified members of clonal complexes from different samples and in consecutive years, demonstrating that the

population, although diverse, is established and stable. Isolates also displayed evidence of frequent recombination. This study describes the distribution and dynamics of a population of endemic *V. cholerae* in an estuary thought to be beyond the northern limits of the range of this species. These data now facilitate the monitoring and detection of invading toxigenic strains and further study of the evolution of this population in response to changing environmental conditions.

MATERIALS AND METHODS

Isolation, serotyping, and genotyping of *V. cholerae*. Water and oyster (*Crassostrea virginica*) samples were collected approximately every two weeks during May to December 2008 and 2009 from two sites in the GBE. The first oyster bed is located near Nannie Island and is within an area classified as approved for shellfish harvesting, and the second oyster bed is located in the Oyster River within an area classified as prohibited because of its proximity to the Durham, N.H. wastewater treatment facility effluent. Oysters were sampled using oyster tongs at similar locations within the same general area of the oyster beds. Sediments were collected with a van Veen grab sampler. The top 2 cm of sediment were scooped into sterile plastic bags. Water samples were collected on site by filling and then capping sterile 1 L plastic bottles at ~30 cm below the water surface. All oyster, sediment and water samples were immediately stored in coolers containing ice packs and brought back to the laboratory for analysis.

Shellfish were cleaned of debris and shucked using aseptic procedures. Tissue was quantitatively transferred to sterile beakers and weighed, then diluted 1:1, 1:2, 1:3 or 1:4, depending on the sample size and shellfish species, with alkaline peptone water (APW, pH 8.6, 1% NaCl) prior to homogenization for 90 s in a Warring blender. Sediment samples were homogenized by hand and 1.0 g wet weight was transferred to 9.0 ml of APW for further dilution. Water samples were shaken for 20 s. Following homogenization, 10, 1.0, 0.1 and/or 0.001 ml volumes of oyster homogenates and water samples were inoculated into multiple tube fermentation (MPN) analysis series with APW selective enrichment for 16 hours at 37°C. Turbid MPN tubes were streaked onto

thiosulfate-citrate-bile salts-sucrose (TCBS) (Massad and Oliver, 1987) and colistin-polymixin B-cellobiose (CPC+) (Warner and Oliver 2007) agar plates. Following incubation, a single representative isolate of each colony morphology type recognized putatively as *V. cholerae* from each environmental sample were re-streaked for isolation onto T-SOY agar. Individuals were further characterized as *V. cholerae* by molecular typing using multiplex PCR for the core genome virulence regulatory genes *toxR* and *tcpI,* as well as the virulence-associated genes used for strain typing *ompU* and *hlyA* (Panicker *et al.*, 2004). Amplicons were analyzed by gel electrophoresis and compared to positive and negative control strains reported in Panicker *et al.* Any isolates from which these genes were amplified were confirmed as *V. cholerae* by 16s V2-3 amplification and sequencing (Liu *et al.*, 2008). Additional accessory virulence genes whose products contribute to disease, including *zot*, *ace*, *tcpA*, and the major toxin encoding gene *ctxA*, were subsequently amplified following published protocols (Chow *et al.*, 2001; Huhulescu *et al.*, 2007; Singh *et al.*, 2001).

Isolates were serotyped using *V. cholerae* poly antiserum (BD Difco, Sparks, MD). Briefly, isolates were grown on LB agar plates and single colonies were transferred by a sterile loop to 1 drop (approximately 40 µl) of antiserum on a glass slide. Glass slides were rotated and observed for agglutination over 5 minutes, and compared to a serotype-01 positive control, *V. cholerae* C6707 (McCarthy and Khambaty, 1994)and a negative non-pandemic control strain (which one).

Analysis of abundance with environmental conditions. Salinity, water temperature, and dissolved oxygen were recorded by two YSI 6-series (Yellow Springs Inc., Yellow Springs, OH) multi-parameter datasondes located near the Oyster River and

Nannie Island oyster beds and managed by the Great Bay System Wide Monitoring Program (SWMP) (http://www.greatbay.org). A YSI 85 was used to measure water temperature, salinity, and dissolved oxygen on site at each sample event; these data were and compared with the sonde data to ensure consistent recording. Daily rainfall totals were also recorded and accessed via the University of New Hampshire Weather Station website (http://www.weather.unh.edu). Multi-variate stepwise regression analysis was used to determine environmental condition correlations with the total maximum MPN, maximum MPN in oyster, and maximum MPN in water for each site at each collection date with JMP version 8.01.1. Effects of environmental factors on *V. cholerae* abundance are reported as the squared partial correlation coefficient, as determined by the freely accessible Pcorr script (http://www.jmp.com/support/faq/jmp2081.shtml). Pearson correlations were performed between each single environmental condition and each MPN value with SPSS PASW Statistic version 18.0.

Phenotypic analysis. All phenotypic assays were performed using standard published protocols. Biochemical tests were performed as described (Choopun). Briefly, lysine and ornithine decarboxylase assays were performed using the Moeller decarboxylase base medium (Difco) with the addition of amino acids at 1% (wt/vol). The methyl red reaction was performed using MR-VP medium (Difco) and incubated at 37°C for 48 hrs, and the Vogues-Proskauer assay was performed on inoculated MR-VP medium at 37°C for 48 hrs. Growth on 0, 6 and 8% (wt/vol) NaCl in nutrient broth at 37°C was determined following seven days of incubation; positive results were confirmed by culture turbidity. Capacity for biofilm formation was measured for cultures grown for 24 hours in HI broth followed by crystal violet staining (Mahoney *et al.*, 2010).

The quantitative hemolysin assay (Mahoney *et al.*, 2010) was performed by incubating defibrinated sheep blood (Northeast Laboratory, Waterville, ME) with overnight cultures of cells conditioned for 24 hrs in HI. Blood cell lysis was assessed by absorbance of cell-free supernatants at 415nm using a Tecan Infinite M200 plate reader (Tecan). Antimicrobial susceptibility was determined by zones of inhibition, as follows. One hundred µl of culture grown in HI media to an OD_{600} of ~1, was spread onto HI agar plates. Two paper discs (BD, Franklin Lakes, NJ) were placed onto each plate and then saturated with antibiotics at the following concentrations: 10 µg gentamicin (Gen), 30 µg kanamycin (Kan), 30 µg chloramphenicol (Chl), 30 µg naladixic acid (Nal), or 30 µg tetramycin (Tet), each of which was replicated three times. The plates were incubated at 37°C for exactly 24 hrs and the maximum zone of inhibition diameter was measured in millimeters.

The collected phenotypic data were used to build a trait matrix, from which a phenogram was produced. Continuous phenotypic data (biofilm levels, hemolytic activity, and antimicrobial susceptibility to 5 antibiotics) were converted into categorical data (pairwise Sidak t-tests, $p < 0.05$), at two or three levels each. The methyl red levels were categorized by either a positive or negative result. The unordered multistate discrete-characters parsimony method (pars) algorithm in the PHYLIP package was utilized to build the phenogram (Felsenstein, 1989).

Phylogenetic characterization. Multi-locus sequence analysis (MLSA) was performed on DNA purified by a CTAB-NaCl precipitation followed by phenol-chloroform extraction (Ausubel *et al.*, 1990), on 5 loci including: *gapA*, *gyrB*, *pyrH*, *recA*, and *topA* using published primers and PCR conditions (Thompson *et al.*, 2005)

PCR was performed with MasterTaq PCR kit (Eppendorf, Hauppauge, NY), with 2.0 mM $MgCl_2$ and a 0.2 μM of each primer. PCR products were analyzed on a 1.2% agarose gel and the remainder of the reactions that yielded a single band of the proper size were treated by ExoSAP (USB, Fremont, CA), and sequenced at the DNA sequencing core facility at the Hubbard Center for Genome Studies at the University of New Hampshire (Durham, New Hampshire) using Applied Biosystems BigDye Terminator Cycle Sequencing Kits (v3.1) and analyzed with an ABI3130 DNA Analyzer.

All of the DNA analysis and phylogeny was completed with MEGA version 4.0 unless otherwise noted (Tamura *et al.*, 2007). Contigs were assembled from the consensus of both forward and reverse sequences of each locus for each strain. The assembled sequences of each locus were then aligned for all strains and the sequences further trimmed to include only overlapping DNA. The five loci were then concatenated, and the sequences were aligned using ClustalW. Phylogenetic trees were constructed by the neighbor-joining algorithm in MEGA version 4.0 with 1,000 bootstrap replacements and modeling substitution with the Jukes-Cantor method.

Additional trees were built and recombination was analyzed by SplitsTree version 4 utilizing the Phi test module (Huson and Bryant, 2006). A non-redundant database was created using NRDB Align (Jolley *et al.*, 2001) and a subsequent allelic profile was determined for the total collection. Using the allelic profile, recombination was analyzed by the LIAN 3.5 linkage analysis program (Haubold and Hudson, 2000). The PHYLIP phyML algorithm was used to build additional habitat trees (Felsenstein, 1989) and used for the adaptML software (Hunt *et al.*, 2008), in which isolates were labeled by location and date collected.

