ABSTRACT

Title of dissertation: Vibrios in the Environment: An Investigation of Environmental *Vibrio vulnificus, Vibrio parahaemolyticus,* and *Vibrio cholerae*

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Vibrio parahaemolyticus, Vibrio vulnificus, and *Vibrio cholerae* are Gram negative bacteria that naturally occur in marine and estuarine environment, both as free-floating cells or attached to chitinous surfaces. Although *Vibrio* spp. are readily isolated from the environment, not all strains are virulent. Therefore, the ability to detect the presence of vibrios is vital, but determination of pathogenicity is equally important. The research reported here was focused on prevalence and characteristics of environmental *Vibrio* species and how the environment provides a natural ecosystem for human pathogens and reservoir of virulence factors. Those objectives were achieved by carrying out intensive sampling over three years, during which water, sediment, and oyster samples were collected from the Chesapeake Bay, Maryland. Detection and molecular characterization of *Vibrio*

parahaemolyticus and *Vibrio vulnificus* were done and the diversity of *V. parahaemolyticus* and *V. vulnificus* isolates from individual oyster samples was investigated. The large-scale populations of *V. parahaemolyticus* and *V. vulnificus* in the Chesapeake Bay and smaller scale populations of individual oysters were analyzed, thereby providing a snapshot of *V. parahaemolyticus* and *V. vulnificus* distribution in the environment. Because antibiotic resistance is an increasing public health concern, antibiograms of *V. parahaemolyticus* and *V. vulnificus* isolates from environmental sources were done to determine antibiotic resistance patterns in environmental isolates. Detection and enumeration of *Vibrio* species are a concern since *Vibrio* spp. can enter viable by nonculturable (VBNC) state. Thus, new and improved *Vibrio* detection methods are needed. This this study the Cholera O1 and O139 SMART II test were investigated for potential use in detecting *V. cholerae* in ballast water treatment systems.

VIBRIOS IN THE ENVIRONMENT: AN INVESTIGATION OF ENVIRONMENTAL *VIBRIO VULNIFICUS, VIBRIO PARAHAEMOLYTICUS,* AND *VIBRIO CHOLERAE.*

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Dedication

I dedicate this work to my husband, parents and sister.
Your love and support has made this possible.

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Chapter 1: Introduction

Human pathogens are everywhere—they can occur in the air we breathe, in the food we eat, and in the water we drink. Although the societal belief is that they are the result of pollution or human interaction, most pathogens are autochthonous and exist ubiquitously in the natural environment.

Members of the genus *Vibrio* are gram negative bacteria that naturally occur in marine and estuarine environments—they persist in the aquatic environment as free floating organisms or by attaching to chitinous surfaces of crabs, shrimp, and zooplankton (Alam et al, 2009). Studies have shown that *V. parahaemolyticus* and *V. vulnificus* display a seasonal pattern, with highest prevalence in warmer summer months when the water temperature and salinity are optimal for their growth.

When environmental conditions are unfavorable, vibrios undergo a protective "viable but nonculturable" (VBNC) state, which allows the bacterial cells to become metabolically dormant (Colwell, 2000). In this state, they are difficult to isolate as they do not grow on routine laboratory culture media. However, when environmental conditions become favorable, typically triggered by warming temperatures and optimum salinity, the cells regain culturability and the capacity to cause infection (Oliver, 2010). *Vibrio* species are typically found in marine and estuarine environments and can be isolated when water temperatures persist between 9 and 31°C, with their ideal temperature being above 18°C and salinity between 15 to 25‰ (Strom and Paranjpye, 2000). Multiple species of *Vibrio* have been readily isolated from water, sediments, and marine organisms, the latter including, shrimp, fish, oysters, and clams (Jones and Oliver 2009).

Vibrio spp. are known to cause diseases and, therefore, the presence of these bacteria and their transmission from the natural environment, are of significant concern. There are at least eleven *Vibrio* species that have been determined to be pathogenic for humans, including *Vibrio vulnificus, Vibrio parahaemolyticus* and *Vibrio cholerae*. *V. vulnificus* and *V. parahaemolyticus* are the more common species and, in the developed world, the causative agents of illness, typically from consumption of raw or undercooked seafood or exposure during fishing or recreational swimming. In contrast, *V. cholerae* is a major contributor to disease in the developing world, where access to safe water and proper sanitation are lacking. In the United States, cases of *Vibrio* infections have been on a constant rise over the past 15 years—according to the Cholera and Other Vibrio Illness Surveillance (COVIS), in 2014, 1,252 cases of *Vibrio* infections (excluding *V. cholerae* O1 and O139) were reported in the United States alone (CDC, 2014). Between 2005 and 2013, there have been 326 reported cases of *Vibrio* related infection in Maryland (Agarwal, 2014). Of non-cholera related *Vibrio* infections, 38.9% (n=129) were traced to *V. parahaemolyticus* and 24.2% (n=80) to *V. vulnificus* (Agarwal, 2014). Although the number of cases per year is relatively low, the economic impact of *Vibrio* related disease is high—in 2013, the USDA estimates that the cost estimate for *V. parahaemolyticus* related disease alone is 43 million dollars per year (USDA, 2017).

Vibrio vulnificus can cause gastroenteritis, primary sepsis (resulting in fever, chills, nausea, hypotensive septic shock), necrotizing fasciitis, and severe wound infections (Horseman and Surani, 2011; Jones and Oliver, 2009). Infection can

occur by consuming contaminated seafood or through exposed, open wounds (Horseman and Surani, 2011). Mortality from septicemia caused by *V. vulnificus* can exceed 50% and death can occur within days of the first signs of infection. The mortality rate for *V. vulnificus* related wound infections is estimated at 25% (Oliver, 2006). The U.S. Food and Drug Administration has reported that an average of 34 cases of *V. vulnificus* related infections occur each year, with the majority reported from the Gulf Coast or of Gulf Coast origin (Horseman and Surani, 2011; Oliver 2006). The CDC reported *Vibrio* infections increased ca. 78% between 1996 and 2006 (Oliver, 2006) and steadily increased in subsequent years (CDC, 2016).

Vibrio vulnificus can be classified into three biotypes—Biotype 1 is related to human infections, Biotype 2 is related to infections of eels, and Biotype 3 was isolated from fish handlers in Israel (Bisharat et al. 1999; Strom and Paranjpye 2000). Biotype 1 can further be classified into genotypes *vcgC* and *vcgE,* representative of clinical and environmental origin, respectively. The main pathogenicity factors detected in *Vibrio vulnificus* include hemolysin cytolysin (*vvhA*), RTX toxin (*rtxA*) and type IV pili (*pilA*) (Johnson, 2013). Similarly*, V. vulnificus* lipopolysaccharide (LPS) of its outer cell membrane has been linked to severe infection resulting in death (Oliver, 2012).

V. parahaemolyticus is one of the leading causes of gastroenteritis associated with seafood consumption in the United States (Newton et al., 2012). This pathogen was first identified in the United States in 1971 in Maryland after three outbreaks of 425 gastroenteritis cases, in total, were found to be associated with consumption of improperly cooked crabs (Molenda et al., 1972).

Subsequently, sporadic outbreaks have occurred throughout the coastal United States (Letchumanan et al., 2014). In some of these cases, *V. parahaemolyticus* was found to cause wound and ear infections or septicemia that were life-threatening for individuals with pre-existing medical conditions (Zhang and Orth, 2013). According to the Centers for Disease Control and Prevention, infections by *V. parahaemolyticus* are estimated to have an annual rate of 4,500 cases per year (CDC, 2006). In August, 2012, a *V. parahaemolyticus* outbreak that included six individuals occurred in Maryland and those outbreak isolates were found to be linked to the O3:K6 pandemic clone of *V. parahaemolyticus* (Haendiges et al., 2014). The major virulence factors for *V. parahaemolyticus* include hemolysin, thermostable direct hemolysin (*tdh*), thermostable related hemolysin (*trh*), and thermolabile hemolysin (*tlh*) (Johnson, 2013). While *tlh* is present in all *V. parahaemolyticus* strains, *tdh* and *trh* are typically associated with clinical isolates (Johnson, 2013). In addition, the Type 3 secretion systems (T3SS1 and T3SS2) are also *V. parahaemolyticus* virulence factors causing enterotoxicity and cytotoxicity (Johnson, 2013).

Vibrio cholerae, the causative agent of cholera, is autochthonous to marine and estuarine environments. Cholera, a disease typified by severe, watery diarrhea, can result in death unless timely rehydration therapy is employed. Of the more than 200 serotypes of *V. cholerae,* most have been associated with mild gastroenteritis and sporadic local outbreaks of cholera (Chatterjee et al., 2009). However, only toxigenic strains of *V. cholerae* O1 and O139 serotypes have been linked to epidemics and pandemics (Chun et al., 2009).

Vibrio spp. have been shown to attach to zooplankton rich in chitin, which serve as food source for the bacteria as they travel into surface waters (Kaneko and Colwell, 1973). Once in the photic zone of surface waters, these heterotrophic bacteria are sustained by dissolved organic matter (DOM) from phytoplankton, supplied by processes of excretion, exudation, and cellular death (Smith et al., 1995). *Vibrio* spp. have been monitored for decades and previous studies have shown that the key determinants for its prevalence are temperature and salinity (Johnson et al., 2012). Typical temperature ranges for *V. parahaemolyticus* and *V. vulnificus* are between 7-36°C and salinity ranges between 5 and 25 ppt (Johnson et al., 2012; Motes et al., 1998). However, when environmental conditions are unfavorable, these bacterial cells can persist in sediments where they can enter the viable but non-culturable (VBNC) state until water temperatures rise, and nutrients become more readily available (Kaneko and Colwell, 1973; Colwell, 2000). This VBNC state allows the bacteria to become metabolically dormant under limiting nutrition conditions (Nowakowska and Oliver, 2012). Once environmental conditions are again favorable, the bacteria can revert from the VBNC state to become metabolically active and culturable again.

The Chesapeake Bay is the largest estuary in the United States and the third largest in the world and produces about 500 million pounds of seafood each year (Chesapeake Bay Program, 2017). Due to its vastly complex ecosystem, the Chesapeake Bay has been the subject of many scientific studies for the past hundred years. The previous studies have shown that *V. parahaemolyticus* and *V. vulnificus* display a seasonal pattern of occurrence, with highest prevalence in warmer

summer months when water temperature and salinity are optimal for bacterial growth. Reports of *Vibrio* infections have been on a constant rise in the United States over the past 15 years. Therefore, determining environmental parameters influencing the cyclic nature of *Vibrio* occurrence in the environment is essential to detect *Vibrio* related disease. In addition, characterization of *Vibrio* spp. is important to track the transmission of virulence factors in the environment. And, equally important, determine occurrence of antibiotic resistance in those organisms in native waters.

Specific Aims

Specific Aim 1: determine the prevalence of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in Chesapeake Bay water, sediment, and oysters and those environmental parameters related to their occurrence.

Specific Aim 2: determine the potential pathogenicity of *Vibrio* spp. isolated from water, sediment and oysters, including assessment of numbers in individual oysters.

Specific Aim 3: Determine the antibiotic profiles of environmental *V. vulnificus* and *V. parahaemolyticus* isolates.

Specific Aim 4: Evaluate Cholera SMART II, a rapid method for detection of *V. cholerae* O1 and O139 and its potential application as a method for testing ballast water.

These specific aims will be addressed in the following chapters. First, the presence of vibrios in seafood is a public health concern because many marine organisms (seafood) harbor *Vibrio* spp., especially oysters, often consumed raw. According to the CDC, *Vibrio* related illnesses are estimated at 80,000 cases

annually, with 100 deaths occurring in the United States each year. Chapters 2-4 deal with detection and characterization of *V. parahaemolyticus* and *V. vulnificus* in water, oyster, and sediment samples collected from the Chesapeake Bay, Maryland.

Although *Vibrio* related gastroenteritis often do not require antibiotics for treatment, in cases where the patients are vulnerable (infants or elderly), having underlying medical condition, or suffering from prolonged illness with high fever, antibiotics are prescribed. The current trend in the increase of multi-drug resistant bacteria, however, is a major concern. Therefore, Chapter 5 investigates the antibiotic resistance patterns of *Vibrio vulnificus* and *Vibrio parahaemolyticus* isolates from the Chesapeake Bay. As mentioned earlier, the vast majority of *Vibrio*-related illnesses are due to consumption of raw or undercooked seafood. For that reason, Chapter 6 addresses the genetic diversity of *Vibrio parahaemolyticus* and *Vibrio vulnificus,* notably those isolates from individual oyster samples. Finally, Chapter 7 provides an evaluation of a newly developed method for rapid detection of *Vibrio cholerae* O1 and O139 for testing ballast waters.

Chapter 2: Detection and Characterization of *Vibrio vulnificus* and V*ibrio parahaemolyticus* in Water, Oyster, and Sediment Samples Collected from the Chesapeake Bay, Maryland

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ABSTRACT:

Vibrio vulnificus and *Vibrio parahaemolyticus* are human pathogens that are responsible for thousands of food-borne and water-borne infections in the United States each year. *Vibrio* spp. are autochthonous in marine and estuarine environments and, therefore, the abundance and distribution of *Vibrio* spp. are critical for disease prevention. In this three-year study, water, oyster, and sediment samples were collected from two sites, the Chester River and Tangier Sound in the Chesapeake Bay. Occurrence of total and pathogenic forms of *V. vulnificus* and *V. parahaemolyticus* were determined using conventional and real-time PCR and a quantitative direct-plating method, followed by DNA colony hybridization. In both the Chester River and Tangier Sound, the two *Vibrio* spp. were present in higher abundance during the summer and fall months; with incidence of *V. vulnificus* peaking during July and October and *V. parahaemolyticus* in July and August. *V. vulnificus* was found to be present in significantly higher numbers in all sample types at both sites. Results of this study showed that chlorophyll-a, dissolved oxygen, and water temperature were significantly associated with *Vibrio* spp. density and incidence of *V. vulnificus* was additionally associated with turbidity and salinity.

INTRODUCTION:

Marine and estuarine *Vibrio* species, notably *V. vulnificus* and *V. parahaemolyticus,* have long been established as native to the aquatic environment and present in the water column, sediment, and in filter feeders, e.g., shellfish (Vezzulli et al., 2010; Pruzzo et al., 2008; Colwell, 1996). The global occurrence of *Vibrio* spp. in rivers, estuarine and coastal waters, and the deep sea is significantly influenced by prevailing environmental conditions and, therefore, can pose a significant public health threat to various countries and cultures (Johnson et al., 2012). Both *Vibrio vulnificus* and *Vibrio parahaemolyticus* can cause mild to severe symptoms leading to health threats, including gastroenteritis and septicemia (Wright et al., 1996; Oliver, 1989; Tamplin and Caspers, 1992).

In the United States, *Vibrio parahaemolyticus* is responsible for approximately 1% of all food borne illnesses and is the leading cause of bacterial gastroenteritis associated with seafood consumption. *Vibrio vulnificus* can cause primary septicemia associated with open cut skin and often from raw oyster consumption or individuals are exposed to contaminated seafood at the seafood during processing centers. The mortality rate of *V. vulnificus* induced septicemia can be as high as 50% or greater and death may occur within 48 hours. In contrast, the mortality of individuals suffering *V. vulnificus* infected wounds is ca. 25%. *V. vulnificus* infected wounds are unsightly, with dangerous tissue damage, and these wound infections occur if an abrasion or open wound is exposed to contaminated seawater. In total, *Vibrio vulnificus* and *Vibrio parahaemolyticus* are responsible for approximately 8,000 food-borne and non-food borne infections per year in the United States, with the majority occurring during the warmer months of the year

when environmental conditions are favorable for their growth (Johnson et al., 2012).

V. parahaemolyticus hemolysin genes have long been epidemiologically associated with disease--the majority of clinical strains demonstrate hemolytic activity (Kanagawa phenomenon) and presence of the genes, therefore, constitutes an important indicator of their pathogenicity (Nishibuchi and Kaper, 1995). *Vibrio parahaemolyticus* produces three types of specific hemolysins, thermostable direct hemolysin (*tdh*), tdh-related hemolysin (*trh*), and thermolabile hemolysin (*tlh*). Of these three hemolysins, both *tdh* and *trh* have been shown to be associated with the pathogenic form of *Vibrio parahaemolyticus,* infection with which results in gastroenteritis in humans. Interestingly, *tlh* is used as a marker to measure the total number of pathogenic *V. parahaemolyticus* in a sample and the hemolysin gene, *vvhA,* is employed to detect the total number of *V. vulnificus* in a sample (Kishishita et al., 1992; Nishibuchi and Kaper, 1995; Johnson et al., 2010; Wright et al., 1993).

Vibrios have been isolated from many different, mainly aquatic organisms, ranging from seabirds, fish, shrimp and seaweed, to filter feeders, such as mussels and oysters. Most seafood requires processing, mainly cooking or freezing, before consumption. Oysters are unique in that they are commonly consumed raw, and the oyster industry enjoys an extensive worldwide market. According to the Natural Resources Defense Council, 35.5 million pounds of oysters are consumed in the US annually. Seafood safety and hygiene, especially for oysters, receive significant attention, notably with respect to pathogenic microorganisms. Because oysters are filter feeders, they are able to concentrate bacteria, especially *Vibrio* spps., to such

an extent that the number of these bacteria in oysters will be much higher than in the surrounding water (Newton et al., 2012). *V. vulnificus* has been suggested to be commensal to oysters, that is, to exist alongside, or more specifically, inside oysters (Oliver, 1989; Tamplin and Capsers, 1992; Iwamoto et al., 2010). The oysters therefore, often act either as a reservoir for *Vibrio* bacteria, serving as a protective niche when environmental conditions become harsh, e.g., winter temperatures, or as "passive concentrators," artificially raising the concentration of *Vibrios* by extracting them from the water column (Vezzulli et al., 2010; Wright et al., 1993). One of the major concerns associated with these bacteria is the increase in cases of *Vibrio* related diseases in America reported by local and state health authorities, as well as the CDC (Newton et al., 2012; Iwamoto et al., 2010). The diseases reported are often those associated with consumption of raw or undercooked seafood and frequently the source of infection proves to be oysters (Slayton et al., 1967; Wallace et al., 1999; Wechsler et al., 1998). This association most likely is reflective of the fact that mollusks often act as reservoirs for the bacteria, with vibrios frequently detected in freshly harvested oyster meat and shell stock (Slayton et al., 1967; Ciij et al., 2002; DePaola et al., 2003).

The human health effect risk that *Vibrio* spp. pose is the most serious component in the relationship of *Vibrio* spp. with mollusks. *Vibrio vulnificus*, for example, is associated with a 50% mortality rate when an infection with *V. vulnificus* develops into septicemia (CDC, 2016; Hilton et al., 2006; Bross et al., 2007). Furthermore, as stated earlier, *V. vulnificus* illness is associated with unsightly and dangerous tissue damage if an abrasion or open wound is exposed to

contaminated seawater and ingestion of oysters containing *V. parahaemolyticus* can result in severe gastroenteritis (Bross et al., 2007; Chatzidaki-Livanis et al., 2006).

In addition to water and shellfish, sediment has also been identified as a reservoir for *V. parahaemolyticus*. It has been demonstrated that the overall population of *Vibrios* spp. can be concentrated in sediment. A study carried out in the Chesapeake Bay by Kaneko and Colwell (1973), discovered *V. parahaemolyticus* is associated with sediment and can survive the near freezing temperatures of the Chesapeake Bay in sediment during the winter months (Kaneko and Colwell, 1973). Similarly, on the West Coast of the U.S. in Washington State, it was shown that large numbers of *V. parahaemolyticus* could be found in sediment (Baross and Liston, 1970). In a parallel study, we reported earlier that the concentration of *V. parahaemolyticus* was higher in sediment than *V. vulnificus*, whereas the opposite was the case for oysters and their surrounding water, indicating *V. parahaemolyticus* survives well in a in sediment rich environment (Johnson et al., 2012). This is an important observation since large numbers of *V. parahaemolyticus* can be found in sediment, with the potential for reintroduction into the water column when sediment is disturbed by currents or mechanical action (Johnson et al., 2012).

Understanding the environmental influence on distribution and growth of *Vibrio* spp. is important, especially with respect to seasonality. Quantification of the public health risk, namely incidence of *Vibrio* related infections by season is very important as has been shown (Paranjpye et al., 2015). Several studies have successfully linked presence of *Vibrio* spp. and epidemiology with predictable

patterns related to changing seasons in the Chesapeake Bay and globally (Paranjpye et al., 2015).

The Chesapeake Bay is an estuarine environment where salinity and nutrients vary depending on the influx of freshwater (Banaker et al., 2011). Previous studies have shown that *V. vulnificus* can be detected over a very wide range of temperature, from 8ºC to 26ºC (Wright et al., 1996). During colder months of the year when the temperature is lower, and the temperature range is narrow, *V. vulnificus* is either not detected or detected only by employing molecular probes rather than culture. In any case, distinct seasonality of *V. vulnificus* in the Chesapeake Bay has been reported (Wright et al., 1996; Kaneko and Colwell, 1973; Kaneko and Colwell, 1975; Kaneko and Colwell, 1975). For *Vibrio parahaemolyticus*, the minimum growth temperature is near or above 17 ˚C and salinity near or below 13 ppt (Kelly and Stroh, 1988). *V. vulnificus* does not grow at salinities above 25 ppt with an optimal growth temperature near 37 ˚C (Kelly, 1982; Motes et al., 1998; Kaspar and Tamplin, 1993; Drake et al., 2007). In addition to physical and chemical variables associated with growth of *Vibrio* spp., other factors such as pressure can influence growth of these bacteria. At greater depths in the water column, there is a statistically significant increase in the number of *V. vulnificus* (Wetz et al., 2014; Wise and Gallagher, 1996).

MATERIAL AND METHODS:

1.1. *Sample Collection:*

Sample Collection: Water, oyster, and sediment samples were collected over a three-year period from June, 2009, to August, 2012 at two locations in the Chester

River (39°05.09'N, 76°09.50'W) and Tangier Sound (38°10.97'N, 75°57.90'W) in the Chesapeake Bay, Maryland (Figure 1). During warmer months of June through August, sampling was carried out twice each month and once each month during September through May. Selected physical and chemical measurements, such as water temperature, conductivity, pH, dissolved oxygen, salinity, total dissolved solids (TDS), and turbidity, the latter measured using a secchi disk, and chlorophyll a, were determined in water samples collected at the surface (1ft) and bottom (1ft from the bottom) at each site, using a digital handheld meter (model 30-25FT; Yellow Springs Instruments, Yellow Springs, OH). In addition, air temperature, weather, wind direction, wind velocity, tide and prior rain events were also recorded. At each site, 12 liters of water, 20-25 oysters, and 80-100g of sediment (collected from oyster boxes or beds) were collected. Samples were maintained in a cool container with chilled packs during transportation to the laboratory at the University of Maryland at College Park, MD. Upon arrival, the samples were stored overnight at 15°C until processing the following morning.

Sample Processing. Details of sample processing have been described elsewhere (Johnson et al., 2010). In brief, water samples were shaken and three volumes (1000ml, 100ml, 10ml), each in triplicate, were inoculated into alkaline peptone water (10× APW, pH 8.5) (111ml, 11ml, 1.1ml, respectively) and incubated at 35ºC for 16-18 hours, with shaking at 30 rpm. Additionally, 5.5 liters of the water samples were filtered through a 20-micron net to collect a 25ml plankton sample. A subset of the filtrate, 200 ml plankton free water, was filtered through a 0.22µm filter and resuspended in 1×PBS to collect bacteria present in the plankton-free

water. Plankton and plankton free samples were enriched with 1×APW and incubated overnight. Oysters were rinsed and scrubbed under running tap water, shucked, and the oyster tissue homogenized 1:1 with 1× phosphate buffer solution (1× PBS; pH 7.4) in a sterile blender for 90 seconds. Homogenized oyster tissue was inoculated (10g, 1g, 0.1g, in triplicate) into 10× APW and incubated at 33°C for 16-18 hours, with shaking at 30 rpm. Sediment samples were weighed and vortexed in equal part 1× PBS, after which 10× APW was added and the samples incubated for 16-18 hours, with shaking at 30 rpm. Simultaneously, samples of water, homogenized oyster tissue and sediment were spread plated onto T1N3 (1% tryptone, 3% NaCl, pH 7.2) and VVA (2% peptone, 3% NaCl, 1% cellobiose, 0.06% bromthymol blue, pH 8.2) agar for colony blot hybridization. For water samples, 1ml was used. For oyster samples, 0.2 grams (final yield 0.01 grams) and 100µl (final yield 0.1g grams) were used. For sediment samples, 0.05 grams, 0.01 grams, 0.005 and 0.001 grams were subjected to analysis. Plates were incubated overnight at 33ºC before colonies were lifted for colony blot hybridization. The following day, an aliquot of each of the overnight samples was collected and DNA was extracted.

DNA extraction. A 1.0 ml aliquot of overnight sample was boiled for 10 minutes at 99ºC, placed on ice, and centrifuged for 10 minutes at 13G. A portion of the supernatant was transferred to a clean, sterile test tube and adjusted to concentration for PCR analysis.

Conventional PCR. Multiplex PCR targeting the *toxR* gene (Bauer and Rørvik, 2007) was used to differentiate *V. parahaemolyticus, V. vulnificus,* and *V. cholerae.*

Subsequent multiplex PCR targeting virulence factors, *tlh, trh* and *tdh* (Bej et al., 1999) and simplex PCR targeting *vvhA*, were performed. All PCR assays were performed using Promega GoTaq Green Master Mix (Promega, Madison, WI). Each reaction tube contained a total of 25µl, including 5µl template DNA. Thermal cycling conditions were as follows: one 10-minute cycle of denaturation at 94°C, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 60 seconds, and final extension for 10 minutes at 72°C. PCR products were stored at 4°C until gel electrophoresis visualization. Sequences, amplicon size and annealing temperatures for each PCR can be found in Table 1. Positive controls included VPTX2103, VPFIHES98, VPAQ4037, and VPF11-3A for *V. parahaemolyticus* and VVBUF for *V. vulnificus*. Sterile water was used as negative controls in all PCR reactions.

Multiplex Real-Time PCR. Real-Time PCR was performed using a similar protocol as described by a previous study performed by Nordstrom et al. to detect total and pathogenic *V. parahaemolyticus,* employing the *tlh, trh, tdh* genes (Nordstrom et al, 2007). Details of the protocol are provided by Johnson et al., 2012. Primers for *tlh, trh, tdh*, internal amplification control (IAC), as well as *tlh* and IAC probes were produced by Integrated DNA Technologies (Coralville, IA). Probes for *tdh* and *trh* were obtained from Applied Biosystems (Foster City, CA). IAC DNA was obtained from BioGX (Birmingham, AL). Sequences for primers and probes can be found in Table 2. For each 25µl reaction, final concentrations for each reactant was as follows: 1× buffer, 5mM $MgCl_2$, 0.4mM dNTPs (equal parts), 0.75µM for *tlh* and IAC forward and reverse primers, 0.2µM for *trh* and *tdh* forward

and reverse primers, 0.150µM *tlh* and IAC probes, 0.075 µM *trh* and *tdh* probes, 2.25 units/µl Platinum Taq polymerase, 2µl IAC DNA and 2µl target DNA. Real time PCR tests was carried out using an AB 7500 thermal cycler (Applied Biosystems, Carlsbad, CA). The cycling parameters consisted of an initial 60 second denaturation step at 95°C and a 45 cycles of 5 second denaturation at 95°C, followed by a 45 second annealing and extension step at 59°C. Fluorescence data were collected at the end of each amplification cycle.

Colony blot hybridization

Growth on T1N3 plates was used for enumeration of *V. parahaemolyticus* (*tlh, trh, tdh*) while VVA plates were used to enumerate *V. vulnificus* (*vvh*). After overnight growth, colonies were lifted and probed with alkaline phosphatase-labeled oligonucleotide probes (DNA Technology A/S, Risskov, Denmark) specific for *vvh, tdh, trh*, and *tlh.* Probe sequences are described in Table 3. After overnight growth, colonies were lifted using 85mm Whatman #541 filter paper. Lysis solution (0.5M NaOH, 1.5M NaCl) was used to lyse cells followed by ammonium acetate buffer (2M) for neutralization. Filters were rinsed twice with 1×SSC before drying prior to probing. Filters were incubated with 1×SSC and 20µl/filter of proteinase K for 30 minutes at 42ºC and rinsed three times with 1×SSC. The filters were added to plastic bags filled with 10ml hybridization buffer (BSA, SDS, PVP-360, 5×SSC) and incubated at 55ºC for 30 minutes. Hybridization buffer was decanted from the bags and fresh hybridization buffer was added, along with *tlh, trh, tdh* or *vvh* probes. Filters were incubated for 1 hour at 50ºC and rinsed twice with 1×SSC/SDS (*tlh,*

trh, vvh) or 3×SSC/SDS (*tdh)*. All filters were rinsed five times with 1×SSC, after which 20ml/filter of NBT/BCIP (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate) solution (Roche, Madison, WI) was added and the filters incubated in the dark at room temperature for 1-2 hours. The reaction was stopped by placing the filters in deionized water for 10 minutes. The filters were allowed to air dry before positive colonies were counted. Positive *V. vulnificus* and/or *V. parahaemolyticus* were described as bluish-gray, purple or dark brown colonies. Negative colonies were colorless, yellow, gray, or light brown.

Water quality analysis. Total suspended particulate matter (SPM), chlorophyll-a (chl-a), and dissolved organic carbon (DOC) samples were collected at each site. For SPM, pre-dried GF/F filters were weighed before ca. 100ml – 200ml of whole sample water was filtered. Filters were dried overnight at 65°C before being reweighed on a high precision scale. For chl-a, whole sample water volumes of between 100ml to 200ml sample water were filtered in triplicate, using 25-mm diameter GF/F filters, and stored at -20°C until analysis. Chlorophyll-a and other pigments were measured in methanol extracts using a Cary model 50 UV–visible-light spectrophotometer. For DOC, whole water samples were filtered using a pre-combusted GFF filter and the collected filtrate was used to rinse equipment. After rinsing, 100ml of whole water was filtered, and the filtrate was used to rinse three individual HDPE bottles three times. After rinsing, the HDPE bottles were filled with freshly filtered water and concentrated HCl was added to convert the inorganic carbon to CO_2 with the bottles uncapped and left standing overnight. Samples were capped the next day and stored at -20°C before measurements were taken using a

Shimadzu TOC-V CSN carbon analyzer equipped with an ASI-V autosampler (Shimadzu Scientific Instruments, Columbia, MD).

1.2. ***Statistical methods:***

Violin plots were used to explore the relative distribution of *Vibrio* genes selected for study (i.e. *tlh, trh, tdh, vvhA*) in the three sample types (water, oyster, and sediment). A violin plot combines a boxplot with kernel densities (Hintze, 1999). Ternary density plots were used to capture abundance of *V. parahaemolyticus* and *V. vulnificus* across each sample type. Water, sediment and oyster samples were used to explore relative incidence of each of the four genes, with respect to sample type. A ternary plot is a barycentric plot of three variables which sums to a constant and graphically depicts the ratios of the three variables as positions in an equilateral triangle.

Incidence of autochthonous *V. vulnificus* containing *vvhA* and *V. parahaemolyticus* containing *tlh, tdh, and trh* at each of the two sampling sites in the Chesapeake Bay, namely the Chester River and Tangier Sound, were depicted using individual circular seasonality plots with combined scatterplots to visualize and detect the seasonality pattern across genes (Barnett et al, 2012). Descriptive seasonal circular plots were adopted to capture the seasonality patterns of *Vibrio* genes for each of the samples and sites in the Chesapeake Bay. The circular seasonal plot, commonly known as a “rose diagram”, is a powerful visual tool to summarize seasonal behavior of pathogens like *V. vulnificus* and *V. parahaemolyticus* and their annual seasonal cycles (Barnett et al, 2012). In the circular rose diagram, the radius is

proportional to the mean of the sample. Rose plots are more effective than traditional scatterplots in the context of seasonality because, for example, they place January and December values side by side, allowing for a clearer depiction of the cyclic nature of seasonal trends. The petals of the rose are representative of the mean density of vibrios in a month. Color gradient of the petals are deeper for low density and improve visibility against the background.

The one-way ANOVA method was used to determine if abundance of the *Vibrio* spp. was different within three scenarios: a) densities differ by sample, e.g., water, oyster, and sediment; b) with respect to genes, e.g., *tlh, tdh, trh*, and *vvhA*; and c) the two sites. The method of analysis has been used widely and has been a staple of statistical modeling for many years (Stahle and Wold, 1989). By comparing variances of the data, ANOVA examines the squares of differences, both between samples or groups, as well as between data sets within samples (Stahle and Wold, 1989). For differences in the mean conditions, the 95% confidence interval plot was employed to determine whether density varied with respect to the three scenarios.

Principal component analysis was used to explore interlinkages of the two *Vibrio* spp. with ecohydrological variables (Pearson, 1901). Principal component analysis, frequently used in biological studies and one of its many strengths is the ability to analyze complex data efficiently without losing the original meaning of the data (Landgrebe et al, 2002; Khan, 2001; Li and Klevecz, 2006; Alter et al., 2000; Holter et al, 2000; Dunteman, 2011; Abdi and Williams, 2010; Jolliffe, 2002). The method can be used to explain variability of data and remove factors not accounting for variation (Dunteman, 2011; Abdi and Williams; 2010; Jolliffe, 2002)

The two-dimensional principal component (PC1 and PC2) loadings plot was employed to determine correlation of the ecohydrological variables measured in this study with the incidence of genes *tlh, tdh, trh* and *vvhA*. The magnitude of the covariation of two variables is determined by their cosine angle, 0º – 180º suggests a positive correlation and 180º – 360º is negative correlation.

2. RESULTS AND DISCUSSION:

2.1. Distribution and abundance of selected *Vibrio* genes across samples

A total of 54 samples were collected monthly for three years, and these yielded violin plots descriptive of the distribution of the selected *Vibrio* genes across samples, based on colony blot hybridization data (Figure 2). The Chester River (CR) and Tangier Sound (TS) sites yielded *V. vulnificus* at densities approximately two-fold higher than *V. parahaemolyticus* across all three sample types, with predominance (~71%) in water. The TS samples showed *tlh* genes were present in large numbers, as was *vvhA*. The *tlh* genes were present in high in number in the oyster (~40%) and sediment samples (45%), and low in water. In contrast, *vvhA* was detected at high densities in water (66%), compared to sediment and oysters. The *vvhA* gene in the CR was present at density (46%) in oyster samples but relatively low in water (24%) and sediment (30%). The *trh* genes were detected least often (5%) in all samples collected at both sites, notably in both locations, especially oyster samples. *Tdh* was least detected (<2%) in water and sediment samples in both the CR and TS. Peak densities of *vvhA*, *trh*, and *tlh* were detected in oyster samples, i.e., 202 cfu/gm and 68 cfu/gm in CR samples and 131 cfu/gm in TS (Figure 2). Results for the *tdh* gene were the opposite, low count of 34 cfu/ml in TS water and sediment samples and 11 cfu/mg oyster sample in TS.

The distribution of the two *Vibrios* spp. in samples was analyzed by plotting ternary densities, using combinations of percent *Vibrio vulnificus* and *Vibrio parahaemolyticus* in water, sediment, and oyster as the three variables (Figure 3). Samples from the CR yielded a triangular proportional display that indicates CR oyster and sediment had high densities. This suggests the two *Vibrio* spp. are concentrated in oysters and sediment. In the TS, both water and sediment samples had moderate concentration of the two *Vibrio* spp. However, the results also indicate oysters are the main reservoir of the two *Vibrio* spp. in the CR and TS.

2.2. **Seasonality of *V. parahaemolyticus* and *V. vulnificus***

Articulating seasonality patterns of selected genes of the two pathogenic *Vibrio* spp. is important for prediction of the potential public health risk. This information provides both timing and intensity of *V. parahaemolyticus* and *V. vulnificus* and allows modeling the public health risk.

Vibrios in the CR are present in highest number in oyster samples during warm summer months (June to August) and in the fall of the year (September to November) (Figure 4). The *tdh* and *trh* genes are detected in higher numbers during summer season, peaking in August and July respectively. In contrast, *vvhA* and *tlh* were detected in large numbers during the fall, peaking in September and October, respectively. *Vibrio parahaemolyticus* was detected in sediment samples in May, peaking at 34/gm, 52/gm, and 39/gm for *tdh, tlh,* and *trh,* respectively. *V. vulnificus* was detected in highest numbers in July, with *vvhA* highest at 145/gm sediment. Vibrios were detected in water samples only in the summer, with *tlh* and *tdh* peaking in June at 32/ml and 26/ml, respectively and *vvhA* present throughout summer

months, with peak density for *vvhA* in July at 80/ml water.

At the TS site, vibrios in oyster samples were higher in density from June to October. Most *V. parahaemolyticus* strains containing *tdh, trh* were common solely in the summer, with *tlh* showing dominance in both summer and fall. The peak density for *tdh* and *trh* was July and *tdh* in August. In contrast, *vvhA* was present in significant numbers only in October, with a peak of 145/gm oyster. *Vibrio* genes in sediment samples showed variable in distribution during summer and fall. *tlh* in sediment showed abundance during these two seasons with a peak of 89/mg in July. However, *tdh* was high in June and *trh* in November respectively. *VvhA* are abundant mainly during early fall, in September and October, with peak density of 95/mg sediment observed in August. Unlike the CR site, *Vibrio* spp. in the water samples in TS were present in large numbers only during May, with *V. parahaemolyticus tdh, tlh, trh* genes at peak densities of 34/ml, 52/ml and 39/ml respectively and *V. vulnificus* (*vvhA*) in July with highest number at 145/ml.

2.3. Variability of *Vibrio* seasonality with respect to sample type, genes, and location

To test whether abundance of *Vibrio vulnificus* or *Vibrio parahaemolyticus*, water, oyster, and sediment differed by site, one-way ANOVA was employed. The previous analysis indicated oysters had highest number of both *Vibrio* spp. One-way ANOVA, with Tukey's post hoc test, indicated only *Vibrio* spp. in water and oysters were statistically significant. ANOVA test results, comparing CR and TS, showed the sites were not significantly different.

2.4. Linkage of *Vibrio* spp. by sample type

Vibrio spp. and genes were examined with respect to environmental

variables (Figure 5). PC loading plots indicated water temperature, air temperature, dissolved oxygen, chlorophyll-a, salinity, and total dissolved solids are representative variables for the two *Vibrio* spp., with respect to sample type (water, sediment, and oyster) and site (CR, TS). The three *V. parahaemolyticus* genes were found to be closely associated with water temperature, air temperature, dissolved oxygen, and chlorophyll across samples. Specifically, *tdh* showed the most consistent relationship with these variables across all three sample types.

The *V. vulnificus* associated *vvhA* gene was associated with salinity, total dissolved solids, and pH, particularly for TS sediment samples and CR water samples.

3. CONCLUSIONS:

Oysters serve as a reservoir for *V. parahaemolyticus* and *V. vulnificus* in the Chesapeake Bay. As filter feeders, oysters filter large quantities of water, therefore, are readily colonized by these bio-accumulated bacteria. During warm months of the year, the overall bacterial load is generally high, and oysters can acquire large numbers of bacteria in general. An interesting discovery from this study is the strong seasonality observed for *Vibrio vulnificus* and *Vibrio parahaemolyticus.* In both the Chester River and Tangier Sound, the two *Vibrio* spp. were present in high abundance in the summer and fall, substantiating findings of previous studies. *Vibrio* populations overall in oysters were higher during June-August and September-November. *V. vulnificus* was present at peak concentrations during July and October and *V. parahaemolyticus* in July and August. Compared to *V. parahaemolyticus, V. vulnificus* was present in significantly higher numbers in all sample types and at both sites in the Chesapeake Bay, a serious threat and more so

than *V. parahaemolyticus* in the Chesapeake Bay. It is important to note that the colony blot method employed in this study is culture dependent. Based on experience, the ability of all *V. vulnificus* present in the samples to grow on standard bacteriological media depends significantly on environmental parameters and colony blot analysis need to take this into account. Results of this study showed chlorophyll-a, dissolved oxygen, and water temperature were significantly associated with *Vibrio* spp. density. Furthermore, incidence of *V. vulnificus* was also associated with turbidity and salinity. The cumulative findings of this and earlier studies are now a significant component of predictive models for these pathogens and incidence of *Vibrio* disease.

Figure 1. Map of Chesapeake Bay showing sampling sites in the Chester River and Tangier Sound.

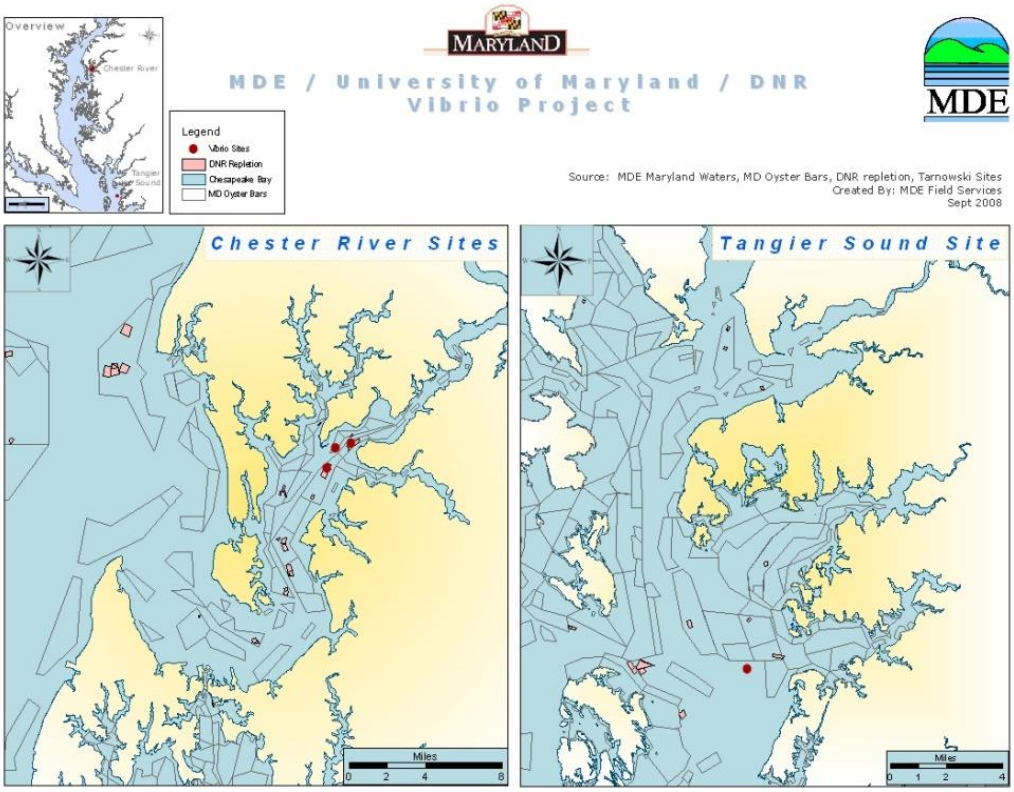


Table 1. List of primers used in this study for conventional PCR, including annealing temperatures and sequences

Primers	**Primer sequence (5' – 3')**	**Amplicon (bp)**	**T_a (°C)**	**Reference**
utox-F	GASTTTGTTTGGCGYGARCAAGGTT		55	(Bauer and Rørvik, 2007)
vptox-R	GGTTCAACGATTGCGTCAGAAG	297		
vvtox-R	AACGGAACTTAGACTCCGAC	640		
vctox-R	GGTTAGCAACGATGCGTAAG	435		
tlh-L	AAAGCGGATTATGCAGAAGCACTG	450	58	(Bej et al., 1999)
tlh-R	GCTACTTTCTAGCATTTTCTCTGC			
tdh-L	GTAAAGGTCTCTGACTTTTGGAC	269		
tdh-R	TGGATAGAACCTTCATCTTCACC			
trh-L	TTGGCTTCGATATTTTCAGTATCT	500		
trh-R	CATAACAAACATATGCCCATTTCCG			
vvh-F	ATTCCAGTCGATGCGAATACGTTG	205	55	(Brasher et al, 1998)
vvh-R	TTCCAACTTCAAACCGAACTATGA			

.

Table 2. Primers and probes used for real-time PCR (Nordstrom et al., 2007)

Primer	**Primer sequence (5' – 3')**
tlh-F	ACTCAACACAAGAAGAGAT CGACAA
tlh-R	GATGAGCGGTTGATGT CCAA
tlh probe	TET-AGAAATACAACAATCAAAACTGA-MGBNFQ
trh-F	TTGCTTTCAGTTTGCTATT GGCT
trh-R	TGTTTACCGTCATATAGGC GCTT
trh probe	TxRED-CGCTCGCGTTCACGAAACCGT-BHQ2
tdh-F	TCCCTTTTCCTGCCCCC
tdh-R	CGCTGCCATTGTATAGTCTT TATC
tdh probe	FAM-TGACATCCTACATGACTGTG-MGBNFQ
IAC-F	GACATCGATATGGGTGCCG
IAC-R	CGAGACGATGCAGCCATTC
IAC probe	Cy5-TCTCATGCGTCTCCCTGGTGAATGTG-BHQ2

Table 3. Probes used for colony blot hybridization.
X = alkaline phosphatase conjugated oligonucleotide 5' Amine-C6

Probe	**Probe sequence (5' – 3')**
tlh	XAAAGCGGATTATGCAGAAGCACTG
trh	XACTTTGCTTTCAGTTTGCTATTGGCT
tdh	XGGTTCTATTCCAAGTAAAATGTATTTG
vvh	XGAGCTACGGCAGTTGGAACCA

Figure 2. Violin plot of *Vibrio* genes detected in water, oyster, and sediment samples.

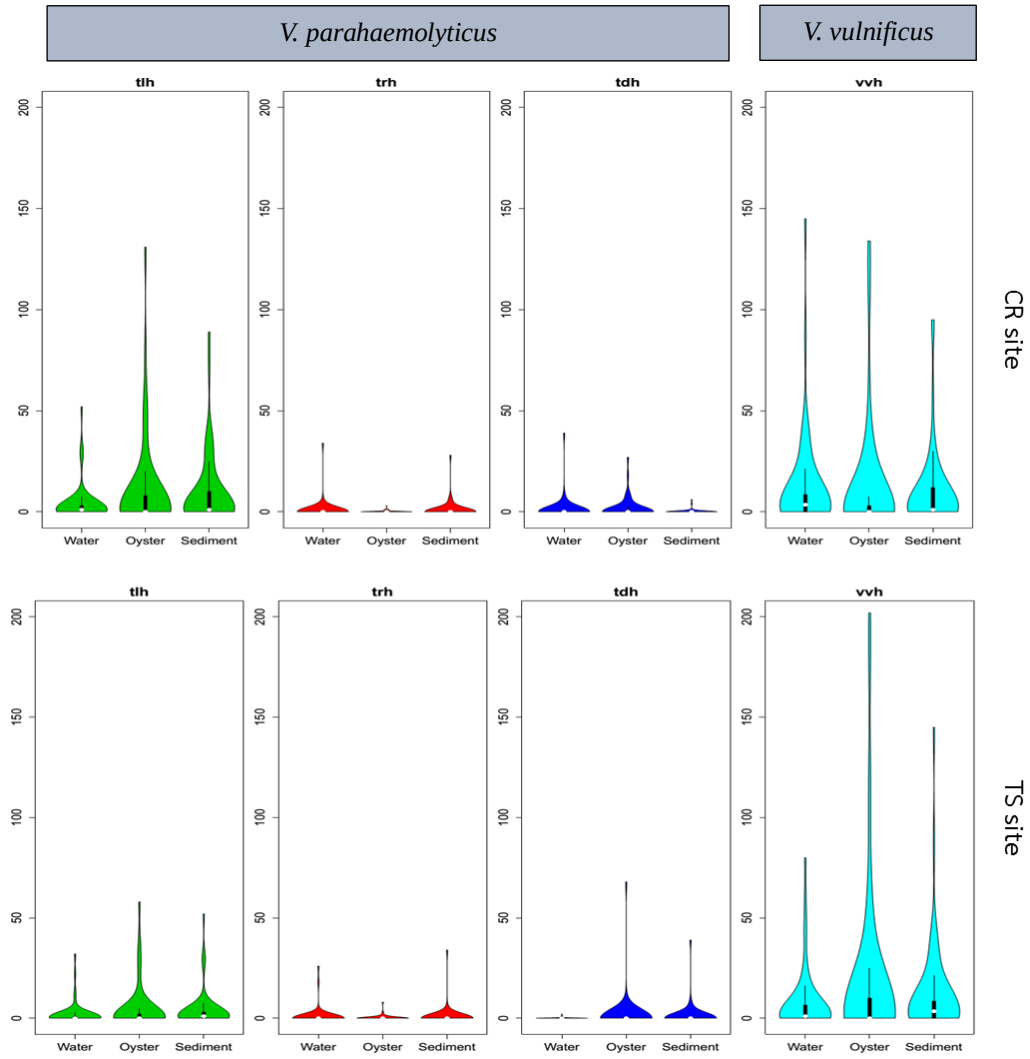


Figure 3. Ternary densities of *Vibrio* detected in water, oyster, and sediment samples. Chester River is on the left and Tangier Sound is on the right.

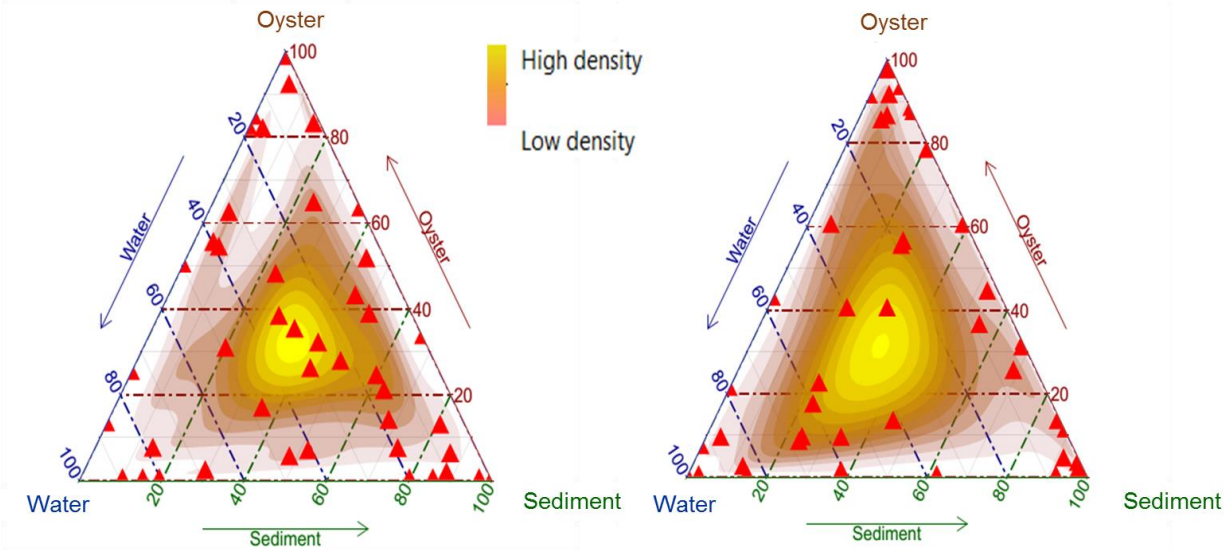


Figure 4. Seasonality of *Vibrio* genes detected in water, oyster, and sediment samples.