RESULTS

Rainfall is the major predictor of abundance of environmental *V. cholerae* in the Great Bay Estuary. Abundance of toxigenic *V. cholerae* typically correlates with warmer temperatures and prevalence usually associates with reduced salinity associated with rainfall (Palit and Batabyal, 2010: Constantin de Magny and Colwell, 2009). In some temperate regions where *V. cholerae* populations have previously been described, these two factors are significant predictors (Louis *et al.*, 2003; Kirschner *et al.*, 2008). However, whether these same factors influence more Northern microbial communities in which *V. cholerae* presence is unexpected has been unknown. In an ongoing study of resident *Vibrio* species in New Hampshire, we for the first time isolated *V. cholerae* from the GBE. From July 2008 to September 2009, 31 bacterial isolates from oysters, sediment, and overlying water from two oyster beds in the GBE were identified as *V. cholerae* by multiplex PCR (Pannicker *et al.*, 2004) (Table 1). Nineteen of these isolates were recovered during 2009 and more frequently from water or oyster.

To evaluate how *V. cholerae* detection associates with environmental factors, we utilized both point-of-collection data and a database of various environmental conditions recorded remotely near the two oyster beds. Although *V. cholerae* was detected only between the months of June and October, isolates were recovered from relatively cold waters, as low as 13.6°C in October 2008. Salinity, temperature and dissolved oxygen each correlated significantly with abundance in pairwise tests (Table 2). Next, a stepwise regression of the highest *V. cholerae* MPN concentration at each collection site and date was performed with the following environmental factors: i) average salinity for 12 hours or 72 hours, ii) average water temperature for 12 hours or 72 hours, and iii) total prior

TABLE 1: Summary of *V. cholerae* collected from the Great Bay Estuary[a]

Isolate	Date	Site	Source	MPN[b]	*toxR*	*ompU*	*hlyA*	*tcpI*
428	7/30/2008	OR	Oyster	$1.5x10^{2}$	+	+	+	-
442	7/30/2008	NI	Oyster	$1.5x10^{1}$	+	-	-	-
466	8/5/2008	NI	Water	$1.5x10^{1}$	+	+	+	+
474	8/5/2008	OR	Water	$2.4\ x10^{1}$	+	-	-	-
476	8/5/2008	OR	Water	$1.5\ x10^{1}$	+	+	+	+
504	8/12/2008	OR	Water	$2.8\ x10^{0}$	+	+	-	-
509	8/12/2008	OR	Oyster	$2.9\ x10^{1}$	+	-	-	-
658	9/19/2008	NI	Water	$3.6\ x10^{-1}$	+	-	-	-
661	9/19/2008	NI	Water	$3.6\ x10^{-1}$	+	-	-	-
684	9/24/2008	OR	Water	$3.6\ x10^{-1}$	+	-	-	-
704	10/8/2008	NI	Oyster	$9.1\ x10^{-1}$	+	+	+	-
705	10/8/2008	NI	Oyster	$3.6\ x10^{-1}$	+	-	-	-
901	6/29/2009	OR	Water	$1.1\ x10^{0}$	+	+	+	-
907	6/29/2009	OR	Oyster	$1.5\ x10^{1}$	+	-	-	-
917	6/29/2009	NI	Water	$2.1\ x10^{0}$	+	+	+	-
925	6/29/2009	OR	Water	$1.5\ x10^{0}$	+	-	-	-
937	6/29/2009	NI	Water	$1.5\ x10^{0}$	+	-	-	-
1063	7/27/2009	OR	Oyster	$3.6\ x10^{0}$	+	+	+	-
1068	7/27/2009	NI	Oyster	$3.6\ x10^{0}$	+	-	-	-
1069	7/27/2009	NI	Sediment	$2.3\ x10^{1}$	+	+	+	-
1070	7/27/2009	NI	Sediment	$2.3\ x10^{1}$	+	-	-	-
1075	7/27/2009	NI	Oyster	$7.3\ x10^{0}$	+	-	-	-
1105	7/27/2009	NI	Oyster	$1.5\ x10^{2}$	+	+	+	-
1106	7/27/2009	OR	Water	$1.5\ x10^{2}$	+	-	-	-
1114	7/27/2009	OR	Water	$2.0\ x10^{0}$	+	-	+	-
1116	7/27/2009	OR	Water	$1.1\ x10^{0}$	+	-	-	-
1118	7/27/2009	NI	Water	$9.1\ x10^{-1}$	+	-	-	-
1173	8/11/2009	OR	Sediment	$2.0\ x10^{2}$	+	-	-	-
1194	8/11/2009	OR	Water	$1.2\ x10^{1}$	+	+	+	-
1200	8/11/2009	OR	Water	$1.5\ x10^{2}$	+	-	-	-
1261	9/1/2009	NI	Sediment	$9.1\ x10^{0}$	+	-	-	-

[a] *zot, ace, tcpA, and ctxA are not included in the table because they were not present in any of the isolates*

[b] *Values are presented in MPN/mL for water samples, and MPN/g for sediment and oyster samples*

rainfall for 72 hours or 96 hours. This analysis revealed a significant correlation between MPN and prior rainfall at both 72 hours and 96 hours (Table 2), explaining 32% and 13% of *V. cholerae* abundance respectively. When the high MPN concentration was considered for oyster and water isolation separately, rainfall for 72 hours prior was the only significant regressor for isolates sampled from water ($p = 0.002$), and only rainfall for 96 hours prior for isolates sampled from oysters ($p = 0.001$). Because 12 hour mean temperature and salinity were no longer significantly associated with MPN, recent rainfall becomes the best predictor of *V. cholerae* incidence in this study: within 72 hours prior to sampling for isolates from water and within 96 hours prior for isolates from oysters. This variation in monitoring timeframes suggests that different environments accumulate *V. cholerae* at different rates, which could influence future predictive models of occurrence.

TABLE 2. Abundance and environmental factor correlations

	Total MPN		Oyster MPN		Water MPN	
	Pearson Correlation[b]	Stepwise Regression[a]	Pearson Correlation	Stepwise Regression	Pearson Correlation	Stepwise Regression
Temperature						
12hr	0.355*	NC	0.230	NC	0.271	NC
72hr	0.389*	NC	0.270	NC	0.266	NC
Salinity						
12hr	-0.369*	NC	-0.355*	NC	-0.236	NC
72hr	-0.461*	0.1768	-0.375*	0.378	-0.367	0.450
Rain						
72hr	0.472**	0.049*	0.112	0.4753	0.623**	< 0.001**
96 hr	0.566**	0.006*	0.555**	0.001**	0.228	0.976
Dissolved O_2						
Percent	-0.333*	NC	-0.261	NC	-0.223	NC
mg/L	-0.355*	NC	-0.247	NC	-0.255	NC
Turbidity	0.112	NC	0.134	NC	0.112	NC

[a] p statistic is given otherwise the environmental condition was excluded from the stepwise model and no correlation (NC) is reported
[b] r statistic is given
* $p < 0.05$
** $p < 0.001$

***V. cholerae* isolates lack pathogenicity markers and belong to a diverse, stable, and recombining population.** *V. cholerae* genotyping and determination of toxigenic potential relies upon a number of markers of virulence and strain type (Mekalanos and Waldor, 1997; Miller and Mekalanos, 1984; Jiang *et al.*, 2003; Hall and Drasar, 1990; Wibbenmeyer *et al.*, 2002). The genes *ompU* and *hlyA* that correlate with serotype were present in 11 isolates, occurring together in each case except for isolate 504 (*ompU*) and 1114 (*hlyA*). Only two isolates were positive for the regulatory genes for toxin coregulated pilus expression (*tcpI*). Virulence-associated genes *zot*, *ace*, *tcpA,* and *ctxA* (encoding cholera toxin subunit A) were absent from all of the GBE isolates. All of the isolates were confirmed as non-O1 serotypes.

To examine the diversity and genetic relationships among these 31 GBE *V. cholerae* isolates, we performed multi-locus sequence analysis with five loci: *gapA*, *gyrB*, *pyrH*, *recA*, and *topA*. Although an existing MLSA scheme is available and has been used to generate a *V. cholerae* database (Garg *et al.*, 2003), it was developed specifically for closely related O1 and O139 serotype strains and failed to amplify the orthologous alleles of our environmental strains. Therefore, we analyzed a subset of the genes developed to type *Vibrio* species more broadly (Thompson *et al.*, 2005), which successfully amplified loci from all collected isolates. In addition, four fully-sequenced pandemic *V. cholerae* strains (0395, M66-2, N16961, and MJ-1236), a *V. mimicus* type-strain (VM603), and *V. parahaemolyticicus* (RIMD 2210633) were included in our analysis to infer their relatedness to these isolates (Fig. 2.1A). The neighbor-joining tree based on concatenated sequences shows a diverse population with two major clades and several several clonal complexes. All O1 clinical strains grouped in the larger and more diverse

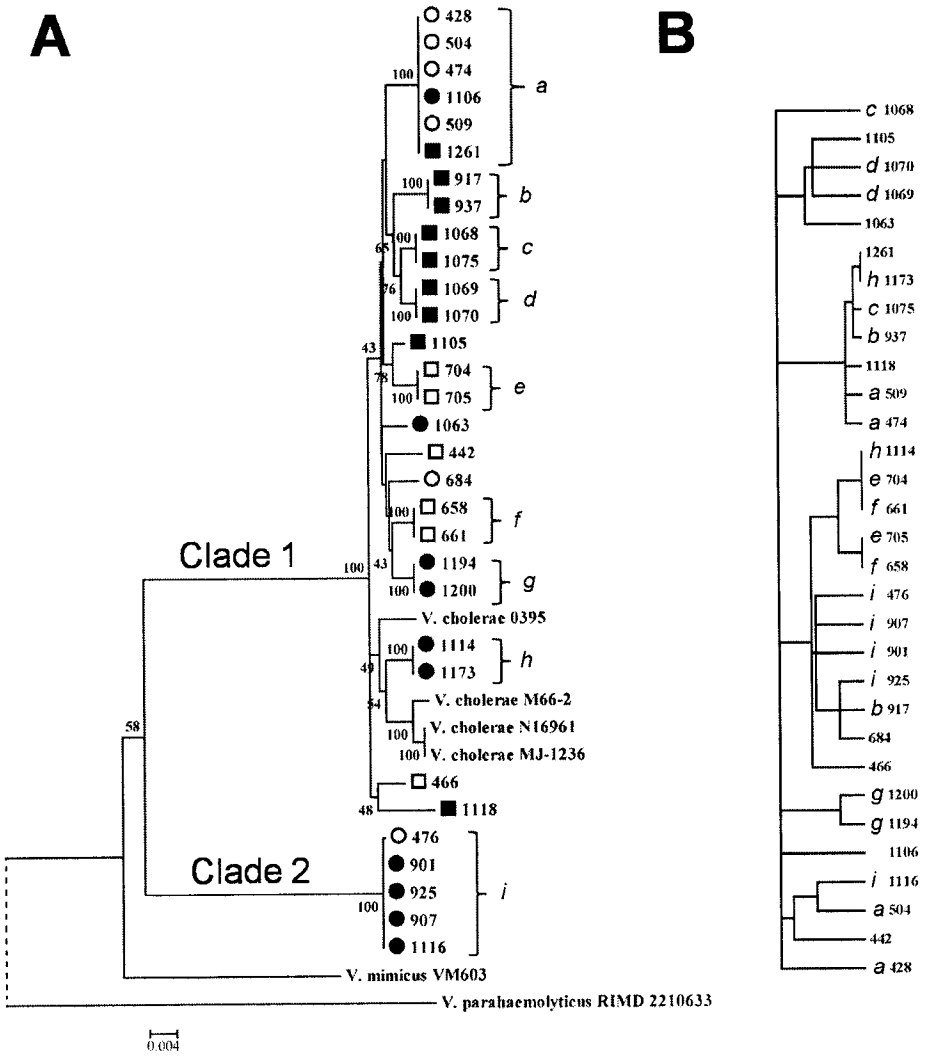

Figure 2.1. Consensus neighbor-joining tree of Great Bay Estuary isolates (A). The tree is based on the concatenated sequences of 5 loci: gapA, gyrB, pyrH, recA, and topA sequences (2,792 bp) using the Jukes-Cantor model of substitution for 31 GBE isolates and 3 serotype-01 El Tor control strains and 1 classical serotype-01. *V. mimicus* VM603 and *V. parahaemilyticus* RIMD 2210633 are included as tree roots for comparison, but the *V. parahaemolyticus* branch is shortened (dashed line) so the tree can be enlarged. Bootstrap values are shown after 1,000 replicates. Bar indicates 0.2% divergence. The circle symbols indicate the isolate was collected from Oyster River, and the square from Nannie Island; an open symbol indicates collection from 2008 and filled from 2009. MLST clonal complexes are labeled with an italic letter to differentiate the clones in the PARS tree (B) which is based on the discrete levels of eight different phenotypes.

clade 1 and most closely with isolates 1114 and 1173, which form an intermediate group between Classical and El Tor strains (Fig. 2.1A). The remaining isolates form a second clade that is more distant from the clinical isolates, less diverse, but nevertheless share 100% identity in the V2-V3 region of 16S rDNA with type strains of *V. cholerae*.. Moreover, these isolates do not cluster with environmental strains of *V. mimicus* (Fig. 2.1A)(Thompson *et al.*, 2008). Phylogenies of each individual gene in our typing scheme mirror the concatenated tree except for *gyrB,* which either indicates unique patterns of recombination or atypical divergence and selective pressures on this locus (data not shown).

We further examined if phylotypes (Fig. 2.1A) persisted year to year and if clones associated with sites. Most phylotypes appear in both years, but some associate with specific sites. To evaluate these associations statistically, we used adaptML(Hunt *et al.*, 2008), which correlates phylogenetic clusters with habitat. This analysis confirmed that over 75% of isolates correlate with site but no single isolate or phylogenetic branch correlates with year (data not shown). Thus *V. cholerae* is endemic and can overwinter in the GBE, wherein different strains persist in different habitats.

Multilocus sequence analysis reflects relatedness among isolates but may improperly define some isolates as clones because only partial sequences of five genes are considered. Although they formed two diverse clades, most isolates collected were apparently clones of at least one other isolate based on the sequence of five genes. However, initial genotyping and phenotyping (Table 1 and Fig. 2.2B) revealed further diversity even within these clonal complexes. Each MLSA clone differed from other clones by phenotype, genotype, or both, and often grouped clones of different multilocus

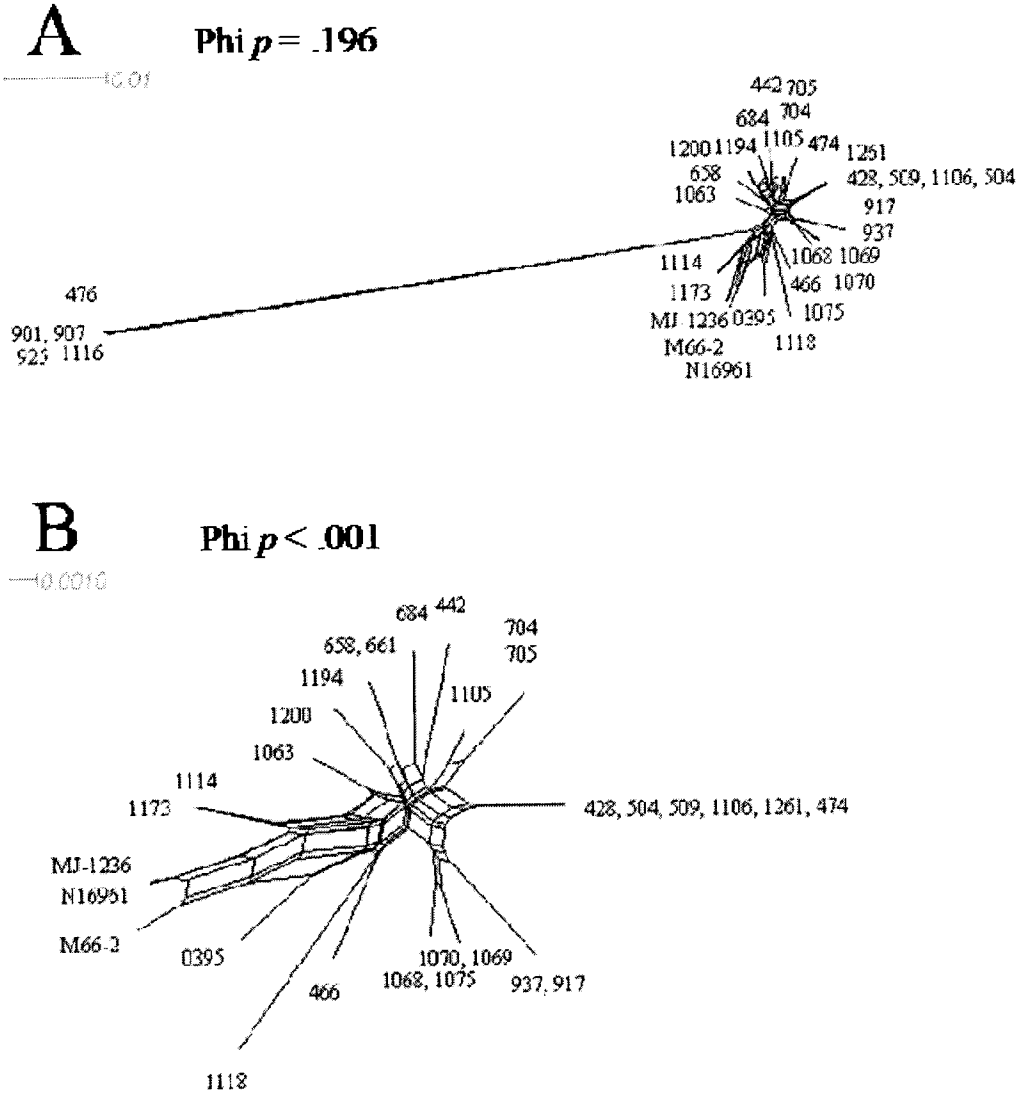

Figure 2.2. Splits Tree and Phi Test of Recombination of GBE isolates. Concatenated split network tree based on five gene loci: *gapA*, *gyrB*, *pyrH*, *recA*, and *topA* sequences (2,792 bp) from 31 taxa, including 3 serotype O1 control isolates. Sequences were concatenated and reconstructed using the SplitsTree4 program (Huson and Bryant, 2006) (A). The *p* values of the phi recombination test (Huson and Bryant, 2006) are shown. (B). The split tree network and corresponding phi test of sequences lacking the outgroup clade.

types (Fig. 2.2B). Thus, even within MLSA clonal complexes, high diversity of *V. cholerae* exists in the GBE.