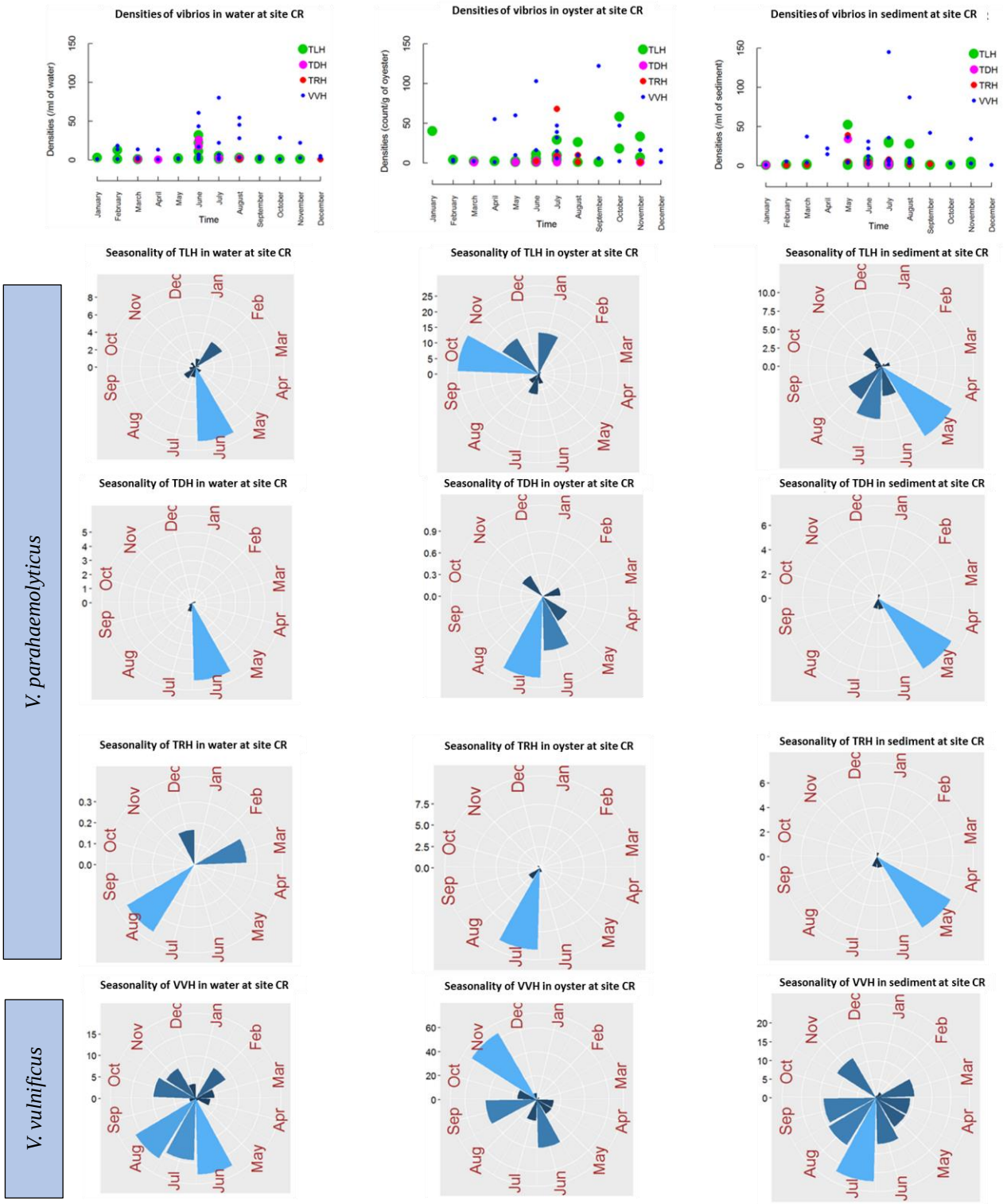
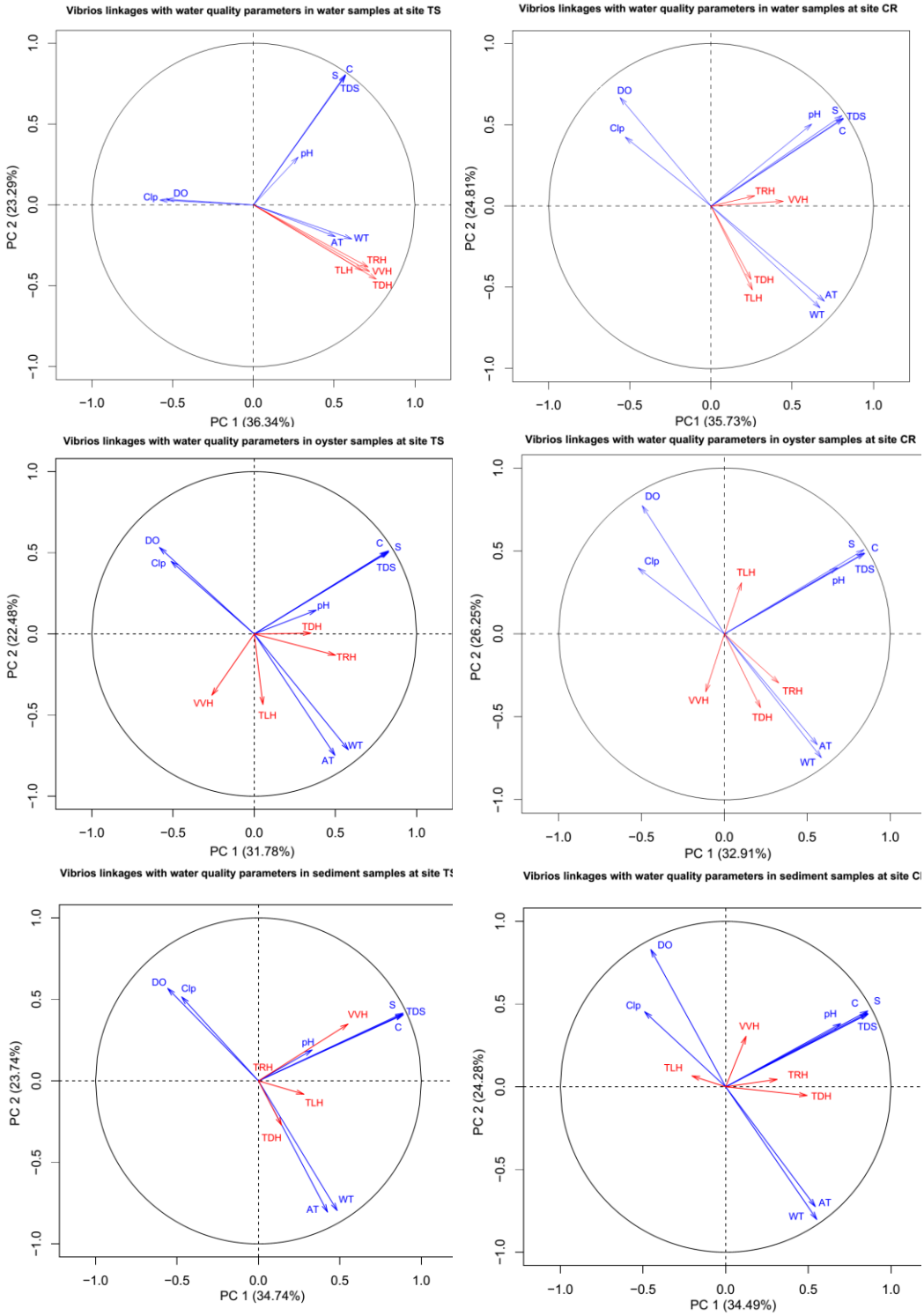


Figure 5. Ecological niches of *Vibrio* genes detected in water, oyster, and sediment samples. Red lines indicate *Vibrio* genes (*tlh, tdh, trh, vvh).* Blue lines indicate environmental parameters (Clp: chlorophyll, DO: dissolved oxygen, S: salinity, C: conductivity, TDS: total dissolved solids, pH, WT: water temperature, AT: air temperature)



Chapter 3: Characterization of Pathogenic *Vibrio parahaemolyticus* isolates from the Chesapeake Bay, Maryland

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Aims; *Vibrio parahaemolyticus* is the leading cause of bacterial gastroenteritis associated with seafood consumption in the United States. Here we investigated the presence of virulence factors and genetic diversity of *V. parahaemolyticus* isolated from water, oyster, and sediment samples collected from the Chesapeake Bay, Maryland.

Methods and Results; Of 2,350 presumptive *Vibrio* isolates collected, more than half were confirmed by PCR as *V. parahaemolyticus,* with ten encoding both *tdh* and *trh* and six encoding only *trh*. Potentially pathogenic *V. parahaemolyticus* were serotyped, with O1:KUT and O3:KUT predominant. Furthermore, PFGE was performed and the constructed dendrogram displayed high diversity, as did results of multiple-locus VNTR analysis.

Conclusions; *V. parahaemolyticus* was readily present on Chesapeake Bay water, oyster, and sediment samples collected during this study. Potentially pathogenic *V. parahaemolyticus* were isolated in fewer numbers and the isolates displayed expansive diversity.

Significance and Impact of the Study; Although characteristics of the pathogenic *V. parahaemolyticus* isolates were highly variable and the percent pathogenic *V. parahaemolyticus* was low, it is important to note that, pathogenic *V. parahaemolyticus* are common to the Chesapeake Bay waters, warranting seafood

monitoring to minimize risk of disease for the public.

Keywords; *Vibrio parahaemolyticus,* Chesapeake Bay, pathogenicity, virulence, environment

INTRODUCTION

Vibrio parahaemolyticus, a halophilic Gram-negative bacterium, is both autochthonous to the marine environment and a causative agent of seafood-related illnesses (Alam et al., 2009). First reported in Japan in the 1950s, *V. parahaemolyticus* has now been recognized as one of the leading causes of seafood-related bacterial gastroenteritis worldwide and accounts for almost 50% of all food poisoning outbreaks in Taiwan, Japan, and Southeast Asia (Alam et al., 2009; Martinez-Urtaza *et al.* 2004). In the United States, *V. parahaemolyticus* is the leading cause of seafood-induced bacterial enteritis, typically related to consumption of raw or undercooked seafood (DePaola et al. 2002). According to the Centers for Disease Control and Prevention, infection by *V. parahaemolyticus* is estimated to have an annual rate of 4,500 cases per year in the United States (DePaola et al., 2002). Illness caused by *V. parahaemolyticus* can occur 3–24 hours after the consumption of contaminated food and symptoms include diarrhea, nausea, vomiting, abdominal cramps, and low-grade fever (Taniguchi et al., 1985). Despite the growing understanding of occurrence and pathogenicity of *V. parahaemolyticus*, the burden of *V. parahaemolyticus* related disease has constantly increased in frequency and range since 2000 (Martinez-Urtaza et al., 2004; Caburlotto et al., 2013).

V. parahaemolyticus is both oxidative and fermentative and occurs naturally

in both marine and freshwater environments, where it interacts with many of the marine and estuarine organisms native to Chesapeake Bay (Caburlotto et al*.,* 2013; Alam et al*.,* 2009). *Vibrio* species are known to concentrate on the gills and in the gut of oysters and other filter-feeding bivalves, leading to a higher risk of infection to humans ingesting raw or undercooked seafood (Froelich et al*.,* 2013). Although not the focus of this study, previous studies have investigated the occurrence of *V. parahaemolyticus* in various fish species, prawn, and shrimp (Pal and Das 2010; Kagiko et al., 2001). Those prior studies have demonstrated environmental parameters most closely associated with occurrence and distribution of *V. parahaemolyticus* are water temperature and salinity (Caburlotto et al*.,* 2013; Kagiko et al., 2001). When environmental conditions are favorable, increased growth of *Vibrio* species in the water column can lead to increased abundance in filter-feeding bivalves and mollusks. Earlier studies carried out in the Chesapeake Bay region have shown *V. parahaemolyticus* is rarely isolated when the water temperature is below 15°C (Kaneko et al., 1973; Caburlotto et al., 2013). However, it is hypothesized that *Vibrio* species can persist in sediment during colder months and can then be released back into the water column once temperatures are conducive for growth, usually in the late spring and early summer. Since *V. parahaemolyticus* can persist in estuarine and marine environments year-round, there is a need to determine when the risk of illness caused by pathogenic *V. parahaemolyticus,* is highest.

Despite their abundance in estuarine and marine environments, the vast majority of *V. parahaemolyticus* isolated from the environment are not pathogenic,

whereas the majority isolated from clinical sources are associated with disease (Shinoda and Miyoshi, 2006). The two major and most commonly referenced virulence factors for *V. parahaemolyticus* are thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) (Alam et al., 2009; Shinoda and Miyoshi, 2006; DePaola et al., 2002; Kagiko et al., 2001; Nishibuchi and Kaper, 1985). The *tdh* gene, which codes for the Kanagawa phenomenon (KP), characterized by β-hemolysis of human erythrocytes, is typically absent (<1%) in environmental isolates whereas more than 90% of clinical isolates are positive (Martinez-Urtaza et al., 2004; Alam et al., 2009).

KP negative clinical *V. parahaemolyticus* isolates were discovered to produce a second hemolysin, *trh*, which unlike *tdh,* is heat labile but immunologically similar to *tdh* (Honda and Iida, 1993). Pandemic O3:K6 strains carry the *tdh* but not the *trh* gene and are generally defined by a positive group-specific PCR (GS-PCR) based on the gene sequences of *toxRS* and ORF8 from the f237 phage (Matsumoto et al., 2000). Thus, it is clear that many studies have investigated pathogenicity of *V. parahaemolyticus,* with low frequency of occurrence of *tdh* and/or *trh* positive strains in environmental samples (DePaola et al., 2002). The objective of this study was to characterize the large number of *V. parahaemolyticus* isolates (1,304) collected from sampling sites in the Chesapeake Bay over a three-year period, focusing on the virulence factors, *trh* and/or *tdh* positive strains, to determine both genomic relatedness its distribution of this *Vibrio* spp. in the Chesapeake Bay.

MATERIALS AND METHODS

Sample Collection., Water, oyster, and sediment samples were collected at two locations in the Chester River (39°05.09'N, 76°09.50'W) and Tangier Sound (38°10.97'N, 75°57.90'W) in the Chesapeake Bay, Maryland from June, 2009, to August, 2012 (Figure 1). During the warmer months of June through August, sampling was done twice each month and once each month during September through May. At each site, 12 liters of water, 20-25 oysters, and 80-100g of sediment were collected. Samples were kept on ice during transport to the University of Maryland, College Park and, upon arrival, stored overnight at 15°C until processing the following morning.

Sample Processing. Details of the sample processing have been described elsewhere (Johnson et al., 2012; Johnson et al., 2010). In brief, collected water samples were shaken and three volumes (1000ml, 100ml, 10ml), each in triplicate, were inoculated into alkaline peptone water (10× APW, pH 8.5) (111ml, 11ml, 1.1ml, respectively) and incubated for 16-18 hours, with shaking at 30 rpm. Oysters were rinsed and scrubbed under running water, shucked, and the oyster tissue homogenized 1:1 with 1× phosphate buffer solution (1× PBS; pH 7.4) in a sterile blender for 90 seconds. Homogenized oyster tissue was inoculated (10g, 1g, 0.1g, in triplicate) into 10× APW and incubated at 33°C for 16-18 hours, with shaking at 30 rpm. Sediment samples were weighed and vortexed in equal part 1x PBS, after which 10x APW was added and the samples incubated at 33°C for 16-18 hours, with shaking at 30 rpm. The following day, a loopful of pellicle from each overnight sample was collected along with a loopful of shaken overnight sample and streaked

individually onto selective media, including CHROMagar™ (CHROMagar, Springfield, NJ), thiosulfate citrate bile salts sucrose agar, TCBS (Oxoid, Ontario, Canada), and *Vibrio vulnificus* agar (VVA). The plates were incubated at 37°C for 16-18 hrs. Presumptive colonies of *V. parahaemolyticus*, based on growth media, were picked and streaked onto LB agar (BD Diagnostic Systems, Sparks, MD) to obtain pure cultures.

DNA extraction and PCR. Presumptive isolates of *V. parahaemolyticus* were inoculated into LB broth, incubated at 37°C for 16-18 hrs. with shaking at 150 rpm. A 1.5 ml aliquot of inoculum was centrifuged for 10 minutes at 13 G and the supernatant discarded. To each pellet, 700µl Tris-EDTA Buffer (TE Buffer; pH 8.0) was added and mixed. Cell suspensions were boiled for 10 minutes at 99°C, after which the samples were allowed to cool on ice for at least 30 minutes before centrifugation for 10 minutes at 13 G. The supernatant was transferred to a clean, sterile tube and adjusted to concentration for PCR analysis. Multiplex PCR targeting the *toxR* gene (Bauer and Rorvik, 2007) was used to differentiate *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae,* and to confirm identification of the isolates. Subsequent PCR targeting virulence factors, *tlh, trh* and *tdh* (Bej et al., 1999), was done for all of the confirmed *V. parahaemolyticus* isolates. PCRs targeting the group specific *toxR* variant, GS-PCR, and opening reading frame, ORF8, were performed (Matsumoto *et al.*, 2000). All PCR assays were performed using Promega GoTaq Green Master Mix (Promega, Madison, WI). Each reaction tube contained a total of 25µl, including 5µl template DNA. Thermal cycling conditions were as follows: one 10-minute cycle of denaturation at 94°C, followed

by 36 cycles of denaturation at 94°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 60 seconds, and final extension for 10 minutes at 72°C. PCR products were stored at 4°C until gel electrophoresis visualization. Sequences, amplicon size and annealing temperatures for each PCR can be found in Tables 1 and 2. Positive controls included VPTX2103, VPFIHES98, VPAQ41037, and VPF11-3A. Appropriate negative controls were included in all PCR reactions.

Storage of Isolates. Confirmed *V. parahaemolyticus* isolates were inoculated into 5 ml LB broth and inoculated for 16-18 hours at 37°C, with shaking at 150rpm. For long term storage, equal volumes of inoculum and 50% glycerol were added to cryovials and the isolates stored at -80°C.

Hemolysis. Cultures of *V. parahaemolyticus* were grown overnight on LB for 18 hours at 37°C, streaked onto 5% sheep blood agar plates, and incubated at 37°C for 18 hr. Green hemolysis was defined as α, β as clear hemolysis, and γ as no hemolysis.

PFGE. Pulsed field gel electrophoresis (PFGE) of *V. parahaemolyticus* DNA was performed using CDC Pulse-Net protocol created by the CDC (Pulse Net United States 2013), as follows.

Gel plug creation and lysis. Cultures were grown for 16-18 hours at 37°C on LB plates and confirmed for purity. A loopful of each broth culture was mixed with 1ml cell suspension buffer (CSB) (100mM Tris: 100mM EDTA, pH 8). The concentration of cell suspension was adjusted to final absorbance of 0.9 ±0.1 at 610 nm. Half of the cell suspension was incubated with 25 µl of 20 mg/ml Proteinase K

for 10 minutes at room temperature. Following incubation, 500 µl of cell suspension was mixed with an equal volume of 1% SeaKem Gold agarose pre-warmed to 55-60°C. The solution was transferred to a gel plug mold, dispensed to avoid bubbles, and allowed to solidify for five minutes at 4°C. Each plug was transferred to individual 50 ml Falcon tubes. Each tube contained 5ml cell lysis buffer (CLB) (50 mM Tris : 50 mM EDTA, 1% *sarkosyl*, pH 8) and 25 µl Proteinase K (20 mg/ml). Tubes containing plugs, CLBr and Proteinase K were incubated in a 54-55°C water bath with shaking at 150rpm, for two hrs. Plugs were washed twice with 10 ml sterilized ultrapure water previously warmed to 54-55°C, with shaking, and temperature conditions as above, for 10 min. Additional washes with TE Buffer (10 mM Tris : 1 mM EDTA, pH 8) were performed a minimum of four times. Plugs were stored at 4°C with 5 ml sterile TE buffer until digestion was complete.

Digestion and Gel Casting. *V. parahaemolyticus* isolates were SfiI digested. *Salmonella enterica* ATCC BAA-664, serving as control, was XbaI digested. Plugs were cut to 2.0 mm wide slices and inserted into individual 1.5 ml Eppendorf tubes containing predigestion master mix consisting of 180 µl sterile ultrapure water and 20 µl 10× restriction buffer per plug. Predigestion of *V. parahaemolyticus* was done with incubation at 50°C. *S. enterica* was incubated at 37°C and after 10 minutes, the predigestion buffer was removed and restriction enzyme master mix added. The restriction enzyme master mix for *V. parahaemolyticus* contained 177 µl sterile ultrapure water, 20 µl 10× restriction buffer, 2 µl BSA (10 mg/ml) and 1 µl SfiI (40 U/µl) per plug. The restriction enzyme master mix for *S. enterica* contained 174 µl sterile ultrapure water, 20 µl 10× restriction buffer, 2 µl BSA (10 mg/ml) and 4 µl

XbaI (10 U/µl) per plug. Plugs were incubated for four hours at 50°C *(V. parahaemolyticus)* or 37°C (*S. enterica*). Following digestion, the restricted enzyme master mix was removed and 200 µl 0.5× TBE was added to each tube and incubated at room temperature for five minutes. Plugs were loaded onto a gel comb, including control plugs of *S. enterica.* A 1% SeaKem Gold Agarose gel was cast in 0.5× TBE, ensuring plug slices did not move. The agarose gel and plugs were allowed to solidify for at least 30 minutes and inserted into an electrophoresis chamber containing 4 L freshly prepared 0.5× TBE adjusted to 14°C, with a flow rate of 1 liter per minute.

CHEF Mapper and Staining. The CHEF Mapper electrophoresis chamber program was set to Auto Algorithm, with a low MW of 78 kb and high MW of 396 kb. After running for 18-19 hrs., the gel was stained in ethidium bromide (10mg/ml) and visualized.

Dendrogram preparation. Restriction patterns were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The background was subtracted and the normalized before fingerprint patterns were typed.

Serotyping. Denken antisera kit containing 13 lipopolysaccharide (O) and 71 capsular (K) sera was used to determine serotypes of pathogenic isolates via slide agglutination. First, *V. parahaemolyticus* isolates were grown overnight at 37°C on 3% NaCl LB agar. Subsequently, a loopful of culture was mixed with 1ml of 90% normal saline. Half of the cell suspension was boiled at 99°C for 2 hrs. and used for O serotyping whereas the remaining suspension was used for K serotyping. (Denka; Seiken Corp., Tokyo, Japan).

DNA Extraction for Sanger Sequencing. *V. parahaemolyticus* isolates were grown overnight in LB broth at 37°C for 16-18 hrs., with shaking at 150 rpm. A 1.5 ml aliquot of inoculum was centrifuged for 10 minutes at 13 g and the supernatant discarded. DNA was extracted using a Qiagen MiniPrep kit, following the manufacturer's protocol (Qiagen, Venlo, Limburg).

MLVA. Multiple locus variable nucleotide tandem repeat (MLVA) analysis was performed for 16 of the *tdh+, trh+ V. parahaemolyticus* strains, employing nine primer sets belonging to both chromosomes 1 and 2. PCR conditions were identical to those described for conventional PCR. After confirmation by PCR, 25 µl PCR product were purified using DNA Clean & Concentrator™-5 (ZymoResearch, Irvine, CA) and amplicons sequenced (Eurofins MWG Operon, Louisville, KY).

RESULTS

Water, oyster, and sediment samples from Tangier Sound and the Chester River in the Chesapeake Bay, collected between June, 2009, and August, 2012, yielded 2,350 presumptive *Vibrio* isolates, of which 1,304 (55%) were confirmed *V. parahaemolyticus* by *toxR* targeted multiplex PCR. The remaining isolates were mainly *Vibrio vulnificus* and *Vibrio cholerae.* All 1,304 *V. parahaemolyticus* isolates possessed the species-specific *tlh* gene. Of all *V. parahaemolyticus* isolates, 16 (1.2%) were potentially pathogenic (Table 3), 10 of which (62.7%) contained both of the virulence encoding genes, *tdh* and *trh,* and 6 isolates (37.5%) were negative for *tdh* and positive for *trh.* The majority of the Chesapeake Bay *V. parahaemolyticus* isolates (83.2%) were recovered from water (whole water, plankton free water, plankton and water), followed by oyster (9.1%), and sediment

(7.7%). Of the sixteen potentially pathogenic *V. parahaemolyticus*, none were isolated from oyster, assumed because of limitations associated with relying on culture based methods.

The majority of presumptively pathogenic *V. parahaemolyticus* strains collected from Tangier Sound were isolated during the colder months of September, December, and January, 2009 to 2011. In contrast, the presumptively pathogenic *V. parahaemolyticus* were isolated from the Chester River during the warmer months of May, June, and August, 2009-2010, except for one strain in September, 2010, and two in December, 2009 (Table 3).

Serotyping was performed on all potentially pathogenic *V. parahaemolyticus* strains and the majority contained O1 antigen, followed by O3 and O5, in that order. Most strains could not be typed for the K antigen using conventional kits and the most frequently occurring serotype was O1:KUT, a serovariant of O3:K6, accounted for 37.5% of strains tested, followed by O3:KUT (18.75%) (Table 3).

PFGE patterns of the 16 potentially pathogenic *Vibrio parahaemolyticus* showed significant diversity, with neither showing identical banding patterns. Similarly, multiple-locus VNTR analysis (MLVA) (Figure 2) showed high diversity with only five falling into a cluster of related strains. None of the strains shared similar MLVA patterns, confirming the diversity detected by PFGE (Tables 3 and 4).

DISCUSSION

During the course of this study, *Vibrio parahaemolyticus* was collected from both locations in the Chesapeake Bay from all sample types and in large numbers. However, of all *V. parahaemolyticus* strains characterized for pathogenicity, based on presence of either *trh* or *tdh*, less than 2% were found to be potentially pathogenic. Although detection of pathogenic *V. parahaemolyticus* was lower than reported by previous investigations for Chesapeake Bay, it is important to note that different methods were used and all of those methods were culture dependent. In this study, all sixteen *tdh*+ and/or *trh*+ were negative for pandemic markers GS and ORF-8 by PCR, therefore, different from pandemic O3:K6 strains. Previous studies reported *V. parahaemolyticus* strains negative for GS-PCR were also negative for ORF-8, the marker for the filamentous phage presumed associated with pandemic genotypes (Alam et al., 2009). These data including serotype, virulence, and genetic traits were for *V. parahaemolyticus* isolated from the estuarine ecosystem of Bangladesh (Alam et al., 2009). The majority of Chesapeake Bay isolates of *V. parahaemolyticus* (83.2%) were from water (unfiltered water, plankton free water, and plankton samples), followed by oyster (9.1%), and sediment (7.7%). Of the sixteen potentially pathogenic *V. parahaemolyticus*, none were isolated from oysters, assumed a result of the limitations associated with culture based methods.

The majority of presumptively pathogenic *V. parahaemolyticus* strains collected from Tangier Sound were isolated during the colder months of September, December, and January, 2009 to 2011. In contrast, the presumptively pathogenic *V. parahaemolyticus* were isolated from the Chester River during the warmer months of May, June, and August, 2009-2010, except for one strain in September, 2010,

and two in December, 2009. Interestingly, all presumptively pathogenic *V. parahaemolyticus* were isolated during the first year of the study, with the last of the isolates collected in September 2010, suggesting environmental factors determine temporal changes in occurrence of *V. parahaemolyticus*. However, more importantly and likely to be the case, is that environmental strains of pathogenic *V. parahaemolyticus* are very difficult to isolate. For example, in a similar study conducted in India, pathogenic *V. parahaemolyticus* were isolated from 59% of samples after enrichment for 18 hours, but the same samples yielded strains negative for *tdh* when conventional methods, followed by PCR, were employed (Deepanjali et al., 2005). A study carried out in Japan found 41.5% of the seawater and 8.5% of organic matter samples were positive for *tdh* and *trh* when MPN followed by PCR was done but *tdh* and/or *trh* positive strains could not be isolated (Alam et al., 2003). Thus, it is concluded that potentially pathogenic strains of *V. parahaemolyticus* are present in the Chesapeake Bay but isolation and culture of these strains remain a challenge.

V. parahaemolyticus isolates associated with disease outbreaks comprise multi-serogroups, with at least 13 O and 71 K serogroups having been reported (Alam et al., 2009). Commercial kits manufactured in Japan are commonly used to distinguish serogroups (Martinez-Urtaza et al., 2004) and the serogroup most frequently isolated from clinical cases is O3:K6, the causative agent of a massive outbreak of diarrhea cases in Kolkata, India, in 1996 and later identified in other parts of the world, including Asia, Africa, Europe, Latin America, and the United States (Alam et al., 2009). Results of previous studies have shown that the *V.*

parahaemolyticus O3:K6 serogroup contains the O3:K6 specific filamentous phage f237 and group-specific (GS) sequences of the *toxRS* operon in addition to ORF-8. These are used as markers to distinguish O3:K6 from other serogroups (Alam et al*.,* 2009). Serotypes O1:KUT, O1:K25, O1:K41, and O4:K68 have been shown to be serovariants of O3:K6 (Martinez-Urtaza et al*.,* 2004). The majority of potentially pathogenic *V. parahaemolyticus* strains contained the *V. parahaemolyticus* O1 antigen, followed by O3 and O5, in that order. Most strains could not be typed for the K antigen using conventional kits and the most frequently occurring serotype was O1:KUT, a serovariant of O3:K6, which accounted for 37.5% of strains tested, followed by O3:KUT (18.75%).

In addition to serotyping, a variety of fingerprinting techniques, including pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA), have been used to profile *V. parahaemolyticus.* Although PFGE is not a new method, few studies have employed PFGE to analyze the diversity of environmental isolates of *V. parahaemolyticus*, especially with respect to geographical distribution. Previous studies employing PFGE have been done in Japan and Bangladesh (Suffredini et al., 2011). Only recently have environmental strains of *V. parahaemolyticus* from more than one European country been characterized using PFGE (Suffredini et al., 2011). Furthermore, very few *V. parahaemolyticus* isolates from the United States, and specifically the Chesapeake Bay, have been subjected to PFGE analysis. A dendrogram constructed using PFGE patterns showed significant diversity among the sixteen strains of *V. parahaemolyticus* isolates of this study, a conclusion also drawn from results of

multiple-locus VNTR analysis (MLVA) (Figure 2). Of the sixteen potentially pathogenic *V. parahaemolyticus* environmental strains typed by PFGE, none had identical banding patterns, not surprisingly given results of past studies showing high genetic diversity among *V. parahaemolyticus* strains. The lack of duplicate banding patterns amongst these strains is important as previous methods have used PFGE to determine ancestry of bacterial strains. Of the sixteen strains, only five fell into a cluster of related strains. Interestingly, those five strains had been isolated from the Chester River on three separate days (the first on May 24, 2010, three on June 14, 2010, and the fifth on August 16, 2010). Given the high diversity among all Chesapeake Bay *V. parahaemolyticus* isolates observed in this study, it is intriguing to note that these five strains formed a related cluster, despite having been isolated over a four-month period. Of the five strains, four were *tdh*+ and *trh*+ and the strain last to be isolated, on June 14 2010, was *tdh*-, an interesting observation since significant strain divergence was observed among those carrying *trh* compared to the *tdh*+ strains. None of the strains shared similar MLVA patterns, confirming the diversity detected by PFGE (Tables 3 and 4). Repeats in the VPTR207 locus were not detected in any of the strains and the least variability was observed at locus VPTR7 of chromosome 1, with most strains carrying four or five repeats. Ultimately, strains of potentially pathogenic *Vibrio parahaemolyticus* are extremely diverse in regard to location, time and sample type.

Ideally, monitoring *Vibrio* species in water, sediment, and oysters should provide a reasonable estimate of the actual occurrence of pathogenic *Vibrio parahaemolyticus* relative to total *Vibrio* spp., if sufficient sampling is done.

However, the requirement for an intensive monitoring regimen, coupled with the difficulty in isolating pathogenic *V. parahaemolyticus* and related pathogens can cause environmental surveillance to remain a serious challenge. However, once patterns of presence of pathogenic *Vibrio parahaemolyticus* in relation to various environmental parameters such as temperatures and salinity, are coupled, an effective monitoring program can be developed to guard the public from *Vibrio* related disease and infection.

In summary, Chesapeake Bay isolates of *V. parahaemolyticus* were found to carry indicators of pathogenicity and were highly diverse genetically. These findings are in concordance with those reported globally. Because potentially pathogenic *V. parahaemolyticus* can be isolated from the Chesapeake Bay, a monitoring program that include *V. parahaemolyticus* would be a wise public health program to prevent an outbreak and reduce the incidence of *V. parahaemolyticus* related illness.

Table 1. List of primers, annealing temperatures, and sequences.

Primers	**Primer sequence (5' – 3')**	**Amplicon (bp)**	**T_a (°C)**	**Reference**
utox-F	GASTTTGTTTGGCGYGARCAAGGTT		55	(Bauer 2007)
vptox-R	GGTTCAACGATTGCGTCAGAAG	297		
vvtox-R	AACGGAACTTAGACTCCGAC	640		
vctox-R	GGTTAGCAACGATGCGTAAG	435		
tlh-L	AAAGCGGATTATGCAGAAGCACTG	450	58	(Bej 1999)
tlh-R	GCTACTTTCTAGCATTTTCTCTGC			
tdh-L	GTAAAGGTCTCTGACTTTTGGAC	269		
tdh-R	TGGATAGAACCTTCATCTTCACC			
trh-L	TTGGCTTCGATATTTTCAGTATCT	500		
trh-R	CATAACAAACATATGCCCATTTCCG			
GS-PCR	TAATGAGGTAGAAACA ACGTAACGGGCCTACA	651	45	(Matsumoto 2000)

Table 2. Description of *Vibrio parahaemolyticus* VNTR loci and primers used for MLVA.

Locus	Chromosome	Primers	Primer Sequence (5' - 3')	Amplicon (bp)	Motif	Reference
VPTR1 VP2892	1	VPTR1-F VPTR1-R	TAACAACGCAAGCTTGCAACG TCATTCTCGCCACATAACTCAGC	255	TATCTC	(Kimura 2008)
VPTR2 VPA1454	2	VPTR2-F VPTR2-R	GTTACCAAACTGGCGATTACGAAG CGGAATTCAGGATCATCCTGAT	615	GCTGTT	(Kimura 2008)
VPTR3 VPA0714	2	VPTR3-F VPTR3-R	CGCCAGTAATTCGACTCATGC AAGACTGTTCCCGTCGCTGA	333	ATCTGT	(Kimura 2008)
VPTR4 VP0446	1	VPTR4-F VPTR4-R	AAACGTCTCGACATCTGGATCA TGTTTGGCTATGTAACCGCTCA	229	TGTGTC	(Kimura 2008)
VPTR5 VP3012.VP3013	1	VPTR5-F VPTR5-R	GCTGGATTGCTGCGAGTAAGA AACTCAAGGGCTGCTTCGG	202	CTCAAA	(Kimura 2008)
VPTR6 VP2226	1	VPTR6-F VPTR6-R	TGTCGATGGTGTTCTGTTCCA CTTGACTTGCTCGCTCAGGAG	312	GCTCTG	(Kimura 2008)
VPTR7 VP2131	1	VPTR7-F VPTR7-R	CAACAGTTCTGCTCTAATCTTCCG CAAAGGTGTTACTTGTTCCAGACG	221	CTGCTC	(Kimura 2008)
VPTR8 VP2956	1	VPTR8-F VPTR8-R	ACATCGGCAATGAGCAGTTG AAGAGGTTGCTGAGCAAGCG	306	CTTCTG	(Kimura 2008)
VP2-07/VPTR16 VPA1455	2	VPTR207-F VPTR207-R	ATCGCTGCTTGAAGAAAATCCTGA T CTAATTTTTCTGGTTGGGCTTGCG	461	TCGTTG	(Kimura 2008)

Table 3. Water and sediment isolates of *V. parahaemolyticus* from the Chester River and Tangier Sound, Maryland.

Strain ID	Area of Isolation	Date of Isolation (M/D/Y)	Source	Serotype	Hemolysis	*tlh*	*tdh*	*trh*	GS	ORF8
TR013-02	Tangier Sound	12/15/09	Water	O1:KUT	β	+	+	+	-	-
TS013-07	Tangier Sound	12/15/09	Water	O1:KUT	β	+	+	+	-	-
CR015-02	Chester River	12/07/09	Water	O1:KUT	β	+	+	+	-	-
CR015-09	Chester River	12/07/09	Water	O3:KUT	β	+	+	+	-	-
TS014-10	Tangier Sound	01/21/10	Sediment	O5:K30	β	+	-	+	-	-
TS014-11	Tangier Sound	01/21/10	Sediment	O5:K3	β	+	+	+	-	-
CR021-01	Chester River	05/24/10	Water	O10:KUT	β	+	+	+	-	-
CR021-06	Chester River	05/24/10	Water	O1:KUT	β	+	+	+	-	-
CR022-06	Chester River	06/14/10	Water	O1:KUT	β	+	+	+	-	-
CR022-08B	Chester River	06/14/10	Water	O1:KUT	β	+	+	+	-	-
CR022-14	Chester River	06/14/10	Water	O1:K68	β	+	+	+	-	-
CR026-19A	Chester River	08/16/10	Water	O1:K58	β	+	-	+	-	-
CR028-01	Chester River	09/13/10	Water	O1:K56	β	+	-	+	-	-
TS026-22	Tangier Sound	09/21/10	Water	O3:KUT	β	+	-	+	-	-
TS026-23	Tangier Sound	09/21/10	Water	O3:KUT	β	+	-	+	-	-
TS026-30	Tangier Sound	09/21/10	Water	O3:K59	β	+	-	+	-	-

Table 4. Number of tandem repeats in sixteen *Vibrio parahaemolyticus* and four reference strains included in this study.

Strain	VPTR1	VPTR2	VPTR3	VPTR4	VPTR5	VPTR6	VPTR7	VPTR8	VPTR207
VPAQ41037	10	22	5	3	7	21	4	10	0
VPF11-3A	9	21	3	3	9	12	4	5	0
VPTX2103	23	14	6	5	7	17	4	8	0
VPFIHES98	16	24	5	6	5	12	4	9	0
TR013-02	20	18	3	1	11	9	4	0	0
TS013-07	5	34	4	2	2	10	5	7	0
CR015-02	1	19	1	0	5	7	4	10	0
CR015-09	10	31	6	2	1	11	4	8	0
TS014-10	4	19	2	3	5	7	4	6	0
TS014-11	9	35	0	0	3	19	4	6	0
CR021-01	12	24	5	1	3	6	4	7	0
CR021-06	12	0	5	2	5	15	4	7	0
CR022-06	12	44	0	1	5	16	4	7	0
CR022-08B	11	34	5	0	5	15	4	7	0
CR022-14	12	46	5	1	5	11	4	7	0
CR026-19A	10	35	2	1	2	8	4	18	0
CR028-01	19	18	5	7	3	20	4	6	0
TS026-22	17	13	5	3	2	19	4	7	0
TS026-23	17	5	5	3	3	18	4	6	0
TS026-30	17	19	5	3	3	18	4	6	0

Figure 1. Map of Chesapeake Bay showing sampling sites in the Chester River and Tangier Sound.

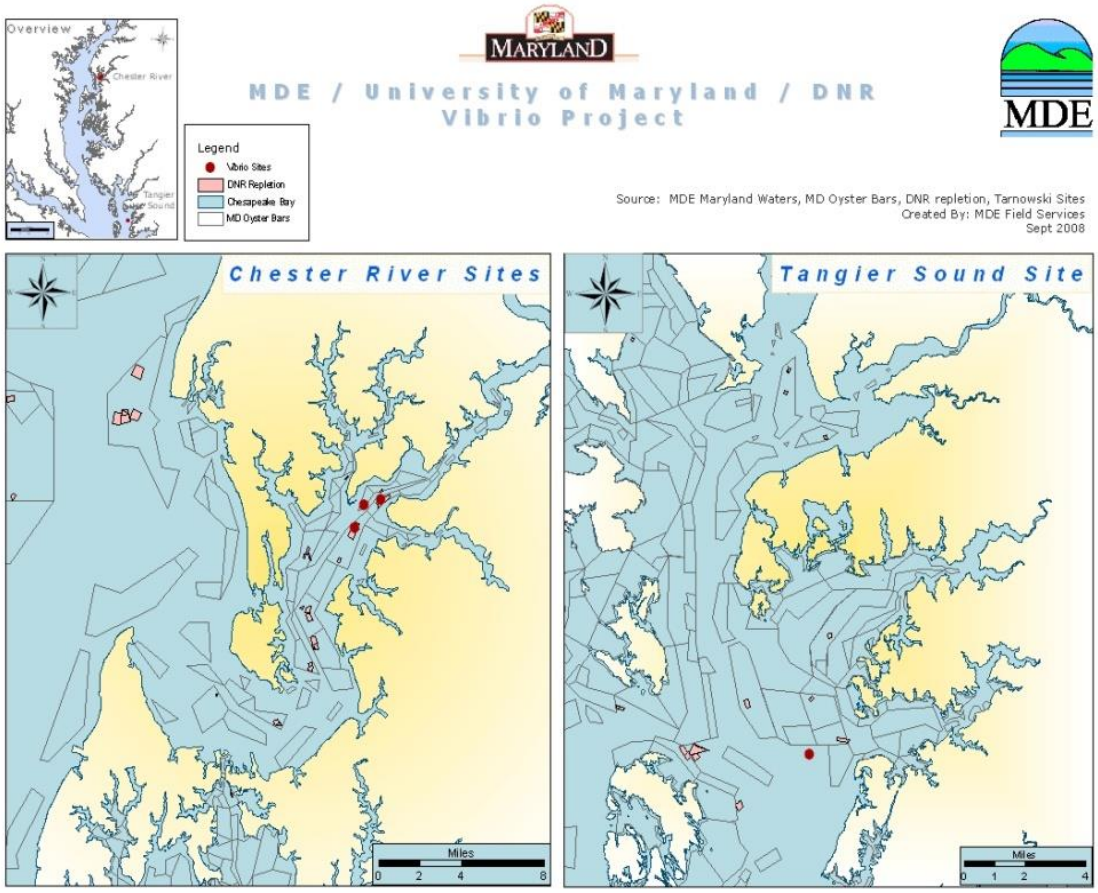
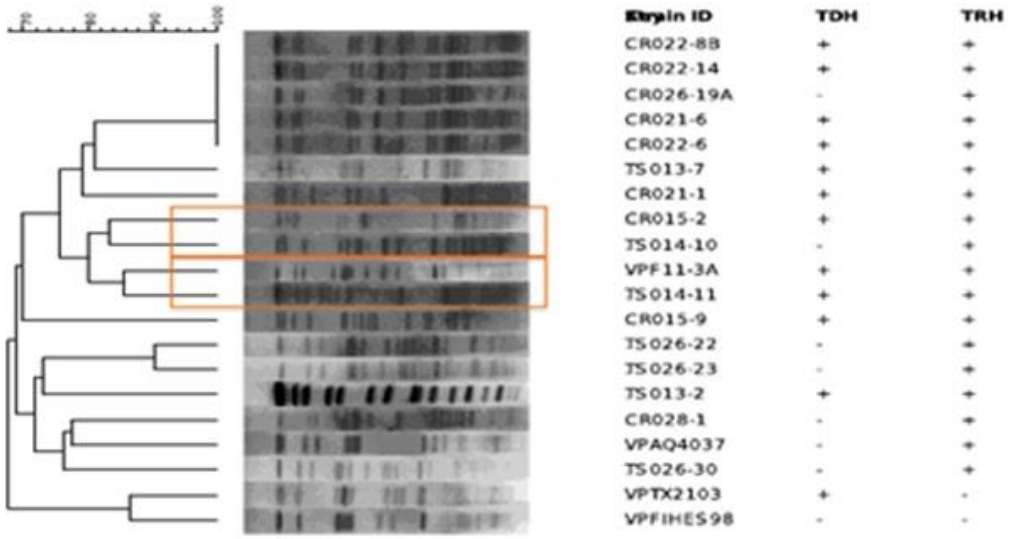


Figure 2. Dendrogram showing PFGE of *Sfi* digested *V. parahaemolyticus* isolates.



Chapter 4: Diversity of Chesapeake Bay Isolates of *Vibrio vulnificus*

Introduction

Vibrio vulnificus, an halophilic, autochthonous marine and estuarine bacterium is the causative agent of several seafood associated infections, including septicemia and gastroenteritis (Oliver, 2006). Infections caused by *V. vulnificus* are typically transmitted via consumption of raw or undercooked seafood or exposure to open wounds in estuarine and coastal waters (Oliver, 1989). *Vibrio vulnificus* is one of the deadliest bacteria known and is responsible for 95% of seafood-related deaths in the United States (Mead et al., 1999). Mortality from septicemia caused by *V. vulnificus* can exceed 50%, with death occurring within days of the first signs of infection (Oliver, 2006). The U.S. Food and Drug Administration (USDA) has reported an average of 34 cases of *V. vulnificus* related infections each year, with the majority of in the Gulf Coast or through a Gulf Coast origin (Horseman and Surani, 2011; Oliver, 2006). In Maryland and Virginia, the Centers for Disease Control and Prevention (CDC) reported 59 illnesses associated with *Vibrio* spp. infections in 2009 (CDC, 2011).

V. vulnificus has been classified into three biotypes based on biochemical characteristics. Biotype 1 is the most common and is responsible for the majority of human infections worldwide (Oliver, 2015). Biotype 2 causes rapidly fatal septicemia but is primarily an eel pathogen and is rarely associated with human cases of *V. vulnificus* (Horseman and Surani, 2011). Biotype 3 is a newly discovered

hybrid of Biotype 1 and 2 and is associated with fish farming in Israel (Jones and Oliver, 2009). The *V. vulnificus* virulence-correlated gene (*vcg)* is highly correlated with geographic source and this gene is used to classify *V. vulnificus* belonging to Biotype 1 (Rosche et al., 2005). Clinical isolates containing common *vcg* sequences have been designated C type (*vcgC)* and environmental isolate sequences are E type (*vcgE*). Ninety percent of clinical isolates of *V. vulnificus* carry the *vcgC* gene and 93% of environmental *V. vulnificus* contain *vcgE* gene (Rosche et al., 2005).

One of the most prominent virulence factors associated with for *V. vulnificus* is an extracellular hemolysin/cytolysin encoded by gene *vvhA*. *VvhA* not only allows release of iron from hemoglobin which serves as a source of nutrient for *V. vulnificus*, but is also responsible for cytotoxic activity and for death of erythrocytes caused by pores produced within the cell membrane (Wright and Morris, Jr, 1991, Kim et al., 2010). The *V. vulnificus vvhA* gene is highly conserved and serves as a species-specific marker for molecular detection of *V. vulnificus*, regardless of biotype or virulence factor (Wright et al., 1999).

Pili are used by many bacteria to attach and then invade the host. The *V. vulnificus* type IV pili are a mechanisms for the adherence to epithelial cells. The operon encoding the pili consists of four genes, *pilABCD,* where *pilA* encodes the pilin protein subunit essential for cell-cell contact which, in turn, is required for cytotoxicity (Gander and LaRocco, 1989).

Another *V. vulnificus* virulence factor is *rtxA1*, a homolog of the *rtxA* toxin gene of *V. cholerae.* The *V. vulnificus* RTX toxin allows formation of pores in the host cell membrane and rearrangement of the cytoskeletal structure, believed to

lead to cellular necrosis by allowing *V. vulnificus* to cross the intestinal epithelium (Jones and Oliver, 2009). In addition, RTX protects the cell from phagocytosis, allowing for increased survival of *V. vulnificus* in the host (Lo et al., 2011). Mutations in *rtxA1* severely decrease virulence of *V. vulnificus* and, therefore, *rtxA1* is believed to be the primary toxin involved in *V. vulnificus* cytotoxicity and virulence (Jones and Oliver, 2009).

The objective of this study was to characterize the virulence factors, *vvha, vcgC, pilA,* and *rtxA,* of a collection of *V. vulnificus* isolates (n=335) collected over a three-year period from the Chester River and Tangier Sound in the Chesapeake Bay.

MATERIALS AND METHODS

Sample Collection., Water, oyster, and sediment samples were collected at two locations in the Chester River (39°05.09'N, 76°09.50'W) and Tangier Sound (38°10.97'N, 75°57.90'W) in the Chesapeake Bay, Maryland, from June, 2009, to August, 2012 (Figure 1). During the warmer months of June through August, sampling was done twice each month and once each month during September through May. At each site, 12 liters of water, 20-25 oysters, and 80-100g of sediment were collected. Samples were kept on ice during transport to the University of Maryland, College Park, laboratory and, upon arrival, were stored overnight at 15°C until processing the following morning.

Sample Processing. Details of the sample processing have been described elsewhere (Johnson et al. 2012; Johnson et al., 2010). In brief, the water samples were shaken and three volumes (1000ml, 100ml, 10ml), each in triplicate, were

inoculated into alkaline peptone water (10× APW, pH 8.5) (111ml, 11ml, 1.1ml, respectively) and incubated for 16-18 hours with shaking at 30 rpm at 33ºC. Oysters were rinsed and scrubbed under running water, shucked, and the oyster tissue homogenized 1:1 with 1× phosphate buffer solution (1× PBS; pH 7.4) in a sterile blender for 90 seconds. The homogenized oyster tissue was inoculated (10g, 1g, 0.1g, in triplicate) into 10× APW and incubated at 33°C for 16-18 hours with shaking at 30 rpm. Sediment samples were weighed and vortexed in equal part 1x PBS, after which 10x APW was added and the samples incubated at 33°C for 16-18 hours with shaking at 30 rpm. The following day, a loopful of pellicle from each overnight sample was collected along with a loopful of shaken overnight sample and streaked individually onto selective media, including CHROMagar™ (CHROMagar, Springfield, NJ), thiosulfate citrate bile salts sucrose agar, TCBS (Oxoid, Ontario, Canada), and *Vibrio vulnificus* agar (VVA). The plates were incubated at 37°C for 16-18 hrs. Presumptive colonies of *V. vulnificus*, based on growth media, were picked and streaked onto LB agar (BD Diagnostic Systems, Sparks, MD) to obtain pure cultures.

DNA extraction and PCR. Presumptive isolates of *V. vulnificus* were inoculated into LB broth, incubated at 37°C for 16-18 hrs. with shaking at 150 rpm. A 1.5 ml aliquot of inoculum was centrifuged for 10 minutes at 13 G and the supernatant discarded. To each pellet, 700µl Tris-EDTA Buffer (TE Buffer; pH 8.0) was added and mixed. Cell suspensions were boiled for 10 minutes at 99°C, after which the samples were allowed to cool on ice for at least 30 minutes before centrifugation for 10 minutes at 13 G. The supernatant was transferred to a clean, sterile tube and

adjusted to concentration for PCR analysis. Multiplex PCR targeting the *toxR* gene (Bauer and Rorvik 2007) was used to differentiate *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae,* and to confirm identification of the isolates. Subsequent PCR analysis was done on all *toxR* positive *V. vulnificus* isolates to test for virulence characteristics. All PCR assays were performed using Promega GoTaq Green Master Mix (Promega, Madison, WI). Each reaction tube contained a total of 25µl, including 5µl template DNA. Thermal cycling conditions were as follows: one 10-minute cycle of denaturation at 94°C, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 60 seconds, and final extension for 10 minutes at 72°C. Sequences, amplicon size and annealing temperatures for each PCR can be found in Table 1. Controls used were two strains of *V. vulnificus,* ATCC 27562 and ATCC 33816.

Results and Discussion

A total of 603 *V. vulnificus* strains were collected between June, 2009, to August, 2012, with highest incidence observed during these warmer, summer months. The majority of *V. vulnificus* were isolated from water samples, with only ten (1.66%) isolated from sediment and two (0.33%) from oyster samples. Both isolates of *V. vulnificus* from oyster samples were collected from the Chester River in June, although during years 2009 and 2012. Isolation of *V. vulnificus* from sediment was comparable for Tangier Sound (n=6) and the Chester River (n=4) and successful isolation was between April and September throughout the study. All *V. vulnificus* isolates were positive for the *V. vulnificus* species specific *toxR* (n=603).