We next evaluated the MLSA data for evidence of recombination by two statistical tests of sequence diversity and association among alleles. Sequence diversity of the entire group of 31 environmental isolates, as measured by the Phi test (Huson and Bryant, 2006)did not demonstrate significant levels of recombination (Figure 2.2A). However, when clade 2 was excluded from this analysis, significant recombination among isolates in clade 1 was observed (Figure 2.2B), consistent with recombination within clade 1 but not between the clade 1 and clade 2. We note that clinical serotype-O1 strains also group within clade 1 (Figure 2.1A) and may be capable of similar levels of recombination. Similar results were obtained using the LIAN test of recombination that is based on allelic profiles, which failed to reject the null hypothesis of linkage equilibrium ($I_A = 0.3204, p < 0.001$).

Although most studies have appropriately focused on regions endemic for *V. cholerae* where outbreaks are prevalent, climate change could expand the range of *V. cholerae* into northern temperate waters. For instance, we have already observed more outbreaks of other pathogens, including *Vibrio parahaemolyticus,* in northern regions not previously thought at risk (http://cdc.gov/). Only 200 reported cases of cholera infections have occurred in the United States since 1997, but alarmingly 90% of these have occurred in the past five years (http://cdc.gov/). Current climate change models predict increasing surface water temperatures and rainfall events in New England (New England Regional Assessment, 2001), and studies demonstrate a direct correlation between these environmental factors and *V. cholerae* abundance (Lobitz *et al.*, 2000; Louis *et al.*, 2003;

reviewed by Rita Colwell, 2006). In this study, we show a strong correlation between *V. cholerae* abundance and rainfall, as well as a seasonal occurrence correlating with water temperature. Although we have not isolated pandemic serotypes of *V. cholerae* or any isolates which harbor the *ctxA* toxin gene, we describe a diverse and recombinogenic natural endemic population of *V. cholerae* in the GBE, which along with other human pathogenic vibrios that reside there may serve as reservoirs for the emergence of infectious strains.

DISCUSSION

Although unlikely, there is still a relevant possibility of emergence of pathogenic biovars or displacement of the existing population in the GBE. For example, in post-natural disaster Haiti an outbreak of non-native Asiatic *V. cholerae* El Tor has emerged where endemic toxigenic strains have not been reported in over 100 years (CDC Morbidity and Mortality Weekly Report, October, 2010; Pan American Health Organization, October, 2010). Similarly, several outbreaks related to unprecedented increases in surface water temperatures have occurred in Peru, a country where cholerae had not been a problem for over a century (Rita Colwell 2006; Martinez-Urtaza *et al.*, 2008). Now, toxigenic *V. cholerae* occurs there seasonally (Lipp *et al.*, 2003). Since the existing GBE population contains representatives that are phylogenetically similar to pandemic strains, the question remains whether any invading virulent strains could survive and persist, especially if surface-water temperatures increase as models suggest. A second concerning possibility is the emergence of new virulent strains here due to lateral gene transfer for which *V. cholerae* is infamous. The most notable emergence of new toxigenic *V. cholerae* was discovered during a 1992 outbreak in Madras, India, when an O-antigen shift resulted in O139 epidemic, however this shift occurred in a toxigenic background (Garg *et al.*, 2003). Alternatively, the emergence of environmental non-O1/non-O139 strains which laterally acquired the *ctxAB* via the CTX prophage have been reported in India, Malaysia, and California but have not been associated with epidemic disease (Maiti *et al.*, 2006; Radu *et al.*, 1999; Jiang *et al.*, 2003). In the Great Bay, long term survival following invasion of a toxigenic strain may not be likely, especially during

years where surface-water temperatures remain cold, but even transient residency could lead to the transfer of virulence genes to this endemic northern population.

Even though the risk of cholera outbreaks in such northern regions with cool water temperatures is by all accounts extremely low, this newly discovered endemic population remains a valuable ecological model. There are only a few rare incidents of non-O1/non-O139 cholera infections from the ingestion of raw shellfish in the United States (Klontz *et* al., 1987; MacRae *et al.*, 1983), and these environmental populations are suspected to have little clinical opportunity for outbreaks. Even so, our analysis revealed considerable recombination among strains, suggesting that even if invading non-endemic virulent strains were unable to displace the existing population, it could transfer virulence genes to this established population. This natural endemic population is currently the northern-most discovered along the Eastern United States seaboard. Due to the GBE's relative isolation, and the extreme environmental conditions that it experiences relative to the greater Gulf of Maine and other known endemic populations, we believe this population serves as an excellent model to explore the ecological and evolutionary dynamics of resident Vibrios. This population is also ideally suited to test effects of climate change on the population structure of *V. cholerae* at the range limit for these potential pathogens.

CHAPTER III

METAGENETIC ANALYSIS REVEALS HOST ASSOCIATED POPULATIONS WITHIN OYSTERS COMPARED TO THE SURROUNDING WATER

INTRODUCTION

The consumption of raw oysters (*Crassostrea species*) is a practice that predates written history and oysters continue to be a prominent vector that exposes human hosts to marine microorganisms. Though most consumers are currently aware of the risks, raw shellfish production remains a lucrative industry in the United States. The Pacific Coast Growers Association that serves Washington, Oregon, California and Alaska alone harvests over 72 million pounds of oysters (excluding shell weight) for over $72 million in sales in just one year (http://www.pcsga.org/). The delicacy, the mineral and vitamin content, and perhaps the novelty of eating raw oysters is incentive enough for consumers to risk certain public health concerns and exposure to seafood-borne pathogens. Oysters and other bivalves are filter feeders, concentrating plankton, bacteria and viruses from overlying waters into their gut. Adequate cooking and other post-harvest processes can effectively sterilize the oyster gut, but consumption of the raw meat delivers a potentially dangerous concentration of marine microorganisms directly into the human enteric system. Because oysters sometimes contain pathogens and are often eaten raw, most cases of seafood borne illness are delivered by oysters (http://www.fda.gov), and disease is most often caused by *Vibrio parahaemolyticus,* a species commonly isolated from the oyster gut (Yeung and Boor, 2004). Other *Vibrios* inhabiting the oyster, which include

Vibrio vulnificus, and *Vibrio cholerae,* can cause isolated incidents of gastroenteritis infection (DeWaal *et al.*, 2006). In the United States, the largest seafood-borne outbreaks caused by live microorganisms result from ingestion of *V. parahaemolyticus* from raw oysters (Daniels *et al.*, 2000; DeWaal *et al.*, 2006). While the vast majority of research focuses on safe oyster harvesting and post-harvesting techniques, as well as microbial diseases that can be delivered by oysters, very little is known about the ecology of the oyster gut microbiota.

Predicting the incidence and abundance of *Vibrios* in oysters and the overlying water is a current research focus that aims to prevent future outbreaks. The Colwell group demonstrated that environmental conditions, including temperature and salinity, correlate with the abundance of *V. cholerae* populations in Chesapeake Bay estuarine waters, and *V. cholerae* is consequently detected in oysters at elevated concentrations (Constantin de Magny *et al.*, 2009; Louis *et al.*, 2003). The correlation between environmental conditions and *Vibrio* incidence is well reported, but correlations between abundance in water and in oysters remains unclear. Many studies in the literature show evidence of this disconnect between abundance of pathogenic *Vibrios* in the water and in the oyster. In New England where *V. cholerae* is rarely detected in the environment, an oyster originating from those waters caused gastroenteritis disease in a patient (MacRae *et al.*, 1983). A Galveston Bay study demonstrated that *V. vulnificus* abundance positively correlated with environmental factors in water and oysters during the summer months, but the pathogen was only detected in the oyster in the fall with no reported correlations to environmental factors (Lin *et al.*, 2003). Oysters void of detectable *V. parahaemolyticus* were collected off the coast of Brazil from overlying water that

contained high concentrations of the pathogen (Sobrinho *et al.*, 2010). This study demonstrated that pathogen incidence in overlying water correlated with incidence in oysters when they were pooled and tested together, however, there appeared to be some variance among individual oysters despite being collected from the same bed at the same time. One possible factor that may influence variance of pathogen incidence and abundance in oysters is the resident microbial community of the oyster gut, which has yet to be explored with new high-throughput and non-culture based methods.