A subset of *toxR*-positive *V. vulnificus* strains were further characterized for *vvha, vcgC/vcgE, pilA* and *rtxA.* The majority of strains (78.5%) possessed *vvhA,* but not every *toxR*-positive *V. vulnificus* isolate was positive for *vvhA* (n=335). The hemolysin gene, *vvhA*, is typically used as a marker for *V. vulnificus,* whereas *toxR* multiplex is used as a rapid and efficient test to distinguish *V. parahaemolyticus, V. vulnificus,* and *V. cholerae.*

Majority of the *V. vulnificus* isolates (71.0%, n=238) were negative for the *vcgC* marker which has been reported to indicate environmental origin (*vcgE*). Results from this study are similar to those of previous studies performed in other parts of the world, that is, almost equal numbers of these two genotypes were isolated from water samples in the U.S. (Warner and Oliver, 2007). In South Korea, *vcgE* accounted for 65% of *V. vulnificus* strains and *vcgC* accounted for 35% of strains isolated from water, oyster, and sediment samples (Kim and Jeong, 2001). Rates of occurrence of *vcgC* in this study were lower than observed in other studies carried out in the United States, where up to 53% of isolates from water were C genotype (Warner and Oliver, 2007). Previous studies have also found that the majority of *V. vulnificus* isolates from oyster samples were E genotype, with approximately 15% belonging to C genotype, perhaps due to selective uptake of *vcgE* by oyster tissue (Warner and Oliver, 2007). In this study, all *V. vulnificus* isolates from sediment and oysters were negative for *vcgC.* It is important to note the low number of *V. vulnificus* isolated from oyster samples (0.33%). It has been reported that the ratio dynamics of *vcgC* and *vcgE* type is highly influenced by the type of environmental reservoir and also community composition (Warner and

Oliver, 2008).

Presence of *pilA* was detected in 17.9% (n=60) of the *V. vulnificus* isolates and all were from water sources. Prior studies that investigated *V. vulnificus* found that 80% of clinical *V. vulnificus* strains possessed *pilA,* while only 30% of environmental isolates carried the *pilA* gene (Gander and LaRocco, 1989). The lack of *pilA* in a *V. vulnificus* strain is associated with overall reduction in biofilm formation and decreased adherence to cells (Paranjpye and Strom, 2005). Nevertheless, *V. vulnificus* strains not possessing *pilA* have been shown to possess surface pili, indicating *V. vulnificus* can produce other types of pili active in attachment (Paranjpye and Strom, 2005). *V. vulnificus* has been shown to produce another type IV pilin, mannose-sensitive hemagglutinin (MSHA), a homolog of the *V. cholerae* MSHA (Yamaichi et al., 1999). Prior studies indicate that *pilA* is important for the incidence of *V. vulnificus* in the Chesapeake Bay oyster, *Crassostrea virginica* (Paranjpye et al., 2007). However, the two *V. vulnificus* strains isolated from oysters in this study did not possess *pilA*.

The *rtxA* gene was detected in 29.3% (n=98) of *V. vulnificus* isolates in this study. All but one strain of *rtxA+ V. vulnificus* were isolated from water samples, with the one *rtxA+ V. vulnificus* not isolated from water was from an oyster sample collected in June, 2009, from the Chester River. Environmental *V. vulnificus* isolates in Mexico were shown to possess *rtxA* 25% consistently (Natividad-Bonifacio et al., 2013).

The environmental *V. vulnificus* isolates obtained in this study revealed a lower incidence of *pilA* as had been reported in previous studies, yet the incidence

of *rtxA* was comparable. It is important to note that this finding could have been influenced by the culture-dependent method of isolating *V. vulnificus* namely on TCBS, VVA and ChromAgar. Similarly, isolation of *V. vulnificus* strains may be clonal, if isolation is from the same culture plate.

Future studies are planned to investigate additional virulence genes, CPS and *vvpE,* which will provide useful information since evasion of *V. vulnificus* from host cell attack is achieved mainly by surface expression of CPS, which prevents phagocytosis by macrophages (Kashimoto et al., 2003).

Figure 1. Map of Chesapeake Bay showing sampling sites in the Chester River and Tangier Sound.

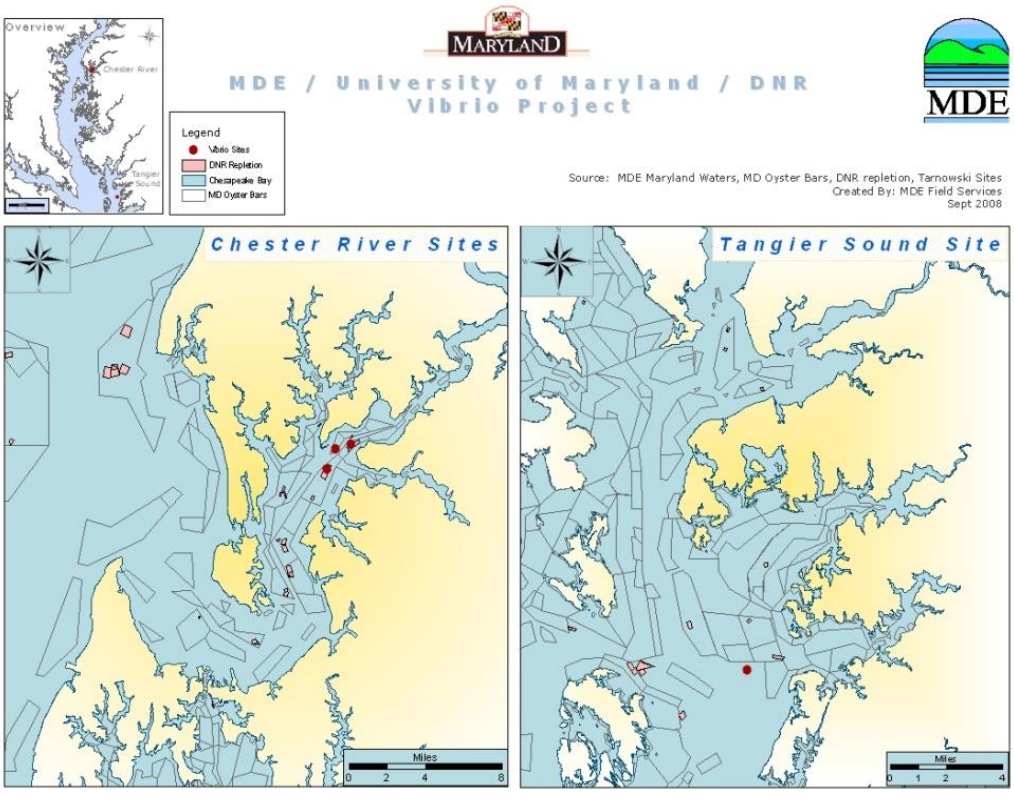


Table 1. List of primers used in this study for conventional PCR, including annealing temperatures and sequences.

Primers	**Primer sequence (5' – 3')**	**Amplicon (bp)**	**T_a (°C)**	**Reference**
utox-F vptox-R vvtox-R vctox-R	GASTTTGTTTGGCGYGARCAAGGTT GGTTCAACGATTGCGTCAGAAG AACGGAACTTAGACTCCGAC GGTTAGCAACGATGCGTAAG	 297 640 435	55	Bauer and Rørvik, 2007
vvh-F vvh-R	ATTCCAGTCGATGCGAATACGTTG TTCCAACTTCAAACCGAACTATGA	205	55	Brasher et al., 1998
vcgC-F vcgC-R	AGCTGCCGATAGCGATCT TGAGCTAACGCGAGTAGTGAG	97	55	Warner and Oliver, 2008b
pilA-F pilA-R	TGGCTGCTGTTGCTATTC GGTCCACCACTAGTACCAAC	217	50	Paranjpye et al., 2005
rtxA-F rtxA-R	CGGGATCCTATGGCGTGAAC-GGCGAAG CGGGATCCAGCAGCCACAA-GCGATTC	1440	61	Kim et al., 2008

Chapter 5: Antibiotic Resistance Profiles of *Vibrio vulnificus* and *Vibrio parahaemolyticus* Isolated from the Chesapeake Bay, Maryland

Abstract

The naturally occurring bacteria, *Vibrio vulnificus* and *Vibrio parahaemolyticus,* are known agents of septicemia and gastroenteritis arising from wound infections, and/or seafood consumption involving contact with contaminated marine and estuarine water. Antibiotics are one of the primary treatments for septicemia and wound infections and CDC guidelines recommend tetracyclines, fluoroquinolones, third-generation cephalosporins, aminoglycosides and folate pathway inhibitors (trimethoprim-sulfamethoxazole) for treatment of *Vibrio* related illness. Antibiotic resistance patterns of autochthonous pathogenic marine and estuarine bacteria are, therefore, important to determine, as well as monitoring the ability of these *Vibrio* spp. to acquire new antibiotic resistances. In this study, 603 *V. vulnificus* and 811 *V. parahaemolyticus* isolates were screened for antibiotic resistance patterns. Of the total number of isolates screened, 26.8% of the *V. vulnificus* and 96.5% of the *V. parahaemolyticus* strains were resistance to at least one antibiotic. Ampicillin and penicillin were the most common antibiotics both *V. vulnificus* and *V. parahaemolyticus* showed resistance.

Of a subset of ampicillin resistant *V. vulnificus,* over 20% were resistant to cefoxitin, a second-generation cephalosporin. Multi drug resistance to at least two antibiotics was observed in over 15% of *V. vulnificus* and 93.1% of *V. parahaemolyticus* tested. Ultimately, accurate and thorough antibiotic resistance profiles are

recommended for *V. vulnificus* and *V. parahaemolyticus* to treat these *Vibrio* infections effectively. Monitoring acquisition of new antibiotic resistances is also an important aspect of this study.

Introduction

Vibrio vulnificus, an halophilic, autochthonous marine and estuarine bacterium is the causative agent of several seafood associated diseases, including septicemia and gastroenteritis (Oliver, 2006). Infections caused by *V. vulnificus* are typically transmitted via consumption of raw or undercooked seafood or through the exposure to open wounds (Oliver, 1989). *V. vulnificus* is one of the deadliest bacteria known and is responsible for 95% of seafood-related deaths in the United States (Mead et al., 1999). Mortality from septicemia caused by *V. vulnificus* can exceed 50%, with death occurring within days of the first signs of infection (Oliver, 2006).

Vibrio parahaemolyticus, a leading cause of seafood-associated illness and death in Japan, but also in the United States, is naturally occurring in marine and estuarine environments, and can be readily isolated from water, sediment, and shellfish when environmental conditions are favorable (Johnson et al., 2010). *V. parahaemolyticus* induced illness is typically transmitted via consumption of raw or undercooked seafood or by exposure to open wounds (Johnson et al., 2010). Both *V. vulnificus* and *V. parahaemolyticus* are naturally occurring in marine and estuarine environments, and are therefore, readily isolated from water, sediment and shellfish when environmental conditions are favorable (Johnson et al., 2010).

Since 2000, cases of *Vibrio* related disease have increased and in 2011, the CDC estimated about 8,000 cases of *Vibrio* infections in the United States annually, of which 4,500 cases were related to *V. parahaemolyticus* and 100 cases to *V. vulnificus* (Scallan et al., 2011). An average of 25 cases per year of *V. vulnificus* infections occur in Maryland (CDC 2006).

The ability of these bacteria and other human pathogens to acquire antimicrobial resistance is a significant public health concern. Although most attention is focused on antibiotic resistance in the hospital setting, the ability of microorganisms found naturally in the environment cannot be neglected because antibiotic resistance determinants are readily exchanged in the aquatic environment (Baquero et al., 2008). Pathogenic bacteria, as well as their antimicrobial resistance genes, have been detected in human, cattle, and swine waste streams, as well as in treated human waste discharged from wastewater treatment plants (Agga et al., 2015; Baquero et al., 2008; Devarajan et al., 2016). It should be pointed out that naturally occurring bacteria release antibiotics into the environment as these are employed for signaling and regulation (Martinez 2008). Furthermore, genetic elements associated with antibiotic resistance are acquired by bacteria to protect themselves against toxic antibiotic compounds. Ultimately, these serve as reservoirs of antibiotic resistance genes in the natural environment (Baquero et al., 2008; Wright, 2007). The combination of clinical pathogenic strains released to the environment and the incidence of naturally occurring bacteria, coupled with their respective antibiotic resistance genes, plays a vital role in the evolution and spread of antibiotic resistance in the natural environment, including estuarine and coastal

waters.

Although *Vibrio* related gastroenteritis is not typically treated with antibiotics, wound infections caused by *V. vulnificus* or septicemia caused by *V. vulnificus* and/or *V. parahaemolyticus* can benefit from antibiotic treatment (Elmahdi et al., 2016). *Vibrio* species can be susceptible to many antibiotics routinely used by humans. However, there has been an increase of resistance in *V. parahaemolyticus* and *V. vulnificus* resistance to ampicillin as a result of excess antibiotic use in human, agricultural and aquaculture settings (Zanetti et al., 2001). Therefore, the ability of naturally occurring *V. vulnificus* and *V. parahaemolyticus* to acquire new antibiotic resistances through agricultural runoff is of interest since they have a significant potential impact on public health, specifically in relation to *Vibrio* contaminated seafood and antibiotic treatment methods for *Vibrio* related disease.

According to CDC recommendations for treatment of *Vibrio* related diseases, the following are included: tetracyclines (doxycycline, tetracycline), fluoroquinolones (ciprofloxacin, levofloxacin, gatifloxacin), third-generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone), aminoglycosides (amikacin, apramycin, gentamicin, streptomycin), and folate pathway inhibitors (trimethoprim-sulfamethoxazole) (CDC 2009). Typical treatment regimens for *V. parahaemolyticus* infections include doses of tetracycline followed by a third-generation cephalosporin (CDC 2009). Tetracycline is the ideal antibiotic as it is believed to inhibit protein synthesis of pathogenic extracellular enzymes, such as proteases and lipases (Elmahdi et al., 2016; Fang, 1992)

Although antibiotic resistance of environmental strains is of increasing interest, only three studies have analyzed the antibiotic resistance profiles of *V. vulnificus* in the United States and ultimately discovered resistances towards doxycycline, tetracycline, aminoglycosides and cephalosporins, apramycin, and streptomycin (Han et al., 2007; Baker-Austin et al., 2009; Shaw et al., 2014). Conversely, only two of the three studies investigated antibiotic susceptibility of *V. parahaemolyticus* in the United States, with the most common resistance to ampicillin and penicillin, with minor resistance to piperacillin and streptomycin (Han et al., 2007; Shaw et al., 2014). Only one study has been done that investigated antibiotic resistance genes detected in oysters. Han et al. (2007) reported only ampicillin resistance in *V. parahaemolyticus* and complete susceptibility in all *V. vulnificus* strain. In all three studies, *V. vulnificus* and *V. parahaemolyticus* strains were all found to be susceptible to cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, and tetracycline (Han et al., 2007; Baker-Austin et al., 2009; Shaw et al., 2014).

In the study reported here, a broader range of antibiotics was investigated for both *V. vulnificus* and *V. parahaemolyticus* isolates from the Chesapeake Bay. These isolates had been collected from a variety of environmental sources (water, oyster, sediment), with the objective to gain a better understanding of the antimicrobial susceptibility patterns in naturally occurring vibrios.

Methods

Sample Collection. Water, oyster, and sediment samples were collected at two

locations in the Chester River (39°05.09'N, 76°09.50'W) and Tangier Sound (38°10.97'N, 75°57.90'W) in the Chesapeake Bay, Maryland, from June, 2009, to August, 2012. During the warmer months of June through August, sampling was performed twice each month, whereas during September through May once each month.

Sample Processing. Details of the sample processing have been described elsewhere (Johnson et al., 2012; Johnson et al., 2010). Briefly, water samples were inoculated with alkaline peptone water (10× APW, pH 8.5) and incubated for 16-18 hours. Oysters cleaned before oyster tissue were homogenized 1:1 with 1× phosphate buffer solution (1× PBS; pH 7.4); homogenized oyster tissue was inoculated into 10× APW and incubated at 33°C for 16-18 hours. Sediment samples were mixed with equal part of 1x PBS, and added to 10x APW before incubation at 33°C for 16-18 hours. Strains were isolated from overnight samples by streaking onto selective media, including CHROMagar™ (CHROMagar, Springfield, NJ), thiosulfate citrate bile-salts sucrose agar, TCBS (Oxoid, Ontario, Canada), and *Vibrio vulnificus* agar (VVA). The plates were incubated at 37°C for 16-18 hrs. Presumptive colonies of *V. parahaemolyticus* and *V. vulnificus*, based on growth media, were picked and streaked onto LB agar (BD Diagnostic Systems, Sparks, MD) to obtain pure cultures.

Confirmation by PCR. DNA from presumptive isolates of *V. parahaemolyticus* and *V. vulnificus* were confirmed using multiplex PCR targeting the *toxR* gene (Bauer and Rorvik 2007) to differentiate *V. parahaemolyticus* and *V. vulnificus*. Subsequent PCR targeting virulence factors, *tlh, trh* and *tdh* (Bej et al., 1999), was performed

on all of the confirmed *V. parahaemolyticus* strains. Similarly, *V. vulnificus* isolates were confirmed by PCR targeting the cytolysin gene, *vvhA*. All PCR assays were performed using Promega GoTaq Green Master Mix (Promega, Madison, WI). Each reaction tube contained a total of 25µl, including 5µl template DNA. Thermal cycling conditions were as follows: one 10-minute cycle of denaturation at 94°C, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 60 seconds, and final extension for 10 minutes at 72°C. Sequences, amplicon size and annealing temperatures for each PCR can be found in Table 1.

Antibiogram. Kirby-Bauer disc diffusion method was used to test resistance according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2010). *Vibrio* strains were grown overnight at 35ºC on LB agar. Isolated colonies of *Vibrio* were suspended in 2 mL of 0.9% saline solution and adjusted to fit a 0.5 McFarland standard before spreading onto Mueller-Hinton agar and adding antibiotic discs. Antibiotics used in this study include ampicillin (AM10), chloramphenicol (C30), ciprofloxacin (CIP5), erythromycin (E15), kanamycin (K30), nalidixic acid (NA30), penicillin (P10), streptomycin (S10), spectinomycin (SPT100), sulfamethoxazole with trimethoprim (SXT), and tetracycline (TE30) (Table 2). Plates were incubated for 16-18 hours at 35ºC before reading a zone diameter. *Vibrio* breakpoints published by the Clinical and Laboratory Standards Institute were used to classify strains as resistant, intermediate or susceptible (Table 3). As official guidelines do not exist for all antibiotics for *Vibrio* species, *Enterobacteriaceae* guidelines were used for nalidixic acid, streptomycin and kanamycin, *Streptococcus* for

erythromycin and *N. gonorrhoeae* for spectinomycin. A subset of ampicillin resistant *V. vulnificus* and *V. parahaemolyticus* strains were further characterized for the following monobactams, carbapenems, and second-, third-, and fourth-generation cephalosporins: aztreonam (ATM30), ceftazidime (CAZ30), ceftriaxone (CRO30), cefotaxime (CTX30), cefepime (FEP30), cefoxitin (FOX30), imipenem (IPM30) (Table 2). Disc diffusion of ampicillin, penicillin and all ß-lactams were performed in triplicate. Clinical and Laboratory Standards Institute *Vibrio* guidelines were used for all antibiotics except for aztreonam and ceftriaxone where *Enterobacteriaceae* guidelines were used.

Results

A total of 603 *V. vulnificus* strains were collected between June, 2009 and August, 2012, with highest prevalence observed during the warmer summer months. The majority of *V. vulnificus* was isolated from water samples, with only ten (1.66%) isolated from sediment and two (0.33%) from oyster samples. Antibiograms were performed on 527 of the 603 isolates in this study. Strains were selected based on location (Chester River and Tangier Sound), date (strains throughout the year were analyzed) and source of isolation (all sediment and oyster strains were analyzed and the rest were from water). The isolates were found to be sensitive to antibiotics, with most common resistance observed was to ampicillin (9.31% resistant and 22.43% intermediate) and penicillin (19.77% resistant and 33.65% intermediate) (Figure 1). Antibiotic resistance is defined as allowing bacterial growth even if the drug is present and is a sign of an ineffective antibiotic.

An intermediate resistance indicates that a higher dose of antibiotic is needed to prevent bacterial growth. Susceptible antibiotic is defined as an antibiotic that prevents bacterial growth if the drug is present and indicates that the antibiotic is effective against the bacteria. Most strains (over 97%) were susceptible to chloramphenicol, ciprofloxacin, sulfamethoxazole with trimethoprim, and tetracycline. Ampicillin resistant *V. vulnificus* showed resistance to various ß lactams, including aztreonam, ceftazidime, ceftriaxone, cefotaxime, and cefoxitin (Figure 2). All ampicillin resistant strains of *V. vulnificus* were susceptible to cefepime and only 1.92% of the strains showed intermediate resistance to imipenem. Of the ampicillin resistant isolates of *V. vulnificus,* none showed similar ß-lactams resistant profiles to each other. The highest percent of resistance was for a second-generation cephalosporin, cefoxitin (21%) followed by monobactam and third generation cephalosporins. For this study, multi-drug resistance, i.e., resistance to more than two antibiotics, in *V. vulnificus* was found in 1.90% (n=10) of strains tested (Table 3). Only 26.8% of the strains tested showed some level of resistance to any of the drugs tested. Of *V. vulnificus* isolates that showed at least one resistance (26.8% of total strains tested), 53.9% (n=76) displayed resistance to one antibiotic, followed by 39.0% (n=55) to two antibiotics, and 1.4% (n=2) to seven antibiotics (Table 3).

During the same sampling time frame as above for *V. vulnificus*, ca. 1,300 *V. parahaemolyticus* isolates were collected, the largest published sampling ever undertaken in the Chesapeake Bay. A subset of 811 isolates collected between June 2009 and May 2011 was analyzed for antibiotic resistance, of which 52.6% were

from Tangier Sound and 47.4% from the Chester River. The majority of strains (73.9%) were collected from whole water (57.7%), followed by water containing plankton (9.4%), and plankton free water (6.9%). The remaining strains were collected from oysters (14.3%) or sediment (11.8%). Most strains were sensitive to all antibiotics tested with, beside resistance to ampicillin (93.2%) and penicillin (96.3%). Of the *V. parahaemolyticus* subset, most isolates were from oysters (97.4%) and sediment (94.8%) and were resistant to ampicillin and penicillin. Following ampicillin and penicillin, the most frequent resistance was to erythromycin (3.0% resistant, 30.0% intermediate), followed by tetracycline (1.6% resistant). Interestingly, 37.9% of the isolates displayed intermediate resistance towards kanamycin. *V. parahaemolyticus* isolates from oyster samples also displayed resistance to erythromycin and spectinomycin. All *V. parahaemolyticus* isolates were susceptible to chloramphenicol and ciprofloxacin. Multi drug resistance was observed 5.5% (n=45) of the *V. parahaemolyticus* isolates tested (Table 5). Of 811 *V. parahaemolyticus* isolates tested, only 3.5% (n=28) were susceptible to all eleven antibiotics (Table 5). A large portion, 93.0% (n=754), of *V. parahaemolyticus* were resistant to both ampicillin and penicillin. Multi drug resistance, resistance towards more than two antibiotics, in *V. parahaemolyticus* was found in 5.5% (n=45) of isolates tested, with 5.3% (n=43) resistant to three antibiotics and only 0.2% (n=2) resistant to four antibiotics. A subset of ampicillin resistant isolates (n=50) were further characterized for ß-lactam resistance and all proved to be sensitive.

Discussion

Overall, the *V. vulnificus* isolates included in this study showed similar antibiotic resistance profiles and were similar to those observed in previous studies, with high resistance found only to ampicillin and penicillin. Unlike previous studies that found all *V. vulnificus* strains susceptible to erythromycin and ciprofloxacin (Baker-Austin et al., 2009), in this study, 2% of *V. vulnificus* were resistant to erythromycin and less than 1% to ciprofloxacin. Although macrolides (erythromycin) are not typically used to treat *Vibrio* related illness, the fluoroquinolones (ciprofloxacin) are one of the CDC recommended treatments (CDC, 2009) and, therefore, determination of resistance to the macrolides should be monitored. Similar to results found in previous studies, none of the *V. vulnificus* isolates of in this study displayed imipenem resistance (Shaw et al., 2014). Only 26.8% of the isolates tested showed some level of resistance to any of the drugs tested, with 19.7% (n=76) displaying resistance to only one of any of the eleven antibiotics tested, indicating that antibiotic resistance, although prevalent in the environment, is not pervasive. Two isolates were found to be highly resistant to many antibiotics (seven out of the eleven antibiotics tested). The first was isolated from the Chester River site in July, 2009, and the second was isolated from Tangier Sound in October, 2009. Both isolates were isolated from plankton unfiltered water sample. The Chester River isolate was resistant to ampicillin, chloramphenicol, erythromycin, kanamycin, nalidixic acid, penicillin, and sulfamethoxazole with trimethoprim. The Tangier Sound isolate was resistant to chloramphenicol, erythromycin, kanamycin, nalidixic acid, penicillin, and sulfamethoxazole with trimethoprim and tetracycline. Important to note, isolates with such high antibiotic

resistance profiles have not been documented in the past and these two were isolated at separate points in time at two different locations. The most common antibiotic resistance profile for *V. vulnificus* was to ampicillin and penicillin.

Of 811 *V. parahaemolyticus* isolates tested, only 3.1% (n=25) were susceptible to all of the eleven antibiotics and a large proportion, 93.0% (n=754), of *V. parahaemolyticus* was resistant to both ampicillin and penicillin. In addition, tetracycline, an antibiotic recommended by CDC to treat *Vibrio* related illness, was the fourth most frequent resistance observed in the *V. parahaemolyticus* isolates in this study. The highest number of antibiotic resistances found in the *V. parahaemolyticus* isolates was four and only for two isolates during the 3-year sampling period. The first was isolated from the Chester River in March, 2011, from a water sample and the antibiotic resistance was to ampicillin, erythromycin, penicillin, and streptomycin. The second was isolated from oysters in May, 2012, in Tangier Sound and was resistant to ampicillin, streptomycin, spectinomycin, and penicillin. As found with *V. vulnificus,* the most common antibiotic resistance of *V. parahaemolyticus* was to ampicillin and penicillin.