Evidence suggests that the oyster-associated microbial community is different from that of the overlying water, but there are some caveats. Studies of the natural bacterial fauna provide evidence that communities within the oyster are more diverse than the overlying water (Pujalte *et al.*, 1999; Brady *et al.*, 1998; Olafsen *et al.*, 1993), but these studies are limited to culture based methods. It is estimated that over 95% of marine bacteria cannot be cultured (Cottrell and Kirchman, 2000; Handelsman et al., 2007), and past studies likely did not capture a large portion of the marine community in the niches explored. An ongoing study shows that relaying oysters in the New Hampshire Great Bay Estuary (GBE) to areas with higher salinity reduces total *V. vulnificus* concentration, but when oysters were treated with sterilized water with high salinity levels, the *V. vulnificus* total concentration did not change (Jones 1994; Yu *et al.*, Vibrios in the Environment 2010 proceedings). These observations suggest that the microbial population present in the water helped exclude the pathogen while the sterilized water failed to do so even at high salinity levels. All of these studies suggest the presence of microbial population interactions within oysters, but new high-throughput and culture

independent methods will clarify nuances of these interactions in ways that were not previously possible.

Advancements in deep sequencing technology allow for comparative microbial community profiling with new depth. Many studies report on the use of metagenetics (deep sequencing of a single species-determining gene) to differentiate populations in multiple niches. Recent approaches amplified variable regions of conserved bacterial genes, such as 16s rDNA, from whole populations and utilized exponentially growing non-redundant DNA taxonomy databases to determine a microbial census (Reviewed in Wooley *et al.*, 2010). Large-scale shotgun 16s rDNA surveys are now being conducted from multiple samples in unison due to improvements in barcoded pyrosequencing methodology (Hamady *et al.*, 2008). One such study compared microbial population differences between terrestrial and aquatic habitats (Tringe *et al.*, 2005). Turnbaugh (2006) used pyrosequencing to explore the effects of host-microbe interactions on population structure in obese versus fit mice and also in humans (Turnbaugh *et al.*, 2006). Multiple researchers investigated the transfer of microbes between different environments using advanced deep-sequencing techniques and others used these methods to study the ability of some community members to either displace or enrich transient species (Hooper *et al.*, 2008; Rawls *et* al., 2006). We hypothesize that by using these techniques, we will determine that the bacterial community in an oyster gut is unique when compared to the community in the water the organism filters and that oysters exhibit complex communities. We also believe that by comparing the metabiome of multiple oysters and water, we will develop specific and testable future hypotheses about

putative indicator species that may correlate with the presence of pathogenic *Vibrios*, or displace them.

In this study, we characterized the microbial community of oysters and the overlying water from the GBE. We collected 20 individual oysters each in 2008 and 2009 and the overlying water from 2009 from two distinct habitats in the GBE, and conducted a comparative metagenetic study using variable 16s rDNA gene region (V2-V3). Sequence reads were grouped into distinct species level taxonomic units . In this preliminary analysis we quantified *V. parahaemolyticus* in individual oysters by a combination of enrichment qPCR and a most probable number test (MPN) (Yu *et al.*, Vibrios in the Environment 2010 proceedings). We then defined the microbial taxa residing in the oyster and overlying water at each site, and investigated the premise that water quality influences microbial diversity. This dataset will be the foundation for future analysis, that requires development of new bioinformatics analysis tools, to examine whether the population profile 1) varies by year or is stable 2) is similar to other estuarine communities examined by similar methods and 3) varies in microbial composition between oysters collected from the same bed.

METHODS

Sample collection and processing. Water and oyster (*Crassostrea virginica*) samples were collected approximately at low tide on September 1st, 2008 and September 1st, 2009 from two sites in the GBE including an oyster bed located near Nannie Island that is within an area classified as approved for shellfish harvesting, and a second oyster bed located in the Oyster River within an area classified as prohibited because of its proximity to the Durham, N.H. wastewater treatment facility effluent outfall. Oysters were sampled using oyster tongs at locations within the same general area of the oyster beds each time. Water samples were collected on site by filling and then capping sterile 50 mL conical tubes at ~30 cm below the water surface. All oyster and water samples were immediately stored in coolers containing ice packs and brought back to the laboratory for processing.

Individual shellfish were cleaned of debris and shucked using aseptic procedures. Whole individual oysters were shucked and transferred into a sterile 50 mL conical tube. An equal weight of alkaline peptone water (APW, pH 8.6, 1% NaCl) was added to the whole oyster and was homogenized with a Tissue Tearor (Biospec Products, Bartlesville, OK) for 30 seconds at medium speed and 60 seconds at high speed. In between each homogenization to minimize cross contamination, the Tissue Tearor was cleared of debris and engorged in 90% ethanol for 30 seconds at high speed, and then washed in filter sterilized water for 30 seconds at high speed. The 2008 oysters were diluted 1 mL of oyster homogenate into 9 mL of APW and incubated for 16 hours at 37°C. For 2009 oysters, 10.0, 1.0, 0.1, 0.01 and 0.001 ml volumes of oyster homogenates and water samples were inoculated into multiple tube fermentation (MPN) analysis series with

APW selective enrichment for 16 hours at 37°C. Following incubation, 1.0 mL of each APW tube was pelleted and DNA was extracted using purification by a CTAB-NaCl precipitation followed by phenol-chloroform extraction (Ausubel *et al.*, 1990). The remaining 9 mL of each APW culture was stored at -80°C, as well as the non-cultured oyster homogenates.

Water samples from the 2009 collection were immediately centrifuged following collection and transfer to the laboratory (~1 hour) in a 5810R centrifuge (Eppendorf, Hamburg, Germany) at 4,000 rpm. The supernatant was discarded and the bacterioplankton containing pellet was frozen at -80°C.

To prepare the metagenetic DNA preparations, first the raw oyster homogenates were thawed on ice for 10 minutes and the top foamy layer (~1 cm) was scraped away with a sterile 15 mL conical tube. A new sterile 15 mL conical tube was used to scrape and collect 1.0 g of each oyster homogenate. The E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA) bead beating kit was used following standard protocols for gram negative and gram positive bacterial DNA isolation from the oyster homogenate. The same bead beating DNA extraction was also performed on the 2009 water sample bacterioplankton containing pellet for each sampling location.

qPCR *V. parahaemolyticus* MPN analysis. The published protocols for thermolabile hemolysin gene (*tlh*) qPCR (Nordstrom *et al.*, 2007) were adapted for scoring MPN results rather than for direct quantification. Briefly, *V. parahaemolyticus* quantification in MPN tubes was performed as reported by Nordstrom *et al.* (2007) with the following modifications: the mastermix consisted of 1X iQ Supermix SYBR Green I (Bio-Rad, Hercules, CA), 5 mM of $MgCl_2$ (Bio-Rad, Hercules, CA), 125 nM of forward

and reverse primers (tlhF – 5'-ACTCAACACAAGAAGAGATCGACAA-3'; tlhR – 5'-GATGAGCGGTTGATGTCCAA-3'), 2 uL of the DNA template, and an iCycler with the MyiQ Single Color Real-Time PCR Detection system with included software (Bio-Rad, Hercules, CA) was used. The qPCR parameters included initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 10 seconds, and primer annealing at 59°C for 15 seconds. The melting curve was performed using the manufacturer's presets (Bio-Rad). Scoring of the MPN tubes as positive or negative was based on whether qPCR starting quantity (SQ) values were below (negative) or above (positive) the threshold value determined by the standard curve and iCycler software. Determination of the MPN tube result based on qPCR was unambiguous because all positive tubes were at least one order of magnitude higher in DNA concentration from the threshold value, and all negative tubes had concentration values of 0. None of the samples had concentrations within one magnitude of order above or below the threshold value and all MPN tube results were confidently assigned. A full MPN scheme was not performed on the 2008 samples, but qPCR as previously described was performed on the 1:10 APW tubes and compared to 2009 samples.

Metagenetic processing and OCTUPUS analysis. The V2 to V3 16s gRNA segment was amplified with standard 16s F8 (5'-AGTTTGATCCTGGCTCAG-3') and R357 (5'-CTGCTGCCTYCCGTA-3') primers with a unique 6 bp MID tag and GS FLX Titanium Primer A (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') or Primer B (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3'). Each sample was amplified in triplicate in 50 uL reactions. In each reaction, 2 uL of molecular grade water and 3 uL of the un-enriched oyster homogenate DNA preparation were added to 45 uL of Platinum PCR

Supermix (Invitrogen, Carlsbad, CA). A negative control also performed in triplicate with 5 uL of molecular grade water was conducted in parallel. The following PCR conditions were used in an iCycler thermocycler (Bio-Rad, Hercules, CA): initial denaturation at 94°C for 90 seconds, 30 cycles of denaturation at 94° for 30 seconds, annealing at 50.7°C for 45 seconds, extension at 72°C for 30 seconds, and a final elongation at 72°C for 3 minutes. The triplicate samples were combined and purified using the MinElute PCR Purification kit (Qaigen, Valencia, CA) following standard protocols. To confirm proper expected amplification of the samples and the absence of amplification in negative controls, bands from 2 uL of each purified sample were visualized on a 1.2% agarose gel.