The frequency of antibiotic resistance observed in *V. vulnificus* during this study was lower than found in previous studies in which 90% of environmental isolates were resistant to at least one antibiotic and 20% to at least five antibiotics (Martinez, 2003). However, the *V. parahaemolyticus* isolates of this study were similar to those of previous studies, with 96.5% (n=783) resistant to at least one antibiotic (Martinez, 2003). However, it should be noted that the previous studies included other bacterial species, in addition to *Vibrio*.

In a previous study Ceccarelli et al. (2015) profiled 307 *V. cholerae* isolates collected during the same sampling period and source as the *V. vulnificus and V. parahaemolyticus* isolates of this study. These *V. cholerae* isolates displayed lower frequencies of antibiotic resistance. Only 13% showed resistance to one or two of the antibiotics tested and only 20 strains showed resistance to both ampicillin and penicillin (Ceccarelli et al., 2015). Multidrug-resistance was not detected in any of the *V. cholerae* isolates. Conversely, *V. vulnificus* and *V. parahaemolyticus* isolates collected at the same time as the *V. cholerae* were multidrug resistant (1.9% and 55.5%, respectively) with the most common to ampicillin and penicillin. All of the *V. cholerae* isolates were sensitive to chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, spectinomycin, streptomycin, sulfamethoxazole-trimethoprim, and tetracycline (Ceccarelli et al., 2015). However, when *V. vulnificus* and *V. parahaemolyticus* strains were included, none of the antibiotics were 100% susceptible for all *Vibrio* spp. The most effective (less than 1% resistant) for *V. vulnificus, V. parahaemolyticus,* and *V. cholerae* were chloramphenicol, ciprofloxacin, nalidixic acid, and sulfamethoxazole-trimethoprim.

Ultimately, occurrence of antibiotic resistant *V. vulnificus* and *V. parahaemolyticus* is increasingly a public health concern because of the impact on *Vibrio* related disease treatment. Treatment of *V. vulnificus* and *V. parahaemolyticus* infections should be preceded by determination of antibiograms to assure that treatment is accurate, rapid, and effective. Given the rapid progression of *V. vulnificus* related disease, it is vital that an effective and speedy treatment method is employed. Similarly, frequent testing for antibiotic resistance in *V. vulnificus* and

V. parahaemolyticus is important to determine if a new resistance arises, to assure public health protection. The increased multidrug resistance reported recently is a serious concern and the morbidity and mortality rates of *V. vulnificus* and *V. parahaemolyticus* related illnesses may also be affected.

Table 1. List of primers used in this study for conventional PCR, including annealing temperatures and sequences.

Primers	**Primer sequence (5' – 3')**	**Amplicon (bp)**	**T_a (°C)**	**Reference**
utox-F	GASTTTGTTTGGCGYGARCAAGGTT		55	Bauer and Rørvik, 2007
vptox-R	GGTTCAACGATTGCGTCAGAAG	297		
vvtox-R	AACGGAACTTAGACTCCGAC	640		
vctox-R	GGTTAGCAACGATGCGTAAG	435		
tlh-L	AAAGCGGATTATGCAGAAGCACTG	450	58	Bej et al., 1999
tlh-R	GCTACTTTCTAGCATTTTCTCTGC			
tdh-L	GTAAAGGTCTCTGACTTTTGGAC	269		
tdh-R	TGGATAGAACCTTCATCTTCACC			
trh-L	TTGGCTTCGATATTTTCAGTATCT	500		
trh-R	CATAACAAACATATGCCCATTTCCG			
vvh-F	ATTCCAGTCGATGCGAATACGTTG	205	55	Brasher et al., 1998
vvh-R	TTCCAACTTCAAACCGAACTATGA			

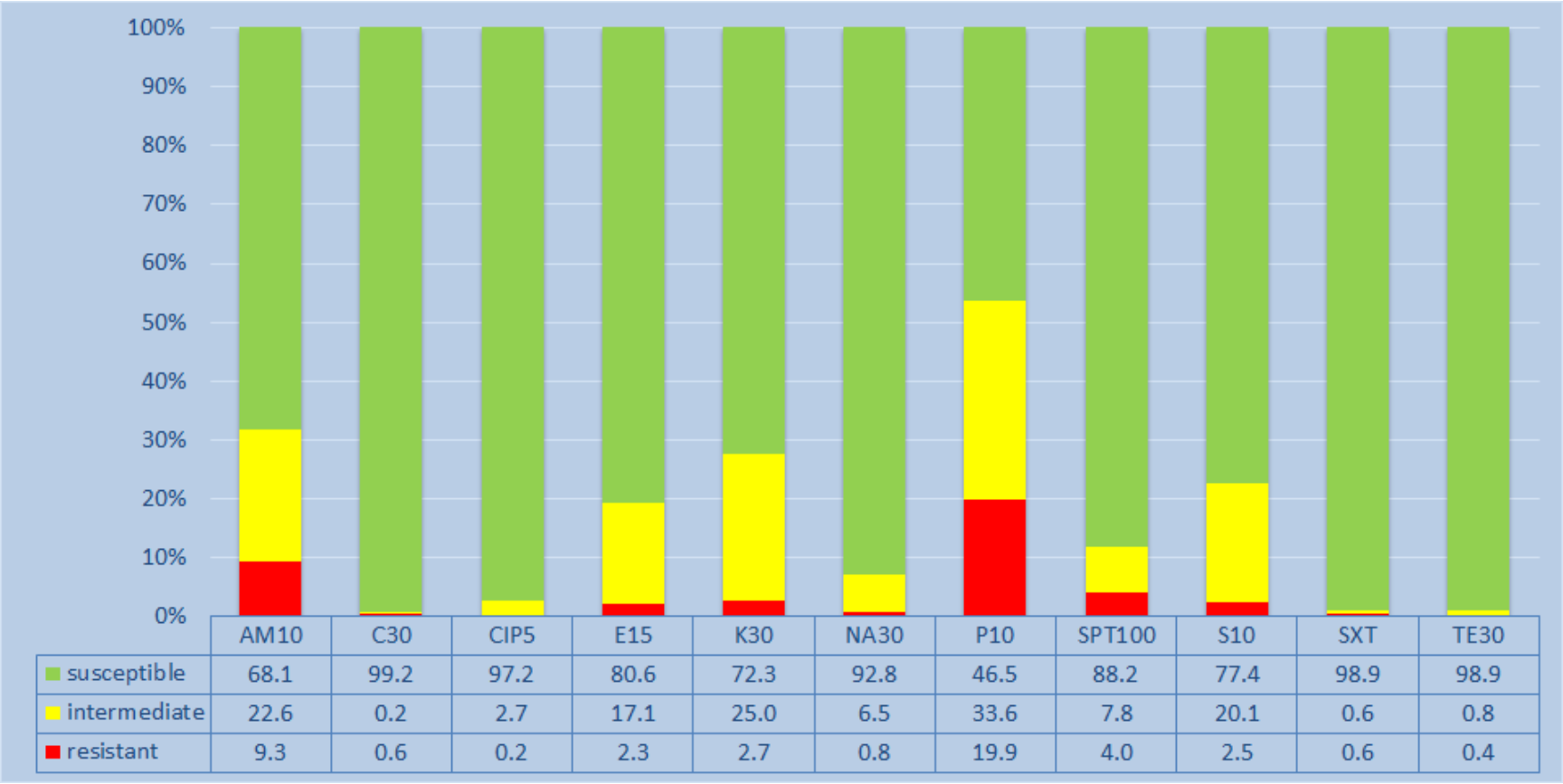
Table 2. Antibiotics and concentrations of the antibiotics used in this study. Antibiotics marked with * were used only in a select number of ampicillin resistant strains. Concentrations of antibiotics follow antibiotic abbreviation in parentheses and are in μg.

β-lactam antibiotics	Penicillin (penams)	Narrow-spectrum	Penicillin (P10)
		Moderate	
		Broad	
		Extended-spectrum	Ampicillin (AM30)
	Cephalosporins (cephems)	First Generation	
		Second Generation	Cefoxitin (FOX30) *
		Third Generation	Cefotaxime (CTX30) * Ceftazidime (CAZ30) * Ceftriaxone (CRO30) *
		Fourth Generation	Cefepime (FEP30) *
		Fifth Generation	
	Carbapenems and Carbacephems		Imipenem (IPM30) *
	Monobactams		Aztreonam (ATM30) *
Fluoroquinolone		First Generation	Nalidixic acid (NA30)
		Second Generation	Ciprofloxacin (CIP5)
Macrolide			Erythromycin (E15)
Aminoglycoside			Kanamycin (K30)
			Streptomycin (S10)
Tetracyclines			Tetracycline (TE30)
Trimethoprim/ Sulfonamides			Sulfamethoxazole with trimethoprim (SXT 23.75/1.25)
Other			Chloramphenicol (C30)
			Spectinomycin (SPT100)

Table 3. Breakpoint guidelines according to the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2010).

Antibiotic Disc Diffusion Susceptibility Testing Breakpoints (mm)									
Antibiotic	AM10	C30	CIP5	E15	K30	NA30	P10	SPT100	S10
S	≥ 17	≥ 18	≥ 21	≥ 21	≥ 18	≥ 19	≥ 17	≥ 18	≥ 15
I	14-16	13-17	16-20	16-20	14-17	14-18	14-16	15-17	12-14
R	≤ 13	≤ 12	≤ 15	≤ 15	≤ 13	≤ 13	≤ 13	≤ 14	≤ 11
Antibiotic	SXT	TE30	ATM30	CAZ30	CR030	CTX30	FEP30	FOX30	IMP10
S	≥ 16	≥ 15	≥ 21	≥ 21	≥ 26	≥ 26	≥ 18	≥ 18	≥ 16
I	11-15	12-14	18 - 20	18 - 20	23 - 25	23 - 25	15 - 17	15 - 17	14 - 15
R	≤ 10	≤ 11	≤ 17	≤ 17	≤ 22	≤ 22	≤ 14	≤ 14	≤ 13

Figure 1. Percent of antibiotic-resistant environmental *V. vulnificus* isolates (n=527).

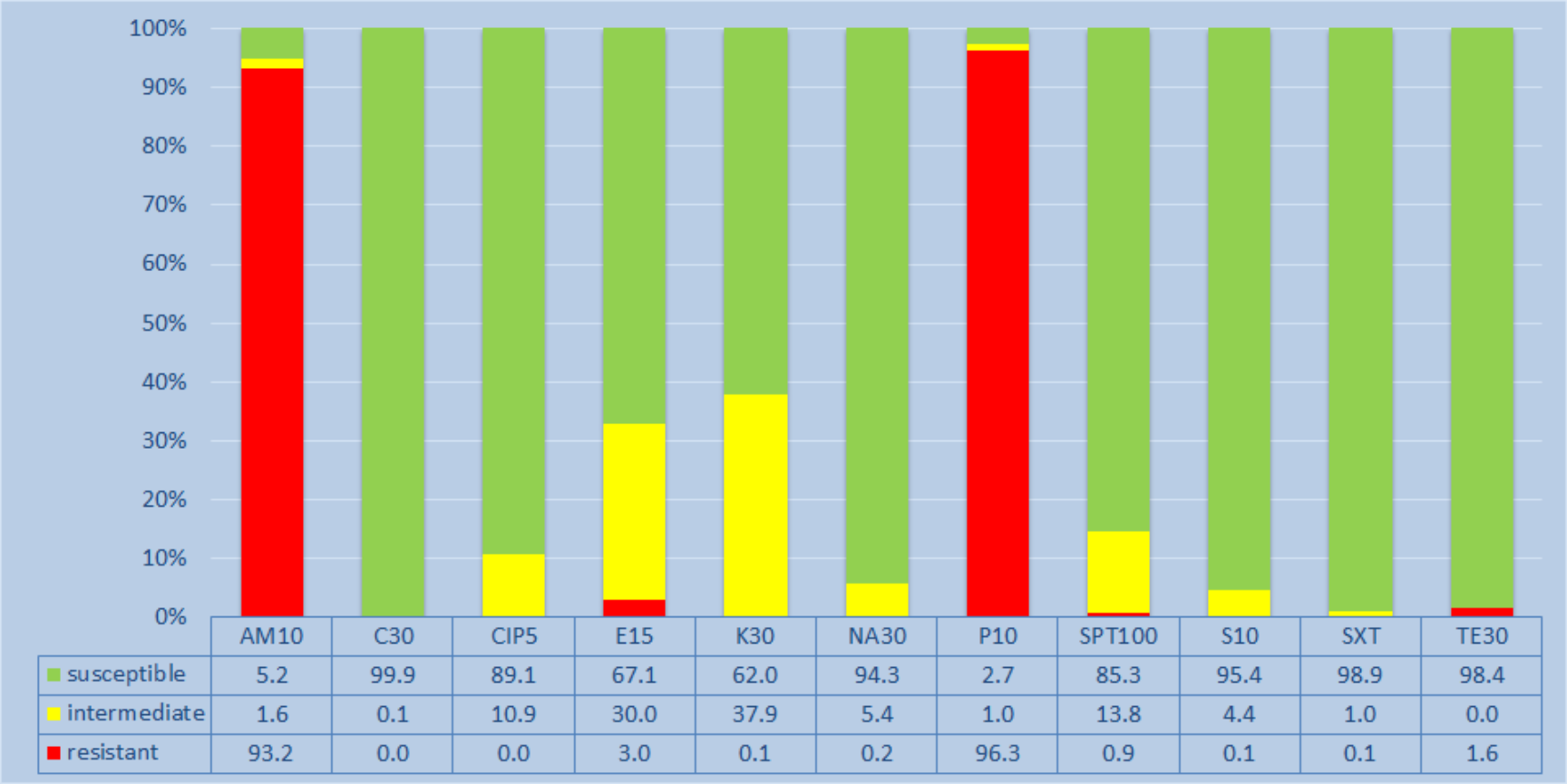


	AM10	C30	CIP5	E15	K30	NA30	P10	SPT100	S10	SXT	TE30
susceptible	68.1	99.2	97.2	80.6	72.3	92.8	46.5	88.2	77.4	98.9	98.9
intermediate	22.6	0.2	2.7	17.1	25.0	6.5	33.6	7.8	20.1	0.6	0.8
resistant	9.3	0.6	0.2	2.3	2.7	0.8	19.9	4.0	2.5	0.6	0.4

Figure 2. Percent of ampicillin-resistant environmental *V. vulnificus* isolates (n=52).

	ATM30	CAZ30	CR030	CTX30	FEP30	FOX30	IMP10
susceptible	63.5	92.3	71.2	75.0	100.0	61.5	98.1
intermediate	28.8	5.8	17.3	15.4	0.0	21.2	1.9
resistant	7.7	1.9	11.5	9.6	0.0	17.3	0.0

Figure 3. Percent of antibiotic-resistant environmental *V. parahaemolyticus* isolates (n=811).



	AM10	C30	CIP5	E15	K30	NA30	P10	SPT100	S10	SXT	TE30
susceptible	5.2	99.9	89.1	67.1	62.0	94.3	2.7	85.3	95.4	98.9	98.4
intermediate	1.6	0.1	10.9	30.0	37.9	5.4	1.0	13.8	4.4	1.0	0.0
resistant	93.2	0.0	0.0	3.0	0.1	0.2	96.3	0.9	0.1	0.1	1.6

Table 4. Number of *V. vulnificus* isolates resistant to more than one antibiotic. * indicates multi-drug resistance

Number of resistances (out of 11 tested antibiotics)	Number of strains possessing multi-drug resistance (n = 527)
0	386
1	76
2	55
3 *	5
4 *	3
7 *	2

Table 5. Number of *V. parahaemolyticus* isolates resistant to more than one antibiotic. * indicates multi-drug resistance

Number of resistances (out of 11 tested antibiotics)	Number of strains possessing multi-drug resistance (n = 811)
0	25
1	28
2	713
3 *	43
4 *	2

Chapter 6 Genetic Diversity of *Vibrio vulnificus* and *Vibrio parahaemolyticus* Isolates from Individual Oysters, *Crassostrea virginica*

Introduction

Vibrio vulnificus, an halophilic, autochthonous marine bacterium is the causative agent of several seafood associated diseases, including septicemia and gastroenteritis. Infections caused by *V. vulnificus* are transmitted by consumption of raw or undercooked seafood or exposure to open wounds (Oliver, 2009). Mortality from septicemia caused by *V. vulnificus* can exceed 50% and death can occur within days of the first signs of infection. Immunocompromised individuals or those with chronic liver disease are at higher risk of fatal infection of *V. vulnificus.* Although *V. vulnificus* is the leading cause of seafood related deaths in the United States, little is known about the genetic diversity of this species of *Vibrio*. Similar to other bacterial species, *V. vulnificus* strains are known to express high genetic heterogeneity. Additionally, past studies have reported that individual oysters are populated by numerous polymorphic strains of *V. vulnificus.* Although many studies have been carried out comparing clinical and environmental strains of *V. vulnificus*, few studies have investigated their genetic diversity within a single oyster.

Vibrio parahaemolyticus, a leading cause of seafood-associated illness and death in the United States, is naturally occurring in marine and estuarine environments and can be readily isolated from water, sediment, and shellfish when

environmental conditions are favorable. *V. parahaemolyticus* induced illness is typically transmitted through consumption of raw or undercooked seafood or exposure to open wounds. Since 2000, cases of *Vibrio* related disease have steadily increased. In August 2012, six Marylanders became ill in a *V. parahaemolyticus* outbreak. Although *Vibrio* related diseases pose a significant health risk, few studies have looked at the genetic diversity of *V. parahaemolyticus*, within a single oyster. Past studies have shown that other *Vibrio* species, including *V. vulnificus*, display high genetic heterogeneity within distinct populations.

Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) is a fingerprinting technique used to distinguish bacterial strains with little to no genetic variation. This is accomplished by amplification of polymorphisms in several Variable-Number Tandem-Repeat (VNTR) loci using PCR (Lindstedt, 2005). These VNTRs are highly polymorphic and can be used to differentiate bacterial strains based on the length of repeat regions (Lindstedt, 2005).

Pulsed field gel electrophoresis, PFGE, is a gel electrophoresis method that produces DNA fingerprints of bacterial genomes and is the current "gold standard" method widely used today. PFGE has been found to produce a high degree of variation in patterns for *V. vulnificus* strains isolated from clinical and environmental settings, compared to other fingerprinting techniques, such as ribotyping (Tamplin et al., 1999).

This study investigates polymorphic strains of *V. parahaemolyticus* and *V. vulnificus* detected within a single oyster collected from the Chesapeake Bay in Maryland. For *V. parahaemolyticus* analysis, MLVA analysis was performed

because robust fingerprinting primers currently exist. For *V. vulnificus*, PFGE was performed rather than MLVA.

Methods

Isolates

Individual oysters were collected in June and July of 2012 from two study sites in the Chesapeake Bay, Maryland, namely Chester River and Tangier Sound. Individual oysters were separately homogenized with 1xPBS and the homogenate spread plated onto TCBS, Thiosulfate Citrate Bile Salts Sucrose Agar, in duplicate and the plates were incubated overnight at 37°C. In addition, an aliquot of each single oyster slurry was incubated overnight at 37°C with 1x alkaline peptone water and a loopful was streaked, in duplicate, onto TCBS. Presumptive *V. vulnificus* and *V. parahaemolyticus* isolates were streaked onto TCBS and single colonies were picked.

PCR targeting species-specific structural gene transmembrane regulatory protein, *toxR,* was performed on presumptive *V. parahaemolyticus* and *V. vulnificus* for confirmation of identification (Bauer and Rørvik, 2007).

DNA extraction and MLVA analysis for *V. parahaemolyticus*

DNA was extracted for sequencing as follows. *V. parahaemolyticus* isolates were grown overnight at 37°C for 16-18 at 150rpm in LB broth. A 1.5ml aliquot of inoculum was centrifuged for 10 minutes at 13K and the supernatant discarded. DNA was extracted with Qiagen MiniPrep kit, following manufacturer's protocol. Clonal relationship of these strains was determined using Multi Locus VNTR

Analysis (MLVA), targeting ten different loci belonging to both Chromosome 1 and 2 (Table 1). PCR assays were performed using Promega GoTaq Green Master Mix (Promega, Madison, WI). Each reaction tube contained a total of 50µl, including 10µl template DNA. Thermal cycling conditions were as follows: one 10-minute cycle of denaturation at 94°C, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 60 seconds, and final extension for 10 minutes at 72°C (Table 1). After completion of PCR, the products were cleaned using ZymoResearch DNA Clean & Concentrator™ and transported to Eurofins MWG Operon for sequencing using Sanger sequencing.

PFGE for *V. vulnificus*

Pulsed field gel electrophoresis (PFGE) of *V. vulnificus* was performed using a slight modification of the CDC Pulse-Net protocol of the CDC (Pulse Net United States 2013). *V. vulnificus* isolates were digested using 40 U NotI or 50 U SfiI enzymes (Promega, Madison, WI) digested. *Salmonella enterica* ATCC BAA-664, serving as control, was XbaI digested. Banding patterns were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

Results and Discussion

From the three rounds of sampling, a total of 38 *V. parahaemolyticus* isolates were isolated, identified, and analyzed by MVLA. From the seven oyster samples, a total of 216 *V. vulnificus* isolates were isolated and confirmed from PCR and a subset comprising 132 *V. vulnificus* isolates were verified and analyzed by PFGE.

All *V. parahaemolyticus* strains were *tlh*+ and *trh/tdh*-. Of the 38 strains, no two strains had identical MLVA patterns. The loci VP2-07/VPTR16 and VPTR18 had no repeats within them for all strains. The largest amount of variability occurred in loci VPTR1, VPTR2, VPTR6, and VPTR8 (Table 2). Similar studies using MLVA yielded similar results, indicating that *V. parahaemolyticus* collected from individual oysters are highly diverse, in fact supporting the hypothesis of a continuous colonization of oysters by heterogeneous environmental *V. parahaemolyticus*, rather than colonization by a single clonal strain followed by amplification of the clone within an oyster.

The majority of the *V. vulnificus* isolates were successfully characterized by PFGE, after genomic DNA digestion with NotI. The results show that the majority of strains were diverse and clustering of NotI digested strains was variable (Figure 2). The majority of *V. vulnificus* with similar banding patterns were, in fact, not isolated from the same oyster. Instead, *V. vulnificus* isolates in individual oysters, after having been collected from the separate trials appeared to be more closely related than *V. vulnificus* isolates from the same oyster. Single outliers were three strains 04-DV-6A, 04-EV-4A, and 04-EV-7A, with identical banding patterns, suggesting theses isolates were clonal. It should be noted that PFGE yields only a limited number of bands per strain, hence distribution of genetic distances is sparse.

In conclusion, the results indicate a continuous colonization of oysters by heterogeneous environmental *V. vulnificus* and *V. parahaemolyticus*, rather than colonization by a single clone, followed by amplification within the oyster. Additionally, observations of such genetic heterogeneity of a bacterial species

within a single oyster indicates that disease caused by this bacterium can result from infection with a population of multiple strains of a *Vibrio* species or with a few highly pathogenic strains of *V. vulnificus* or *V. parahaemolyticus* within a single oyster. These findings support active monitoring of harvested oysters for human consumption to safeguard public health.

Table 1. Primers used for MLVA analysis of *V. parahaemolyticus* isolates.

Locus	**Chr**	**Primers**	**Primer Sequence (5' -> 3')**	**Product Size (bp)**	**Repeat motif**
VPTR1 VP2892	1	VPTR1-F VPTR1-R	TAACAACGCAAGCTTGCAACG TCATTCTCGCCACATAACTCAGC	255	TATCTC
VPTR2 VPA1454	2	VPTR2-F VPTR2-R	GTTACCAAACTGGCGATTACGAAG CGGAATTCAGGATCATCCTGAT	615	GCTGTT
VPTR3 VPA0714	2	VPTR3-F VPTR3-R	CGCCAGTAATTCGACTCATGC AAGACTGTTCCCGTCGCTGA	333	ATCTGT
VPTR4 VP0446	1	VPTR4-F VPTR4-R	AAACGTCTCGACATCTGGATCA TGTTTGGCTATGTAACCGCTCA	229	TGTGTC
VPTR5 VP3012.VP3013	1	VPTR5-F VPTR5-R	GCTGGATTGCTGCGAGTAAGA AACTCAAGGGCTGCTTCGG	202	CTCAAA
VPTR6 VP2226	1	VPTR6-F VPTR6-R	TGTCGATGGTGTTCTGTTCCA CTTGACTTGCTCGCTCAGGAG	312	GCTCTG
VPTR7 VP2131	1	VPTR7-F VPTR7-R	CAACAGTTCTGCTCTAATCTTCCG CAAAGGTGTTACTTGTTCCAGACG	221	CTGCTC
VPTR8 VP2956	1	VPTR8-F VPTR8-R	ACATCGGCAATGAGCAGTTG AAGAGGTTGCTGAGCAAGCG	306	CTTCTG
VP2-07/VPTR16 VPA1455	2	VPTR207-F VPTR207-R	ATCGCTGCTTGAAGAAAATCCTGAT CTAATTTTTCTGGTTGGGCTTGCG	461	TCGTTG
VPTR18 VP2529	1	VPTR18-F VPTR18-R	ATCGATGAAGAAAATGCCATTGCTG CTATGGAGAAGCCTTCAGGGAAAGTTTT	231	TCCAAGA

Table 2. Subset of VNTR patterns of *V. parahaemolyticus* isolates picked from ChromAgar.

Strain	VPTR1	VPTR2	VPTR3	VPTR4	VPTR5	VPTR6	VPTR7	VPTR8	VPTR18	VPTR207
VPAQ41037	10	22	5	3	7	21	4	10	0	0
VPF11-3A	9	21	3	3	9	12	4	5	0	0
VPTX2103	23	14	6	5	7	17	4	8	0	0
VPFIHES98	16	24	5	6	5	12	4	9	0	0
10-DC-2A	13	46	5	4	0	14	6	19	0	0
10-DC-2B	13	46	5	3	8	14	6	19	0	0
10-DC-3A	0	44	2	1	1	4	0	7	0	0
10-DC-3B	0	26	2	2	1	14	6	9	0	0
10-DC-3C	12	28	4	2	7	6	2	9	0	0
10-DC-3D	19	30	2	4	6	10	0	15	0	0
10-DC-4A	9	45	4	3	2	15	0	10	0	0
10-DC-4B	0	29	4	1	1	7	0	5	0	0
10-DC-4C	9	46	4	3	2	15	6	10	0	0
10-DC-4D	5	30	8	0	9	11	0	9	0	0

Figure 1. PFGE gel of NotI digested *V. vulnificus* isolates from a single oyster. Lanes 1, 8 and 15 are positive controls.