A 10 ng/mL multiplexed sample was prepared for the Roche Genome Sequencer FLX System using Titanium Chemistry (454 Life Sciences, Branford, CT). Each sample was quantified for DNA concentration using a NanoDrop 8000 (Thermo Scientific, Wilmington, DE) and weighted and combined as follows: 75% of the total concentration consisted of equal proportions of the 20 2009 oyster samples and two 2009 water samples, and 25% of the total concentration consisted of equal proportions of the 19 2008 oyster samples. This multiplexed mixture was cleaned up using the AMPure XP Purification (Beckman Coulter Genomics, Danvers, MA) as follows: 720 uL of the AMPure beads was added to 450 uL of the multiplex mix in a 1.8 mL microcentrifuge tube and incubated for 5 minutes at room temperature. The tube was placed in an appropriately sized magnetic particle collector and left idle for ~5 minutes until the supernatant cleared and a pellet formed adjacent to the magnet. The supernatant was discarded with a pipette and the magnetic bead pellet was washed twice with 70% ethanol incubated for 30 seconds each time. The pellet was dried for 10 minutes at 37°C, and then

resuspended in 20 uL elution buffer EB from the MinElute kit. The pooled tagged single-stranded pyrosequencing library underwent fusion PCR and pyrosequencing using a Roche 454 FLX Pyrosequencer (454 Life Sciences, Branford, CT) according to the manufacturer instructions at the University of Illinois W.M. Keck Center High-Throughput DNA Sequencing center.

The 454 pyrosequencing reads were processed using the OCTUPUS pipeline (Way Sung and Kelley Thomas, unpublished). Briefly, the algorithm compresses reads into operational taxonomic units (OCTUs) with 97% similarity, blasts the OCTUs against the NCBI non-redundant database assigning the best hit nomenclature, and then displays the distribution of each OCTU per MID tagged sample. The following formula was used to calculate the cutoff for well-sampled OCTUs: (3 x standard deviation of the total number of reads per each OCTU) / (square root of the total number of OCTUs). The resulting list was used for comparative taxonomic distribution graphs.

RESULTS AND DISCUSSION

Oysters and overlying water have different population structure. We determined the relative abundance of taxa from the microbial communities of 39 oysters and 2 overlying water samples with 16s rDNA sequencing. These data were used in three ways: 1) to determine the microbial community in oysters compared to overlying water, 2) to compare the community of oysters with high and low concentrations of *V. parahaemolyticus* infestation, and 3) to compare the diversity of the microbial communities between the two sites. We processed 1,405,413 multiplexed 16s rDNA reads for 19 oysters from 2008, 20 oysters from 2009, and 2 overlying water samples from each site in 2009. The final metasequencing sample was weighted for the 2009 collection (75%) since *V. parahaemolyticus* quantification was more precise that year than in 2008. The OCTUPUS algorithm categorized reads into 29,771 taxonomic units (OCTUs) based on 97% similarity, which is within the bacterial species definition of similarity for 16s rDNA. Only 34,211 reads (< 2.5%), or 17,915 OCTUs (~60%) were flagged as chimeras, which are hybrid sequences due to polymerase extension errors. Although rejection of the flagged chimeras reduced the number of OCTUs, they only comprised a very small portion of the total reads and elimination of chimeras did not compromise the analyses. Before we conducted the community analysis, we optimized the data to obtain a dataset of well-sampled OCTUs. We used three standard deviations below the mean OCTU abundance as the cutoff statistic to determine the minimum reads expected for well-sampled taxonomic units. The OCTUs that fit this statistical requirement contained at least 28 reads, which reduced the dataset to 1,323,289 reads (> 94% of the total reads) and 1,475 OCTUs (< 5% of the original OCTUs assigned). This

optimization of the dataset eliminated rare population representatives to avoid overestimation of diversity.

The total distribution of taxa were consistent with metagenetic analyses of other marine and estuarine environments (Fig. 3.1), particularly the abundance of cyanobacteria, marine proteobacteria, bacteriodetes and actinobacteria found in the water column and associating with marine hosts in these other studies (Dinsdale *et al.* 2008; Venter *et al.*, 2004; Tringe *et al.*, 2005; Gilbert *et al.*, 2008). The overwhelming majority of reads mapped to 10,000 OCTUs that were characterized only as environmental samples. As is common with metagenetic studies, we could not determine taxonomic identity of these "environmental samples" due to a lack of available cultured representatives of these groups in the existing databases (Cottrell and Kirchman, 2000; Handelsman et al., 2007). The most abundant identifiable taxa across all samples included proteobacteria, bacteriodetes, cyanobacteria, and actinobacteria. Other abundant taxa were differentially proportioned in various samples, correlating either with site or substrate.

We first compared the population structure, as phyla distributions, within oysters to the overlying water from the 2009 collection. If oysters simply concentrate water-borne bacteria and there is no interaction or population structure within the host, the proportion of community members within the oyster should reflect that of the water. Oysters from GBE exhibited multiple fold differences in phyla abundance of the pooled oyster fauna compared to the overlying water (Fig. 3.1). Taxa that were more abundant in oysters at both GBE sites included the cyanobacteria (4 fold more abundant at NI, 2 fold at OR), the firmicutes (10 fold greater at both sites), the planctomyces (which

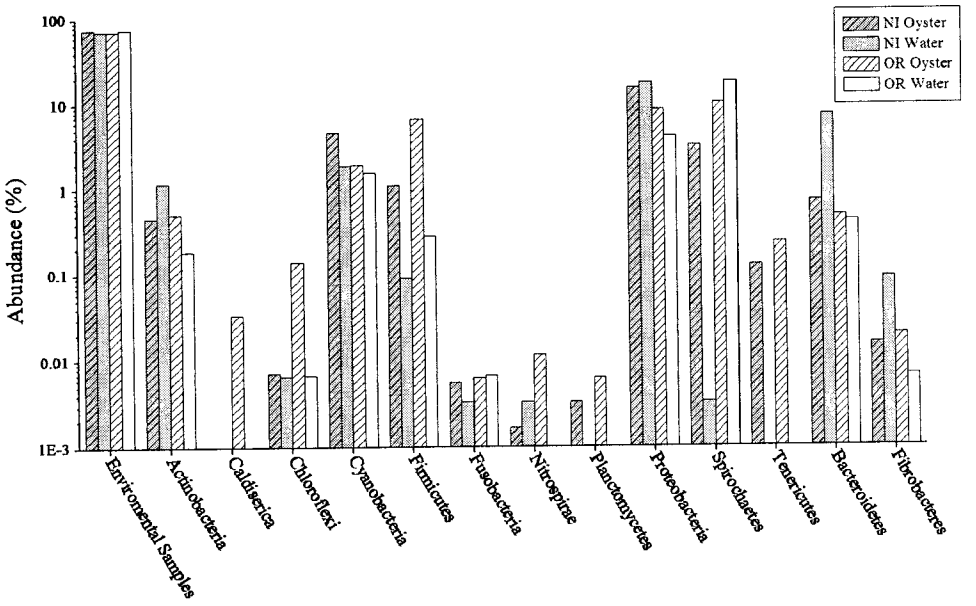

Figure 3.1: Water versus oyster abundances of different phyla per site. The total percentage of reads representing different phyla for the water sample and combined 10 oysters per the sites Nannie Island and Oyster River . The graph is presented on a logarithmic scale to visualize rarer representatives in the populations.

comprised ~0.01% of the oyster metapopulation but were not detected in water), and the tenericutes (which comprised >0.1% of the oyster metapopulation but were not detected in the water) (Fig 3.1). Phyla, such as planctomyces, which represented a very low proportion of the total distribution, were possibly under-sampled. All other large and consistent differences at both sites supported our hypothesis that oysters do not have microbial communities that reflect filtered bacterioplankton but appear to contain host-specific microfauna.

We observed some large differences in phyla abundance between the oysters and the overlying water that are inversely proportional at the two sites. For example, bacteroidetes in the NI samples are present in 10-fold greater abundance in the water compared to the oysters, comprising a very large portion of the metapopulation, 10% and 1%, respectively (Fig 3.1). The opposite is true at the OR site where bacteroidetes are 2-fold more abundant in the oyster than in the water. The disparity between the sites likely excludes a direct biological interaction of this phyla and the host. Similarly, fibrobacteres are 8-fold more abundant in NI water, but 5 fold more abundant in OR oysters (Fig 3.1). When we consider the distribution of proteobacteria classes, the epsilonproteobacteria vary the most between the samples (Fig. 3.2). These inconsistent phyla distribution differences may reflect variance of pathogen concentrations in oysters and warrants further future investigation.