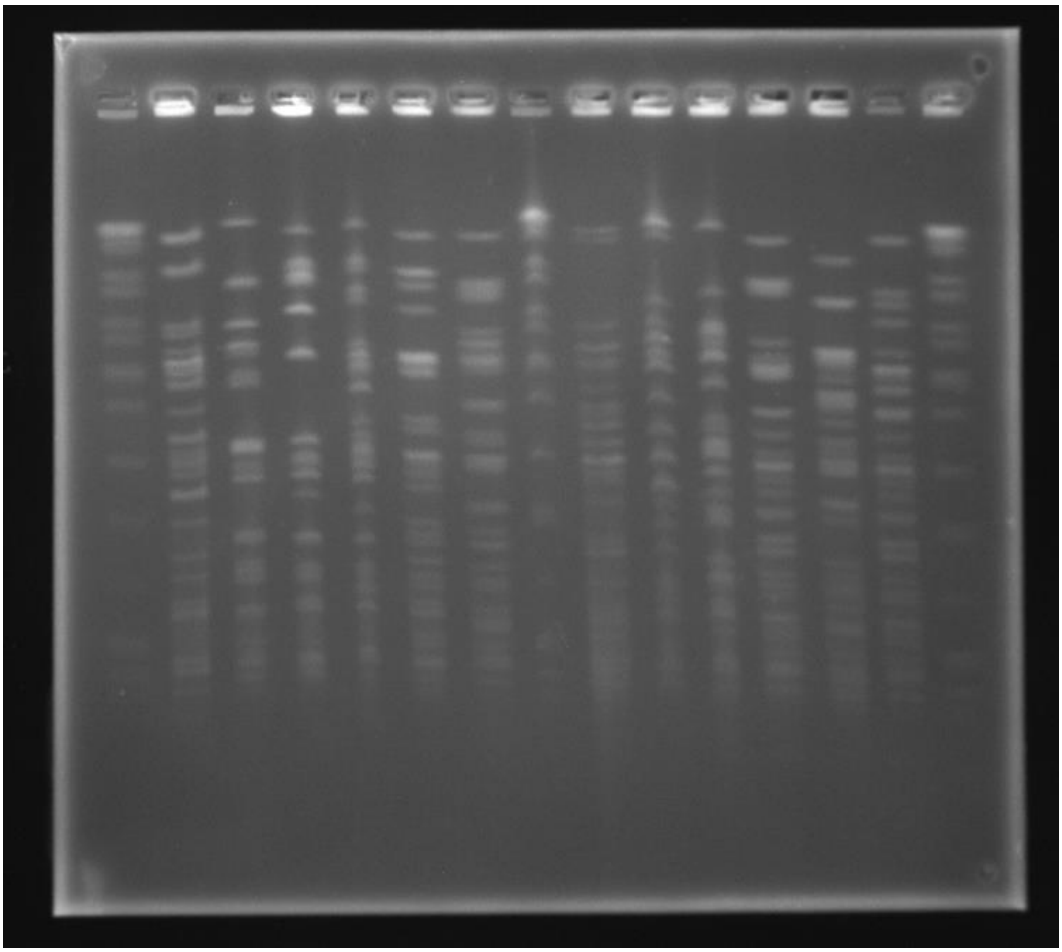
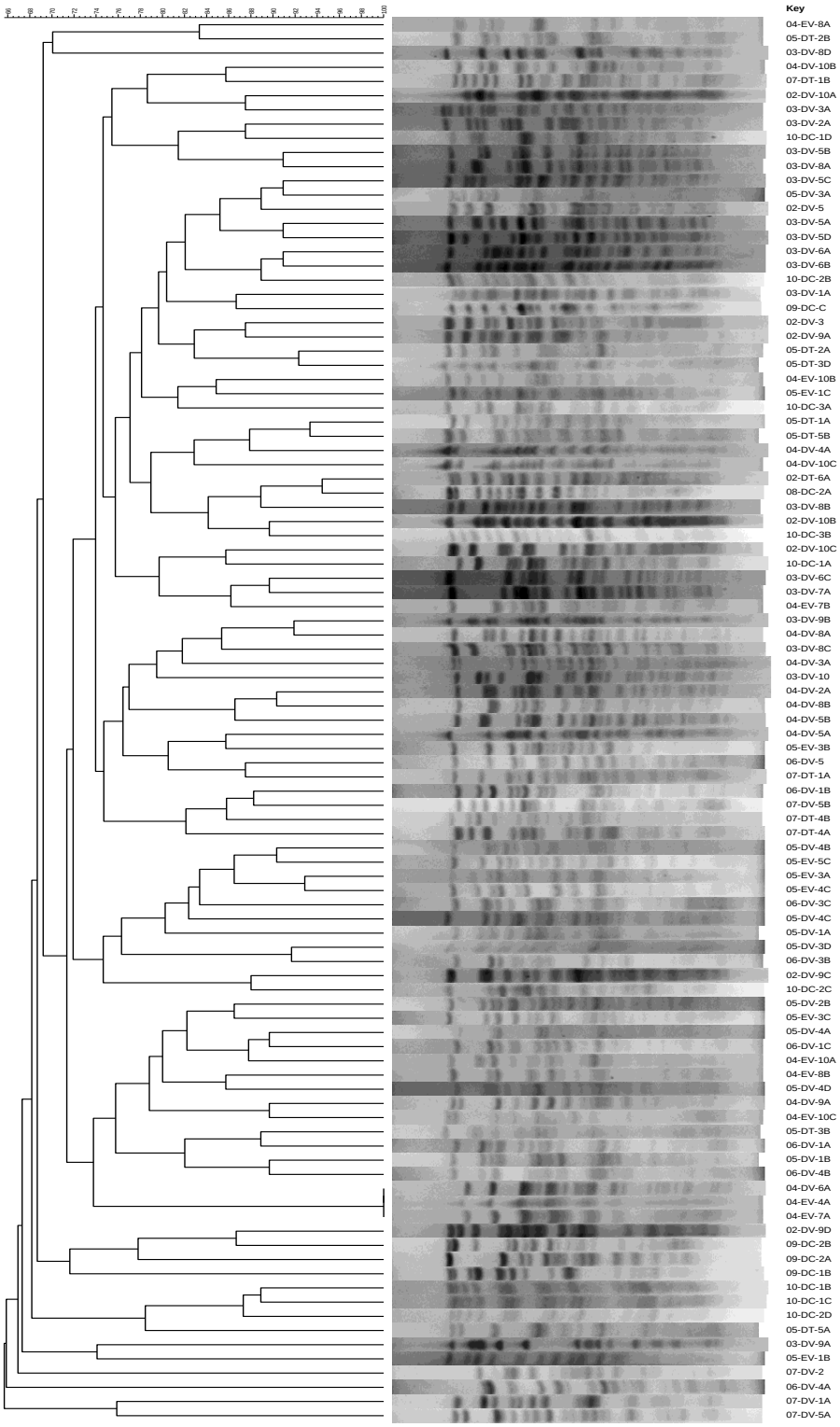


Figure 2. Dendrogram of NotI digested *V. vulnificus* isolates.



Chapter 7: Cholera SMART II Test for Rapid Detection of *Vibrio cholerae* in Ballast Water

Introduction

The water in ship ballast tanks is used primarily to stabilize ocean-bound shipping vessels, balance cargo load and maintains safe operation of the ship. The extra water that is taken on board allows the ship to achieve an enhanced stability and decreases overall stress on the vessel (Satir, 2008). Once a vessel arrives in port to unload cargo, the ballast tanks are filled with water and discharged later, once the vessel reaches its next destination and loads new cargo. The tanks of ships empty of cargo are filled with water that serves as ballast and later discharged when the ship reaches their destination or is loaded with new cargo. Although ballast water allows enhanced safety for seafaring vessels, the water stored in ballast tanks contain a variety of organisms that can be transported nationally and internationally hence introducing non-native species, (Endresen et al., 2004; Rozen and Belkin, 2001). A variety of non-native organisms have been introduced around the world, including the infamous zebra mussel, mitten crab, water flea and the bacterium, *V. cholerae,* to various parts of the world in discharged ballast water (Ruiz, 2000). These non-native organisms can establish themselves in their new environment and ultimately alter or impact the natural ecosystem. The introduction of new species, including potentially pathogenic organisms, may therefore, pose a threat to the local marine ecological system. The impact caused by these non-native organisms introduced via ballast water can be extensive and is one of the many major threats

to the world oceans today.

Therefore, to prevent these organisms from transmitting from one environment to another via ballast water, a variety of methods have been devised and are being investigated, including physical filtration, treatment with oxidizing chemicals, and/or UV irradiation to remove or inactivate pathogens, in the ballast water is before being released to the new environment (Ruiz, 2000). Our laboratory, in collaboration with the Maritime Environmental Research Center (MERC, www.maritime-enviro.org), has participated in testing ballast water management system (BWMS) performance, including evaluation of bacterial response to the various treatments.

Current ballast water discharge regulations include limits of allowable levels of fecal coliforms, including *Escherichia coli* and *Enterococcus spp.,* and toxigenic *Vibrio cholerae*. Accurate detection and enumeration of pathogens persisting after treatment is, therefore, critical for determining the efficiency of BWMS. There are several EPA approved standard operating protocols available for detection of *E. coli* and *Enterococci*, but none are known to be effective for detection of *V. cholerae* in ballast water. Existing regulations allow <1 CFU toxigenic *V. cholerae* per 100 mL and this typically is determined by colony blot hybridization, DFA, or PCR (as described in the EPA ETV Protocols, 2010). However, such methods can be time consuming and, in practice, difficult to perform in the field outside of a laboratory, especially onboard of a ship. In this study, the Cholera O1 SMART II and Cholera O139 SMART II (New Horizons Diagnostics, Columbia, MD) for water were employed to determine efficacy to detect *V.*

cholerae and for potential application in ballast water treatment systems. Cholera SMART II tests are rapid, lateral flow, colorimetric immunoassays designed for detection of *V. cholerae* O1 or O139.

Methods

This study was divided into multiple parts as outlined below:

SMART II for *V. cholerae* O1 and O139 detection

The Cholera O1 SMART II was evaluated to determine efficacy. For this test, nine clinical strains of *Vibrio cholerae* O1, nine environmental *Vibrio cholerae* O1 and six environmental *Vibrio cholerae* non-O1 were grown overnight in 1x APW and tested using the Cholera O1 SMART II. Cholera O139 SMART II was tested by using two clinical *V. cholerae* O139 and two environmental *V. cholerae* O139. *V. cholerae* O139 strains were grown overnight in 1x APW.

To use Cholera O1 or O139 SMART II test strips, three drops of overnight culture were placed in the sample well (S) of the SMART II lateral flow device. After three minutes or after the sample was absorbed into the sample well, two free falling drops of Chase Buffer were added to the sample well. Results were recorded after 15 minutes (no longer than 30 minutes) post sample addition, namely, observation of the development of color in the Control (C) and Test Line (T).

Determining cell concentrations of *V. cholerae* O1 and O139

For in-depth tests, two environmental *V. cholerae* O1, two clinical *V. cholerae* O1, two environmental *V. cholerae* O139, and two clinical *V. cholerae* O139 strains were used. Cultures were grown overnight in LB broth at 37°C. Cell concentration

was determined by a spectrophotometer, Genesys 10S (ThermoScientific, Pittsburgh, PA) to calculate OD_{600} of undiluted overnight cultures. In the next step, 100µl of diluted overnight culture (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were plated onto LB agar overnight at 37°C and colonies were enumerated. This process was repeated three times to determine the OD_{600} for each of the eight strains. The OD_{600} values were used in subsequent tests to estimate amending tests with 1cfu/100ml *V. cholerae.*

SMART II for testing *V. cholerae* O1 and O139 in natural water

After initial determination of the specific number of cells of pure cultures of environmental and clinical *V. cholerae* O1 and O139 strains were added to water collected from Port Covington (Baltimore, MD) and Lake Artemisia (College Park, MD). Cultures of environmental and clinical *V. cholerae* O1 and/or O139 were grown in LB broth overnight at 37°C. Natural whole water samples were collected from Port Covington (Baltimore, MD), Lake Artemisia (College Park, MD) at two-time points. The water samples (500ml) were filtered using membrane filters, resuspended in 100ml of 1x APW, and amended with 10^1, 10^2, 10^3,10^4 environmental or clinical *V. cholerae* O1 and *V. cholerae* O139. Samples, in duplicate, were incubated for six hours at 35°C and tested using SMART II. The samples were stored at room temperature for four days and retested using SMART II at 1 day, 2 days and 4 days. Positive and negative controls were included in the SMART II kit at each stage of testing.

SMART II for testing *V. cholerae* O1 and O139 in unfiltered natural water with

overnight incubation

Cultures of environmental *V. cholerae* O1 and/or O139 were grown in LB broth overnight at 37°C. Natural whole water samples were collected from Port Covington (Baltimore, MD), Lake Artemisia (College Park, MD) at two-time points and 100 ml of whole unfiltered water samples were added to pre-dried APW bottles (Hardy) and amended with environmental or clinical *V. cholerae* O1 and *V. cholerae* O139 isolates (10^{-2}, 10^{-1}, 10^{0}, 10^{1}). Samples amended with 10^{-2} cells were prepared in quintuplicate and were equivalent to 1cfu/100ml. All samples were incubated overnight at 35°C and tested using Cholera O1 or O139 SMART II. For overnight samples that gave negative Cholera O1 or O139 SMART II results, a loopful of sample was streaked onto TCBS to determine detect culturable cells, if any. For all overnight samples, regardless of positive or negative result, 1ml of sample was boiled to extract DNA with which to validate results using PCR targeting *ompW,* in addition to O1 and/or O139 coding genes (Hoshino, 1998). In addition, 1ml of sample was preserved with formaldehyde for enumeration by DFA.

PCR

All PCR assays were performed using Promega GoTaq Green Master Mix (Promega, Madison, WI). Each reaction tube contained a total of 25µl, including 5µl template DNA. Thermal cycling conditions were as follows: one 10-minute cycle of denaturation at 94°C, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 60 seconds, and final extension for 10 minutes at 72°C. PCR products were stored at 4°C until

gel electrophoresis visualization. Sequences, amplicon size and annealing temperatures for each PCR can be found in Table 1.

DFA

Fixed samples were analyzed using Cholera O1 DFA kit and Bengal O139 kits (New Horizon Diagnostics, Arbutus, MD) as follows. In duplicate, 5 µl of sample, were applied onto a 10-well slide (provided in kits) along with positive and negative controls. Samples were air dried for one hour and 5 µl of methanol was added to fix samples to the slides. Next, 10 µl of reconstituted Cholera O1-DFA Reagent or Bengal O139 DFA Reagent was added to each well. Slides were placed in a covered, moist chamber at 35°C for 30 minutes, after which the slides were rinsed with 1X PBS and dried. Fluorescent mounting medium was applied to each well. Slides were read using a fluorescent microscope at 1000X with oil immersion. Positive cells were characterized through a bright fluorescent cell wall and black interior (Figure 1).

SMART II for testing *V. cholerae* O1 and O139 in ballast water

Ballast sample water was collected from the MERC barge located in Port Covington (Baltimore, MD) and Joint Expeditionary Base (Norfolk, MD) during uptake (control water only) and discharge (control and treated water) days. Treated ballast water collected during shipboard testing at other locations (Annapolis, Japan, Canada) was also included in the analysis.

Whole water (100ml) was added to pre-prepared dehydrated APW bottles (Hardy Diagnostics, Santa Maria, CA) or pre-prepared 400ml APW concentrate bottles

(Hardy Diagnostics, Santa Maria, CA). Samples were incubated overnight at 35°C and tested using Cholera O1 or O139 SMART II. From overnight samples, 1ml of sample was boiled to obtain DNA to validate results, using PCR targeting *ompW* in addition to O1 and/or O139 coding genes (Nandi, 2000; Hoshino et al., 1998) and 1ml of each sample was preserved with formaldehyde and analyzed using DFA.

Results

Initial Results of SMART II test for *V. cholerae* O1 and O139 detection

Results of the initial trials demonstrated that SMART II was effective in detecting as few as 10^3 cells after incubation for six hours and 10^2 after four days at room temperature (Figure 2 and 3). No difference was observed in results obtained for the environmental and clinical *V. cholerae* O1 or O139 strains.

SMART II for testing *V. cholerae* O1 and O139 in filtered natural water collected from Port Covington (Baltimore, MD) and Lake Artemisia (College Park, MD)

The results demonstrated that incubation for six hours was not sufficient to detect *V. cholerae* when using the Cholera O1 and O139 SMART II tests since only 25 (66%) were positive when spiked with 10^1 cells after 6 hours of incubation at 35ºC. However, after 24-hour incubation, positive tests with lower numbers of bacterial inoculum could be observed. For Cholera O1 SMART II, using a 25ml sample after 24 hours incubation at 35ºC, 17% were positive at 10^{-2} addition. For Cholera O139 SMART II, 25ml sample volume after 24 hours at 35ºC, yielded 33% positive with 10^{-2} addition. When the sample volume was increased to 100ml, the positive results

were between 50% for Cholera O1 SMART II and 75% for Cholera O139 SMART II with 10^{-2} addition and with overnight incubation. Samples yielding negative Cholera SMART II results were also negative by both PCR (*ompW* and O1/O139) and DFA (O1 or O139), indicating that originally the samples were not inoculated with enough *V. cholerae.* Increasing the incubation time to two or three days did not change the results. The optimal incubation time was 24 hours. Cross-reactivity was not observed between the tests.

SMART II for testing *V. cholerae* O1 and O139 in unfiltered natural water collected from Port Covington (Baltimore, MD) and Lake Artemisia (College Park, MD)

Unfiltered water and filtered water gave similar results. Samples inoculated with *V. cholerae* O1 or O139 (equivalent to 1cfu/100 ml) showed 65% of the samples were positive using *V. cholerae* O1 or O139 SMART II tests, after 24 hours incubation at 35ºC. Samples that gave negative results for Cholera SMART II tests were also by both PCR (*ompW* and O1/O139) and DFA (O1 or O139) indicating that originally the samples were not inoculated with enough *V. cholerae*. In addition, some growth was observed on TCBS plates that gave negative Cholera SMART II results, but the growth was not consistent with typical *V. cholerae* colonies, i.e., likely were contaminated. Hardy dried culture media is not a sterile product, which accounts for probable colonies appearing on TCBS.

SMART II for testing *V. cholerae* O1 and O139 in ballast water

The majority of trials testing ballast water using prepared pre-dried APW (Hardy Diagnostics, Santa Maria, CA) were negative (Table 3). However, some trials provided false positives (as confirmed by PCR and DFA). It was determined this was due to the Hardy Diagnostics pre-dried APW not being sterilized which lead to false positives. In comparison, all trials testing ballast water using prepared APW concentrate, a sterile liquid media that gets diluted to 1% with the addition of 100ml of water, were negative using Cholera O1 or O139 SMART II, PCR (*ompW* and O1/O139) and DFA (O1 or O139).

Discussion

Based on our results, Cholera O1 and O139 SMART II were judged effective for detecting *V. cholerae* 1cfu/100ml. Although, not all samples were positive by the Cholera SMART II test, samples with negative results were also negative by both DFA and PCR. Similarly, when there was no growth on TCBS of colonies typical of *V. cholerae,* ie: no *V. cholerae* cells were present and high enough concentrations of *V. cholerae* were not inoculated in the beginning.

One serious issue is that cross reactivity was observed when pre-dried APW was employed for *V. cholerae* detection. This issue was successfully resolved by switching to Hardy APW concentrate, a sterile liquid media that is diluted to 1% with the addition of 100ml of unfiltered water.

Since a regulation limit has been set for culturable *V. cholerae*, but not for the total number of *V. cholerae,* the SMART II can be used for ballast water testing. Suggested future directions include investigating the impact of salinity on SMART

II sensitivity. Methods currently used to detect specific ballast water indicators, e.g., *E. coli* and Enterococci, require changes in SOP, depending on type of water tested. For example, the IDEXX *E. coli* detection method, namely the IDEXX Colilert kit, was developed for freshwater testing. Brackish water and seawater require a dilution step to reduce interference of environmental factors, such as salinity, and particulates, etc., and the Coliliert-18 test is preferable for *E. coli* detection in marine waters.

Current protocols for *V. cholerae* detection in ballast water discharge waters are time consuming and laborious. Both DFA-DVC and RNA/DNA colony blot hybridization are routinely used for detection of *V. cholerae* O1, but these methods require overnight incubation, as well as hours of microscope observation (DFA-DVC) or extensive effort in developing blots (colony blot hybridization). Although conventional PCR can be effective and a relatively rapid method to detect *V. cholerae*, PCR does not provide quantitative data and cannot be used to determine viability and/or culturability. The Cholera SMART II test is simple to use and requires only an incubator for equipment, to achieve a relatively rapid and accurate detection of *V. cholerae.* Thus, it can be concluded to be a useful method for validating ballast water treatment system safety.

Table 1. List of primers used in this study for conventional PCR, including annealing temperatures and sequences.

Primers	Primer sequence (5' – 3')	Amplicon (bp)	T_a (°C)	Reference
ompW-F1	CACCAAGAAGGTGACTTTATTGTG	588	64	Nandi et al., 2000
ompW-R	GAACTTATAACCACCCGCG			
O139F2	AGCCTCTTTATTACGGGTGG	449	55	Hoshino et al., 1998
O139R2	GTCAAACCCGATCGTAAAGG			
O1F2-1	GTTTCACTGAACAGATGGG	192		
O1R2-2	GGTCATCTGTAAGTACAAC			
VCT1	ACAGAGTGAGTACTTTGACC	308		
VCT2	ATACCATCCATATATTTGGGAG			

Table 2. Isolates of *Vibrio cholerae* O1 and O139 included in the study.

V. cholerae Strain	Serotype	Source	Test Used
267	O139	Environmental (zooplankton)	Cholera O139 SMART II
442	O139	Environmental (phytoplankton)	Cholera O139 SMART II
MO10	O139	Clinical	Cholera O139 SMART II
MO45	O139	Clinical	Cholera O139 SMART II
241	O1	Environmental (water)	Cholera O1 SMART II
320	O1	Environmental (phytoplankton)	Cholera O1 SMART II
MJ1236	O1	Clinical	Cholera O1 SMART II
B33	O1	Clinical	Cholera O1 SMART II

Figure 1. Positive *V. cholerae* O1 cells detected by the New Horizon Diagnostics DFA kit.

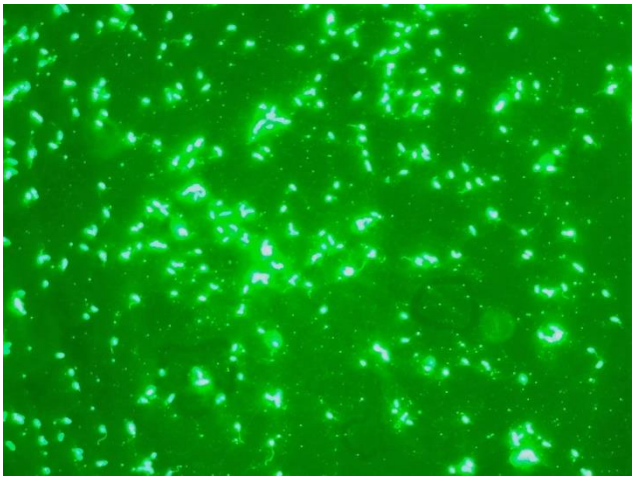
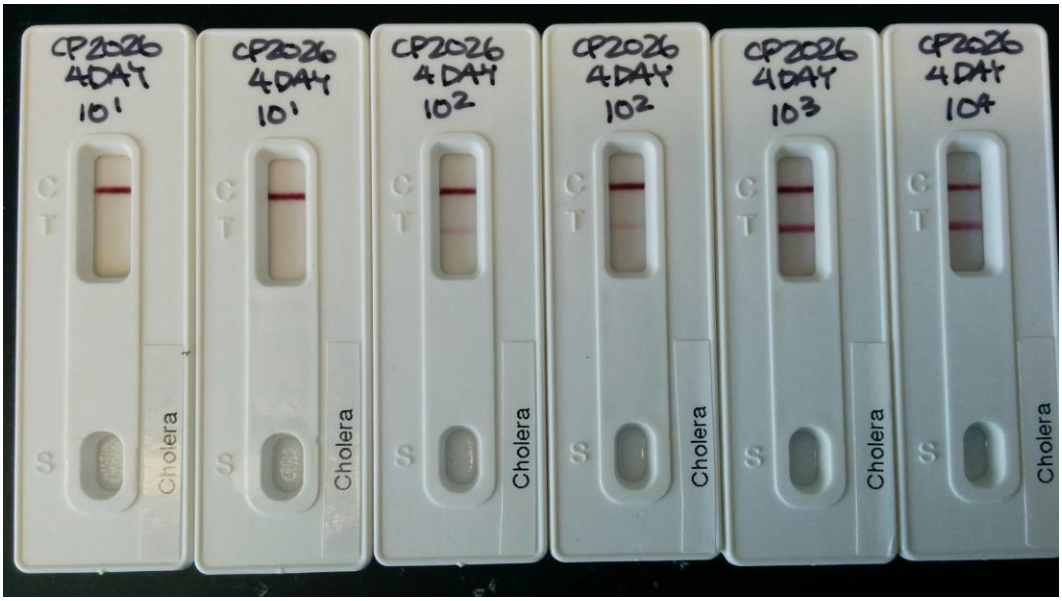


Figure 2. Results of Cholera SMART II after 6 hours incubation at 35°C.

Control line = appearance of control line indicates a valid test. If no control line appears, this indicates an invalid test.
Test line = indicates a positive result if both control line and test line display distinct red lines.
Sample well = area where water samples and chase buffer are added

Figure 3. Results of Cholera SMART II after four-day incubation at room temperature.