Some differences between the oyster and water population may have potential biological explanations indicative of direct host-microbe interactions. Planctomycetes sampled in oysters but not in water belong to the Mollicutes class that lack cell walls and experience genome reduction typical and proven to be a result of parasitic and symbiotic

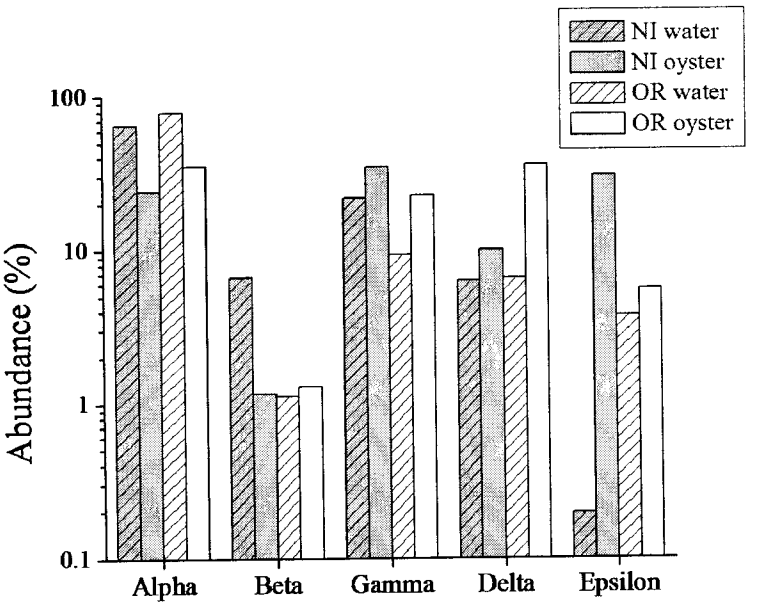

Figure 3.2: Water versus oyster abundances of different Proteobacteria classes per site. The total percentage of reads representing different Proteobacteria classes for the water sample and combined 10 oysters per the sites Nannie Island and Oyster River . The graph is presented on logarithmic scale to visualize rarer representatives in the populations.

interaction with a host. Mollicutes have been shown to readily associate with many marine organisms including shrimp (Krol *et al.*, 1991) and marine bryozoans (Boyle *et al.*, 1987). Planctomycetes could have been under-sampled in water, but we can now specifically sample oysters and water and enrich for these particular organisms to explore the possibility that there is a symbiotic or parasitic relationship. Firmicutes have been previously shown to have an association with oyster shell decomposition (Math *et al.*, 2010), and here we show an enormous preference of the phyla with the living host compared to the water. Some of these population structural differences have clear possibilities for a host-interaction; future work will explore if these associations can affect human pathogen loads that differ in the host.

Water microbial communities differ between two Great Bay sites. We compared the water community between both sites to determine if wastewater effluent and correlating environmental conditions (Yu *et al.*, Vibrios in the Environment 2010 proceedings) at the OR affects the distribution of taxa compared to NI. Many of the taxa are distributed similarly between the two sites, but actinobacteria, proteobacteria and bacteroidetes are each present in 10-fold higher abundances at the NI (Fig. 3.1). The difference in proteobacteria is particularly interesting, because that phylum contains many of the pathogens that are associated with oyster contamination, including Vibrios. Nannie Island is generally accepted as a cleaner site where recreational permits are issued to oyster collection, and the OR bed is prohibited for this purpose. Spirochetes are 30-fold more abundant in OR water, the largest taxa variance discovered from all of the comparisons (Figure 3.1). This phylum is usually found in high abundance in wastewater effluent (Ben-Dov *et al.*, 2008; McGarvey *et al.*, 2006), and the organisms are

predominantly anaerobic, which could explain why these organisms are more abundant in the lower oxygenated OR water (Yu *et al.*, Vibrios in the Environment 2010 proceedings). There are some dramatic community differences between the two sites on the level of phyla distribution, but future work will make comparisons on a species or phylogenetic level to further elucidate these different populations.

***V. parahaemolyticus* concentration varies between oysters but not between sites.** We examined the degree of variance of *V. parahaemolyticus* concentrations in oysters collected from the same site. We sampled oysters from two ecologically distinct sites in the GBE, determined the level of *V. parahaemolyticus* contamination, and sequenced the total oyster microbiota. On September 1st 2008 and September 2nd 2009, ten oysters per site were collected and processed individually from Oyster River and Nannie Island, and *V. parahaemolyticus* concentrations determined. Although qPCR quantification on oyster-homogenate enrichment was conducted on all 40 oysters collected in both years, a full MPN was only conducted for the 2009 collection. DNA SQ ranged between 0.031 to 3.420 pg (Table 3.1 and Fig. 3.3). The MPN for individual 2009 oysters ranged from 0.36 MPN/g to 750 MPN/g (Table 3.1), and were statistically categorized into two groups: high and low abundance of *V. parahaemolyticus* (pairwise Sidak t-tests, $p < 0.05$). Oysters at both sites vary in their infestation of *V. parahaemolyticus* when analyzed individually by this method, varying by 326x at NI and 60x at OR (Table 3.1). The restricted oyster site, OR, in fact contained oysters with lower concentrations of *V. parahaemolyticus* (average = 7 MPN/g), compared to the approved site, NI, which contained the most contaminated oysters (average = 154 MPN/g). Since we report a large range of oyster contamination at each site, standard methods that pool

Table 3.1: Concentration of *V. parahaemolyticus* based on MPN analysis, enrichment qPCR, and metagenetic analysis

Sample Designation		MPN/g[a]	1:20 APW qPCR (pg)	Metagenetic Distribution
2009 Nannie Island				
	1	21.00	0.106	0.0029%
	2	240.00	0.229	0.0056%
	3	240.00	0.616	0.0029%
	4	240.00	0.088	0.0040%
	5	4.300	0.466	0.0046%
	6	21.00	0.264	0.0089%
	7	9.30	1.161	0.0107%
	8	9.30	0.819	0.0086%
	9	750.00	0.544	0%
	10	2.30	0.343	0.0015%
2009 Oyster River				
	11	9.30	0.788	0%
	12	2.10	0.500	0%
	13	9.30	0.672	0%
	14	0.92	0.232	0%
	15	7.50	1.106	0%
	16	0.92	0.126	0.0019%
	17	15.00	1.980	0%
	18	2.300	0.183	0%
	19	21.00	0.286	0%
	20	0.36	0.031	0%
2009 Nannie Island Water[b]				0.0097%
2009 Oyster River Water[b]				0.0064%
2008 Nannie Island				
	1		0.041	0%
	2		0.070	0%
	3		0.204	0%
	4		0.074	0%
	5		0.028	0.0121%
	6		0.056	0%
	7		0.020	0%
	8		0.073	0%
	9		0.107	0%
	10		0.033	0%
2008 Oyster River				
	13		0.066	0%
	14		1.800	0%
	15		0.114	0%
	17		0.035	0%
	18		0.035	0%
	19		3.420	0.0120%
	20		0.242	0%
	21		1.190	0%
	22		0.437	0%

[a]MPN was not calculated for 2008 samples

[b]MPN and APW enrichment qPCR was not performed for the water samples

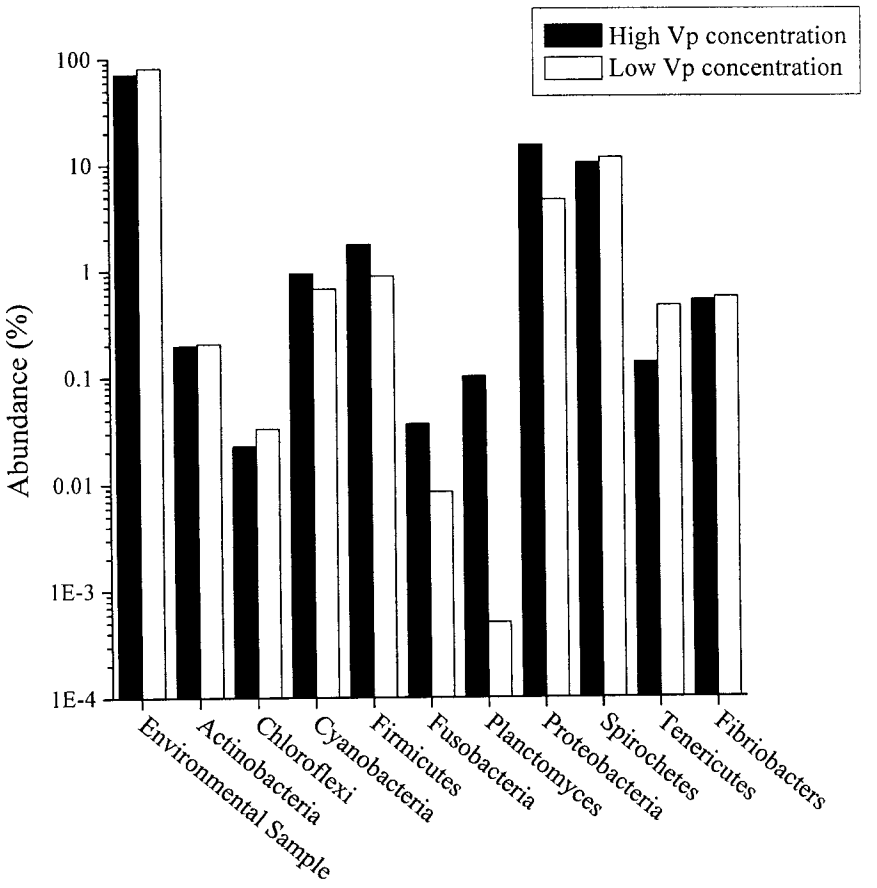

Figure 3.3: High versus low *V. parahaemolyticus* concentration oysters. The total percentage of reads representing different phyla for 8 elevated *V. parahaemolyticus* contaminated oysters and combined 12 low-contaminated oysters from the 2009 collection. The graph is presented on a logarithmic scale to visualize rarer representatives in the populations.

multiple oysters before performing an MPN evaluation may fail to reveal unsafe oysters present because the average concentration is within a safe threshold.

Before metagenetic community analysis of high and low contaminated oysters, we evaluated the validity of qPCR enrichment quantification that is the only available culture based enumeration for the 2008 collection. A direct comparison of qPCR on enrichments and the MPN from 2009 oysters demonstrated a poor correlation between the two methods (Pearson -0.059, $p = 0.805$) (Table 3.1 and Fig. 3.3), thus the 2008 oysters cannot be categorized by varying levels of *V. parahaemolyticus* based solely on qPCR of the enrichment. There is a positive correlation between 2009 MPN and abundance of the *V. parahaemolyticus* specific OCTU according to the metagenetic sequencing (Pearson 0.358, $p = .025$) (Table 3.1), but a Pearson's correlation approaching 1.0 is desirable to justify quantification from metagenetic data. Thus, qPCR of enrichment tubes is not an accurate measure of *V. parahaemolyticus* concentration, and metagenetic quantification correlates with the MPN enumeration, but not positively enough to confidently categorize the 2008 oysters.

Microbial communities differ between oysters with high and low *V. parahaemolyticus* concentrations. After we determined the oyster microbial population is unlike that of the overlying water, we performed similar comparisons between two sets of oysters with elevated or low *V. parahaemolyticus* concentrations determined by the 2009 MPN analysis (Table 3.1). Unlike the vast differences observed between the oyster and overlying water populations, high and low contaminated oysters differ only by one phyla. The proteobacteria in low-contaminated oysters comprise 5% of the population, whereas they comprise 15% of the population in the high-contaminated oysters (Fig. 3.4).

The low-contaminated oyster population contains 10% more environmental sample designated OCTUs, and these uncultured taxa may correlate with the displacement of *V. parahaemolyticus*.

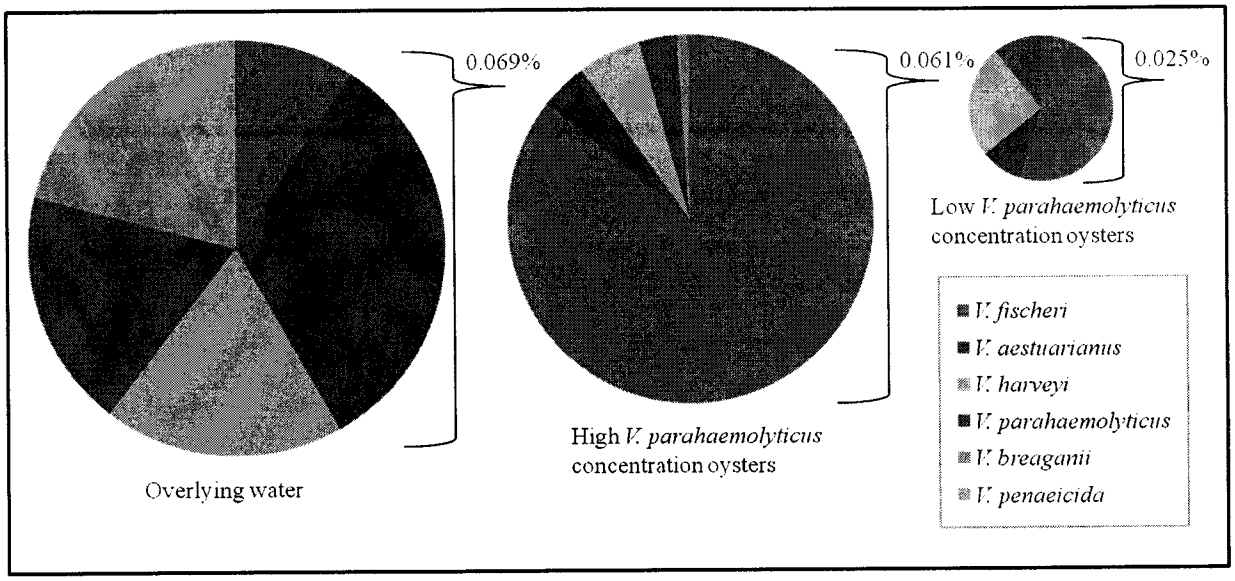

Figure 3.4: Distribution of total *Vibrio* in the overlying water, high and low *V. parahaemolyticus* concentration oysters. The pie charts reflect the relative abundance of the *Vibrio* species in each catagory, and the actual pies are sized relative to the total *Vibrio* abundance in each category. The number over each brace reports the abundance of total *Vibrio* in each category.

To determine if other Vibrios co-colonize the oyster along with *V. parahaemolyticus*, we evaluated the distribution of *Vibrio* in the high-contaminated, low-contaminated, and overlying water metapopulations. *V. fischeri*, a symbiont of many marine organisms (Nyholm and McFall-Ngai, 2004; Guerrero-Ferreira and Nishiguchi 2010; Mandel *et al.*, 2009), is found in the disproportionately high concentrations within the oysters compared to the overlying water, but is most abundant in the low-contaminated oysters (Fig 3.5). *V. aestuarianus*, a pathogen of the Pacific Oyster {Labreuche *et al.*, 2010}, is present in the oyster samples, but is most abundant in the overlying water. *Vibrio* diversity is highest in the low-contaminated oysters, but species

overlying water. *Vibrio* diversity is highest in the low-contaminated oysters, but species are more evenly distributed in the high-contaminated oyster (Fig. 3.5). The abundance of total *Vibrio* in the low-contaminated oysters is nearly 3 fold higher despite an average 10 fold greater abundance of *V. parahaemolyticus* in the high-contaminated sample. Based on these data, an elevated *Vibrio* concentration and a high proportion of *V. fischeri* colonization are consistent characteristics with each oyster evaluated in the low-contamination dataset.

FUTURE DIRECTIONS

Future directions of this work will specifically address hypotheses about how biological interactions within oyster influence population structure, with the caveat that the available data are limited to two sites over two years. For instance, analysis of the distribution of populations in individual oysters will provide further insight into commonalities of population structure specific to the host, as well as differences between individual oysters. We evaluated which taxa correlate with oysters with varying levels of *V. parahaemolyticus* contamination, and further analysis may identify whether specific taxa potentially associate with the pathogen. Additional bioinformatic pipelines will be written to perform these nuanced comparisons. With additional analysis, we will also address other specific questions, such as whether the microbial populations are stable and capable of persisting throughout harsh winters, or if structure varies from year to year. Further optimization of the bioinformatic pipeline will allow us to compare this data to the broader reported metagenetic data, to determine how the structure and diversity of our dataset compares to other marine environments.

Although we did not capitalize upon the fact that individual oyster metagenomes were sequenced for this study and instead used combined data, future analysis will take advantage of this data capacity. For instance, in our current approach utilizing pooled data, even a single anomalous oyster could result in misidentification of taxa correlating with *V. parahaemolyticus* abundance. Such approaches require additional script creation. To determine diversity between oysters and to account for any diversity within the environmental sample OCTUs, we will use phylogenetic comparisons using available algorithms which require some preliminary data processing and script creation. The

UNIFRAC module measures β-diversity using phylogenetic information specifically designed to compare communities from different populations (Lozupone and Knight, 2005). A benefit of this tool is total population OCTU comparison, including the uncultured environmental taxa, and assigns a confidence *p* value to differences between different populations. Once written, different grouping of the data can be arranged to address specific questions about community composition within and between sites, and between years.

Public databases and a growing collection of metagenetic reports will allow us to put the GBE population structure into the context of the global microbial community. Data already suggests that *V. parahaemolyticus* in the GBE is the most diverse ever described, and possibly one of the most recombinant microbial populations ever discovered (Ellis and Cooper unpublished). This is unexpected in a temperate estuarine environment where conditions change rapidly and drastically, generally thought to purge diversity. Our study will allow us to determine if this highly diverse species is indicative of a highly diverse population as well. In a new world where the environment is hugely influenced by human activity, it is imperative that we understand what affects we as a species have on the microbial communities which are the source of potentially devastating emerging pathogens.

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