Control line = appearance of control line indicates a valid test. If no control line appears, this indicates an invalid test.
Test line = indicates a positive result if both control line and test line display distinct red lines.
Sample well = area where water samples and chase buffer are added

Table 3. Ballast water sample, type of Hardy Diagnostics APW media used and results of SMART II, DFA and PCR. Hardy pre-dried and concentrate are alkaline peptone media (APW) that is 1% APW after addition of 100ml. Hardy APW concentrate is a sterile media.

Sample Water	Hardy Media	Cholera O1 SMART II	Cholera O139 SMART II	DFA (O1/O139)	PCR (ompW)	PCR (O1/O139)
Baltimore – 1	Pre-dried	+	-	- / -	-	- / -
Baltimore – 2	Pre-dried	-	-	- / -	-	- / -
Baltimore – 3	Concentrate	-	-	- / -	-	- / -
Norfolk – 1	Concentrate	-	-	- / -	-	- / -
Norfolk – 2	Concentrate	-	-	- / -	-	- / -
Canada – 1	Concentrate	-	-	- / -	-	- / -
Japan – 1	Concentrate	-	-	- / -	-	- / -
Japan – 2	Concentrate	-	-	- / -	-	- / -
Annapolis – 1	Concentrate	-	-	- / -	-	- / -

Chapter 8: Summary and Conclusions

Results of the research reported here contribute to the overall knowledge of *Vibrio* spp. in estuarine and marine environments, specifically the Chesapeake Bay, as a natural reservoir of pathogenic *Vibrio* species.

The ecology of *V. parahaemolyticus* and *V. vulnificus* was explored during a three-year study that included measurement of environmental parameters influencing the occurrence and incidence of the human pathogenic *Vibrio* species. Notably, *V. parahaemolyticus* carrying *trh* and *tdh* genes was most abundant in samples collected from the Chester River and Tangier Sound during the warm summer months of the year (June to August), but total *V. parahaemolyticus (tlh)* and *V. vulnificus (vvha)* could be isolated throughout the year, being most abundant in the fall (September to November). The incidence of *V. parahaemolyticus* genes associated with pathogenicity was observed to be correlated with water temperature, air temperature, dissolved oxygen, and chlorophyll. Isolation of *V. vulnificus* was found to be correlated with salinity, total dissolved solids, and pH. Statistical analysis revealed that overall results for the Chester River and Tangier Sound did not differ significantly, which was not unexpected since the single major difference in environmental parameters of the two sites was salinity, which typically varied 3-4ppt.

During the course of this three-year study, *Vibrio parahaemolyticus* was isolated at both locations from all sample types and in relatively large numbers.

However, of all *V. parahaemolyticus* isolates characterized for pathogenicity, i.e., presence of either *trh* or *tdh*, less than 2% were concluded to be potentially pathogenic and none of these had been isolated from oyster samples. The majority of the total collection of Chesapeake Bay *V. parahaemolyticus* isolates (83.2%) was from water samples, followed by oyster (9.1%) and sediment samples (7.7%). The majority of presumptively pathogenic *V. parahaemolyticus* isolates collected from Tangier Sound were obtained during colder months of September, December, and January. In contrast, the majority of presumptively pathogenic *V. parahaemolyticus* were isolated from the Chester River during warmer months of the year, May, June, and August. All sixteen presumptively pathogenic strains of *V. parahaemolyticus* displayed substantial diversity in their genetic complement.

A significantly smaller number of culturable *V. vulnificus* isolates was collected during the same time frame and not all *V. vulnificus* that were confirmed to be *toxR*+ were positive for *vvhA,* reported to be a species identification marker. The majority of *V. vulnificus* isolates were from water and the incidence of the virulence markers, *vvhA, vcgC, rtxA* and *pilA* was similar to that reported in previous studies. *V. vulnificus* was less frequently isolated from sediment (1.65%) and oyster (0.33%) samples and those isolates did not contain virulence markers.

Given that *V. parahaemolyticus* and *V. vulnificus* can be readily isolated from the environment, it is important for public health reasons that antibiograms of these bacteria be done to obtain knowledge for effective treatment of infection and illness. Overall, the *V. vulnificus* isolates of this study showed similar antibiotic resistance profiles to those reported in previous studies, with significant resistance

to both ampicillin and penicillin. Only 3.1% (n=25) of the *V. parahaemolyticus* isolates tested for antibiotic resistance were susceptible to all of the eleven antibiotics included in the tests and a large proportion, namely 93.0% (n=754) of the *V. parahaemolyticus* isolates were resistant to both ampicillin and penicillin, indicating antibiotic resistance is common in *V. parahaemolyticus*. Tetracycline, an antibiotic recommended by CDC to treat *Vibrio* related illnesses, was the fourth most frequent resistance observed in the *V. parahaemolyticus* isolates obtained in this study. Clearly, occurrence of these antibiotic resistant forms of *V. vulnificus* and *V. parahaemolyticus* are increasingly becoming a public health concern. Treatment of *V. vulnificus* and *V. parahaemolyticus* infections require antibiogram analysis for accurate, rapid, and effective treatment. Similarly, testing for antibiotic resistance in *V. vulnificus* and *V. parahaemolyticus* should be done to determine if new resistance is occurring, another measure of public health importance and safety.

The diversity of the *Vibrio* spp. isolated from single individual oysters has not been carefully studied in the past. This kind of information could have a profound impact given that many *Vibrio* related diseases are caused by the consumption of raw or undercooked seafood. Both PFGE and MLVA analysis of *V. parahaemolyticus* and *V. vulnificus* isolates showed these isolates from the Chesapeake Bay, Maryland, were highly diverse in each oyster, leading to the conclusion that there is a continuous colonization of oysters by heterogeneous environmental *V. vulnificus* and *V. parahaemolyticus* and not a colonization by a single clone, followed by amplification of that clone within the oyster. Additionally, the genetic heterogeneity of a bacterial species within a single oyster indicates that

an infection caused by this bacterium can be the result of a mixed population of multiple strains of *Vibrio* species or a few highly pathogenic strains of *V. vulnificus* or *V. parahaemolyticus* from a single oyster. Although the total number of cases in the U.S. due to *V. vulnificus* and *V. parahaemolyticus* infection are low, an outbreak resulting from these bacteria could cause major economic impacts in the region.

It is extremely important and useful to be able to accurately detect the presence of *V. cholerae, V. vulnificus* and *V. parahaemolyticus* in order to address actions that need to be taken. Based on the results reported here, the Cholera O1 and O139 SMART II test were found to be effective in detecting as low a number as 1cfu/100ml of *V. cholerae*. Similarly, it should be pointed out when a sample was Cholera SMART II negative, those samples were also negative by DFA and PCR when using APW concentration. Similarly, no growth on TCBS was concluded to indicate *V. cholerae* was not isolated from the samples. Since the regulation limit is for culturable *V. cholerae* and not total *V. cholerae,* SMART II has the potential to serve usefully in ballast water testing.

In summary, *V. parahaemolyticus* and *V. vulnificus* can be readily detected in Chesapeake Bay water, oysters, and sediment samples, with isolation from water being most frequent. Active monitoring for *Vibrio* spp. allows an estimate of actual occurrence of potentially pathogenic *Vibrio* species and can be used to safeguard the public from exposure of *Vibrio* spp. during periods of high incidence in the Chesapeake Bay. Similarly, the highly diverse nature of environmental *V. parahaemolyticus* and *V. vulnificus* and their ability to readily acquire new antibiotic resistances calls for continuous monitoring of the antibiograms of the

isolates. The results of this study have contributed to understanding of the occurrence, incidence, and distribution of potentially pathogenic *Vibrio* spp. in the coastal United States and in the aquatic environment in general, ultimately aiding the public to make educated decisions to prevent illness caused by these bacteria.

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Curriculum Vitae

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EDUCATION

B.S. in Biological Sciences, Specialization in Microbiology (2005 – 2009)
Department of Chemical and Life Sciences, University of Maryland

Ph.D in Environmental Molecular Biology and Biotechnology (MSEH)
(2012 – present)
Marine Estuarine Environmental Sciences (MEES)
College of Computer, Mathematical and Natural Sciences, University of Maryland

PROFESSIONAL EXPERIENCE

Research Graduate Assistant
Maryland Pathogen Research Institute
August 2012 – present
40 Hours/Week

Faculty Research Assistant
Maryland Pathogen Research Institute
June 2009 – August 2012
40 Hours/Week

- Identified environmental determinants favorable for presence and transmission of pathogenic *Vibrios* throughout the United States in collaboration with Louisiana State University, University of Southern Mississippi, NOAA Fisheries Service Northwest Fisheries Science Center and United States Food and Drug Administration
- Evaluated ballast water management systems (BWMSs) adherence to IMO and USCG ballast water discharge standards, including zooplankton, phytoplankton and heterotrophic bacteria (total bacteria, *Escherichia, Enterococci* and *Vibrio*)
- Analyzed environmental and clinical samples collected from Haiti during the Haitian Cholera Outbreak beginning in 2010 to determine the possibility of an environmental influence
- Established signature sequences for possible bacterial bioterrorism agents, including *Vibrio parahaemolyticus*, *Campylobacter jejuni* and *Clostridium botulinum*, with Department of Homeland Security and Canon U.S. Life Sciences
- Trained high school interns (5) and undergraduate students (4) on various laboratory techniques, including media preparation, streaking/spreading plates, DNA extraction, PCR, gel electrophoresis, IDEXX, membrane filtration, confocal microscopy, DNA/RNA colony blot hybridization, serotyping, real time PCR, pulse field gel electrophoresis (PFGE), MLVA, antibiogram, and general lab safety

Internship, The Johns Hopkins University Applied Physics Laboratory
May 2008 – August 2008
40 Hours/Week

- Improved and utilized MAGIchip microarray assay to detect various pathogenic bacteria, including *Bacillus anthracis*, for ultimate use by police force and antiterrorist organizations

Internship, Smithsonian Institution National Museum of Natural History
October 2006 – January 2007
10 Hours/Week

- Assessed conditions of terrestrial mammalian fossils collections and evaluated efficiency of various conservation techniques to effectively preserve samples from the environment

BOOK CHAPTERS

- Huq, A., B.J. Haley, E. Taviani, **A. Chen**, N.A. Hasan, R.R. Colwell (2012) Detection, Isolation, and Identification of *Vibrio cholerae* from the Environment. Unit 6A.5.1-6A.5.51. *Current Protocols in Microbiology, Supplement 26*. Wiley Online Library.

PUBLISHED PEER-REVIEWED PUBLICATIONS

1. Ceccarelli, D., **A. Chen**, N.A. Hasan, S. Rashed, A. Huq, and R.R. Colwell. 2015. Vibrio cholerae non-O1/non-O139 carrying multiple virulence factors and V. cholerae O1 in the Chesapeake Bay, Maryland. Appl. Environ. Microbiol. 81(6):1909-18. doi:10.1128/AEM.03540-14
1. Kahler, A.M., B.J. Haley, **A. Chen**, B.J. Mull, C.L. Tarr, M. Turnsek, L. Katz, M.S. Humphrys, N. Freeman, J. Boncy, R.R. Colwell, A. Huq, and V.R. Hill. 2015. Environmental Surveillance for Toxigenic Vibrio cholerae in Surface Waters of Haiti. Amer. J. Trop. Med Hyeg. 92(1): 118-125. doi:10.4269/ajtmh.13-0601
2. Urquhart, E. A., B.F. Zaitchika, S.D. Guikemab, B.J. Haley, E. Taviani, **A. Chen**, M.E. Brown, A. Huq, R.R. Colwell. 2015. Use of Environmental Parameters to Model Pathogenic Vibrios in Chesapeake Bay. J Environmental Informatics. 26(1):1-13. doi:10.3808/jei.201500307
3. Haley, B.J., S.Y. Choi, C.J. Grim, T.J. Onifade, H.N. Cinar, E. Taviani, N.A. Hasan, A.H. Abudllah, L. Carter, S.N. Sahu, M.H. Kothary, **A. Chen**, R. Baker, R. Hutchinson, C. Blackmore, T.A. Cebula, A. Huq and R.R. Colwell (2014) Genomic and phenotypic characterization of *Vibrio cholerae* non-O1 isolates from a US Gulf Coast cholera outbreak. PLoS One. 9(4):e86264.
4. Haley, B.J., T. Kokashvili, A. Tskshvediani, N. Janelidze, N. Metashvili, C.J. Grim, G. Constantin de Magny, **A. Chen**, E. Taviani, T. Eliashvili, M. Tediashvili, C.A. Whitehouse, R.R. Colwell and A. Huq (2014) Molecular diversity and predictability of *Vibrio parahaemolyticus* along the Georgian

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5. Johnson, C.N., J.C. Bowers, K.J. Griffitt, V. Molina, R.W. Clostio, S. Pei, E. Laws, R. Paranjpye, M.S. Strom, **A. Chen**, N.A. Hasan, A. Huq, N.F. Noriea III, D.J. Grimes, R.R. Colwell (2012) Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington, United States. *Applied and Environmental Microbiology*. 78(20):7249-57.
6. Hasan, N.A., S.Y. Choi, M. Eppinger, P.W. Clark, **A. Chen**, M. Alam, B.J. Haley, E. Taviani, E. Hine, Q. Su, L.J. Tallon, J.B. Prosper, K. Furth, M.M. Hoq, H. Li, C.M. Fraser-Liggett, A. Cravioto, A. Huq, J. Ravel, T.A. Cebula, R.R. Colwell (2012) Genomic diversity of 2010 Haitian cholera outbreak strains. *Proceedings of the National Academy of Sciences of the United States of America*. 109(29):E2010-2017.
7. Taviani, E., M. Spagnoletti, D. Ceccarelli, B.J. Haley, N.A. Hasan. **A. Chen**, M.M. Colombo, A. Huq, R.R. Colwell (2011) Genomic Analysis of ICEVchBan8: an Atypical Genetic Element in *Vibrio cholerae*. *Federation of European Biochemical Societies (FEBS) Letter*s. 586(11): 1617-1621.
8. Haley, B.J., **A. Chen**, C.J. Grim, P. Clark, C.M. Diaz, E. Taviani,, N.A. Hasan, E. Sancomb, W.M. Elnemr, M.A. Islam, A. Huq, R.R. Colwell, E. Benediktsdóttir (2011) *Vibrio cholerae* in an Historically Cholera-Free Country. *Environmental Microbiology*. 4(4):381-389.

MANUSCRIPTS IN PREPARATION

1. **Chen, A**., R. Khan, S. Cavanaugh, A. Huq, A. Jutla, R.R. Colwell (2017) Detection and Exploratory Analysis of Pathogenic Vibrio vulnificus and Vibrio parahaemolyticus Across Water, Oyster and Sediment in the Chesapeake Bay, Maryland (In review)
2. **Chen, A.**, N.A. Hasan, B.J. Haley, E. Taviani, S.Y. Choi, R. McKay, M. Tarnowski, K. Brohawn, C.N. Johnson, D.J. Grimes, R.R. Colwell and A. Huq (2017) Characterization of Pathogenic *Vibrio parahaemolyticus* from the Chesapeake Bay, Maryland. *Applied and Environmental Microbiology* (In review)
3. **Chen, A**., D. Ceccarelli, N.A. Hasan, S.M. Rashed, A. Huq and R.R. Colwell (2017) Antibiotic Resistance Profiles of *Vibrio vulnificus* and *Vibrio parahaemolyticus* Isolated from the Chesapeake Bay, Maryland (In preparation)
4. **Chen, A.**, N.A. Hasan, B.J. Haley, E. Taviani, R. McKay, D. White, M. Tarnowski, K. Brohawn, S.M. Rashed, M. Alam, A. Huq and R.R. Colwell (2017) Genetic Diversity of *Vibrio vulnificus* Isolated Within Individual Oysters from the Chesapeake Bay, Maryland (In preparation)
5. **Chen, A**., N.A. Hasan, P.W. Clark, D. Ceccarelli, A. Huq and R.R. Colwell (2017) Isolation and Characterization of *Vibrio vulnificus* Isolated from the Chesapeake Bay, Maryland (In preparation)
6. Clark, P., B.J. Haley, N.A. Hasan, **A. Chen**, E. Taviani, E.J. Sancomb, J. Prosper, M.J. Figueras, A Huq, R.R. Colwell (2017) *Aeromonas caviae*

Isolated from Patients Symptomatically Diagnosed with Cholera During the 2010 Haitian Cholera Outbreak (In preparation)

7. Jutla, A., **A. Chen**, R. Colwell, and A. Huq (2017) Linking *V. vulnificus* in Chesapeake Bay with Climatic Processes using Bayesian Logistical Models (In preparation)

PRESENTATIONS

1. **Chen, A.** and A. Huq. A 3-Year Study on the Pathogenic *Vibrios* Associated with Oysters and the Oyster Growing Environment in the Chesapeake Bay. 66th Interstate Seafood Seminar. April 16-18. Virginia Beach, VA

CONFERENCE POSTERS

1. **Chen**, **A.,** S.M. Rashed, M.N. Tamburri, A. Huq R.R. Colwell (2016) Cholera SMART II Test for Rapid Detection of *Vibrio cholerae* in Ballast Water. ASM Microbe 2016, Boston, Massachusetts, USA.
2. **Chen**, **A.,** N.A. Hasan, A. Huq, R.R. Colwell (2016) Genetic Diversity of *Vibrio parahaemolyticus* Within Individual Oysters. Vibrio2016, Brest, France.
3. **Chen**, **A.,** D. Ceccarelli, N.A. Hasan, A. Huq, R.R. Colwell (2015) Antibiotic Susceptibility of *Vibrio parahaemolyticus* Isolated from Chesapeake Bay, Maryland. 115th General Meeting Amer Soc for Microbiol, New Orleans, Louisiana, USA.
4. **Chen**, **A.,** N.A. Hasan, D. Ceccarelli, S.M. Rashed, A. Huq, R.R. Colwell (2014) Antibiotic Resistance Profiles of *Vibrio vulnificus* Isolated from the Chesapeake Bay, Maryland. 114th General Meeting Amer Soc for Microbiol, Boston, Massachusetts, USA.
5. Ceccarelli, D., **A. Chen**, N.A. Hasan, S.M. Rashed, A. Huq, R.R. Colwell (2014) Aquatic *Vibrio cholera* non-O1/non-O139 carry cholera toxin and virulence associated genes. 114th General Meeting Amer Soc for Microbiol, Boston, Massachusetts, USA.
6. Ceccarelli, D., **A. Chen**, M. Tarnoswski, K. Brohawn., A. Huq, R.R. Colwell (2014) Distribution of Virulence Genes in Environmental Vibrio cholera strains Isolated From Oysters, Sediments and Water in The Chesapeake Bay, Maryland. Vibrio2014, Edinburgh, Scotland.
7. **Chen**, **A.,** N.A. Hasan, B.J. Haley, E. Taviani, R. McKay, D. White, M. Tarnowski, K. Brohawn, S.M. Rashed, M. Alam, A. Huq, R.R. Colwell (2013) Genetic Diversity of *Vibrio vulnificus* Within Individual Oysters. 113th General Meeting Amer Soc for Microbiol, Denver, Colorado, USA.
8. **Chen**, **A.,** N.A. Hasan, B.J. Haley, E. Taviani, S.Y. Choi, S. Hiner, R. McKay, D. White., M. Tarnowski, K. Brohawn, C.N. Johnson, D.J. Grimes, A. Huq, R.R. Colwell (2012) Characterization of Pathogenic *Vibrio parahaemolyticus* from the Chesapeake Bay, Maryland. Public Health Research at Maryland 2013, College Park, Maryland, USA.
9. Haley, B.J., N.A. Hasan, S.Y. Choi, E. Taviani, **A. Chen**, A. Huq, R.R. Colwell (2012) Genomic analyses demonstrates cholera outbreaks are caused by heterogeneous V. cholerae strains. 14th International Symposium

on Microbial Ecology, Copenhagen, Denmark.

10. **Chen, A.,** N.A. Hasan, B.J. Haley, E. Taviani, S.Y. Choi, M. Tarnowski, R. McKay, K. Brohawn, C.N. Johnson, D.J. Grimes, A. Huq, R.R. Colwell (2012) Characterization of Pathogenic *Vibrio parahaemolyticus* from the Chesapeake Bay, Maryland. 112th General Meeting Amer Soc for Microbiol, San Francisco, California, USA.
11. Hasan, N.A., D.W. Fadrosh, A.S. Abdullah, A.G. Longmire, S.Y. Choi, **A.J. Chen**, A. Huq, T.A. Cebula, R.R. Colwell (2012) Generation of Genetic Diversity in *Vibrio cholerae* during Limited Laboratory Passage 112th General Meeting Amer Soc for Microbiol, San Francisco, California, USA.
12. Kahler, A.M., B.J. Haley, B. J. Mull, J. Narayanan, **A. Chen**, N. C. Purcell, R. R. Colwell, A. Huq, V. R. Hill (2012) Evaluation of Human Pathogens in Surface Waters of Haiti. 112th General Meeting Amer Soc for Microbiol, San Francisco, California, USA.
13. Haley, B.J., E. Taviani, **A. Chen**, A. Phillippy, A. Huq, R.R. Colwell, I. Knight (2011) Validation of Unique Signature Discovery in Select Agents and an Emerging Pathogen. ASM Biodefense and Emerging Diseases Research Meeting, Washington, District of Columbia, USA.
14. **Chen, A.,** N.A. Hasan, B.J. Haley, E. Taviani, P.W. Clark, M. Tarnowski, R. McKay, K. Brohawn, C.N. Johnson, D.J. Grimes, A. Huq, R.R. Colwell (2011) Virulence Factors of *Vibrio parahaemolyticus* in the Chesapeake Bay, Maryland. 111th General Meeting Amer Soc for Microbiol, New Orleans, Louisiana, USA.
15. Clark, P.W., J.B. Prosper, **A. Chen,** B.J. Haley, N.A. Hasan, E. Taviani, E.J. Sancomb, K. Furth, A. Huq, R.R. Colwell (2011) Intestinal Microflora of Cholera Patients in Haiti. 111th General Meeting Amer Soc for Microbiol, New Orleans, Louisiana, USA.
16. Haley, B.J., N.A. Hasan, E. Taviani, **A. Chen**, A. Huq, R.R. Colwell (2011) Endemic *Vibrio cholera* in a Cholera-Free Region in the Absence of Either Cholera or Ship Ballast Discharge. 111th General Meeting Amer Soc for Microbiol, New Orleans, Louisiana, USA.
17. Haley, B.J., E. Taviani, J. Choi, C.J. Grim, **A. Chen**, P. Clark, L. Sancomb, M. Tamburri, R.R. Colwell, and A. Huq (2011) Comparison of Methods for Quantifying Bacterial Indicators in Water from Urban Brackish Environment. 111th General Meeting Amer Soc for Microbiol, New Orleans, Louisiana, USA.
18. Taviani, E., M. Spagnoletti, D. Ceccarelli, B.J. Haley, N.A. Hasan, **A. Chen**, M.M. Colombo, A. Huq, R.R. Colwell (2011) Genomic analysis of an Integrative Conjugative Element (ICE) identified in a clinical *V. cholera* O37: a new ICE or a Genomic Island? 111th General Meeting Amer Soc for Microbiol, New Orleans, Louisiana, USA.
19. **Chen**, **A.,** N.A. Hasan, D. White, S. Harvey, M. Tarnowski, R. McKay, K. Brohawn, C.N. Johnson, D.J. Grimes, A. Huq, R. R. Colwell (2010) Occurrence of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Chesapeake Bay waters, sediments and oysters. Vibrios in the Environment,

Biloxi, Mississippi, USA.

20. Haley, B.J., **A. Chen**, C.M. Diaz, P. Clark, A. Huq, R.R. Colwell, E. Benediktsdóttir. (2010) Endemic *Vibrio cholerae* Populations in an Historically Cholera-Free Country. Vibrios in the Environment, Biloxi, Mississippi, USA.
21. Taviani, E., **A. Chen**, B. Haley, N.A. Hasan, A. Huq and R.R. Colwell (2010) Geographical distribution and molecular characterization of Integrative Conjugative Elements in environmental *Vibrio cholerae*. Vibrios in the Environment, Biloxi, Mississippi, USA.
22. Clark, P., B.J. Haley, **A. Chen**, N.A. Hasan, E. Taviani, A. Huq, R.R. Colwell (2010) Occurrence of Human Pathogenic Vibrios Associated with Polychaetes in Oyster Beds in the Chesapeake Bay. Bioscience Day, University of Maryland, College Park, USA.
23. Hasan, N.A., **A. Chen**, S. Harvey, D. White, R. McKay, M. Tarnowski, K. Brohawn, CN. Johnson, DJ. Grimes, A. Huq, R.R. Colwell (2010) Environmental Investigations of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *V. cholerae* in Oyster, Water and Sediment in the Chesapeake Bay. 110th General Meeting Amer Soc for Microbiol, San Diego, CA, USA.

SKILLS

- Technical: SAS, PERL (basic), Python (basic), MS Office, Windows and Linux experience
- Laboratory: general microbiology skills, maintaining sterile environment, DNA extraction, PCR, real time PCR, PFGE, MLVA, gel electrophoresis, microscopy (light, confocal – Zeiss), colony blot hybridization, membrane filtration, IDEXX, serotyping, antibiogram

PROFESSIONAL DEVELOPMENT

- Quantitative Microbial Risk Assessment Interdisciplinary Instructional Institute, QMRA III (August 2016) Michigan State University
- Bioinformatics Crash Course (July 2014) University of Maryland, Bioinformatics Core
- Bioinformatics: Life Sciences on Your Computer (June 2014, with distinction) Coursera, Johns Hopkins University

GRANTS

- Sigma Xi William Procter Prize Grant-in-Aid of Research, 2013

ACADEMIC AWARDS

- University of Maryland Scholars Program, 2005 – 2007
- Earth, Life, and Time Scholars Citation, 2007

PROFESSIONAL ASSOCIATIONS

- American Society for Microbiology (ASM), 2011 – present
- American Society for Microbiology Maryland Branch Ltd., 2013 – present
- International Society for Infectious Disease (ISID), 2012 – present