ABSTRACT

Title of Document: ECOLOGICAL AND EXPOSURE ASSESSMENT OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* IN AREAS OF IMPORTANCE FOR HUMAN USE IN CHESAPEAKE BAY

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Current microbial surveillance of water quality in marine and estuarine environments focuses on fecal indicator concentrations to determine suitable conditions for swimming or fishing, including commercial harvest of seafood. However, there are many pathogens in our waters, such as *Vibrio vulnificus* and *V. parahaemolyticus*, and it remains unclear how well fecal indicator surveillance protects the public from infection. This dissertation studied *V. vulnificus* and *V. parahaemolyticus* at locations in Chesapeake Bay where human contact is likely, in order to quantify dermal transmission to humans, describe the impact of storms on pathogen concentrations in oysters, and quantify antimicrobial resistance. Swim studies at four public beaches in Chesapeake Bay in 2009 and 2011 were the first of

their kind to quantify *Vibrio* exposure by recreating swimmers and to qualify exposure in terms of dermal dose. Estimated exposures correlated with surface water *Vibrio* concentrations and suggested that the public could be exposed to *V. vulnificus* and *V. parahaemolyticus* at rates that may cause illness. To better protect human health, estimates of non-consumption dose-response would be helpful in completing a quantitative microbial risk assessment to calculate relative risk of swimming in waters known to harbor *Vibrio* bacteria.

Oysters, water, and sediment were sampled at an aquaculture facility before and after Hurricane Irene impacted the Chesapeake Bay in 2011. Results indicated no difference in *Vibrio* uptake between oysters positioned on floats and on bottom sediments, but showed a difference in *Vibrio* species uptake, with *V. parahaemolyticus* increasing 1 day post-Irene, unlike *V. vulnificus*. This study suggests that storm events may increase *V. parahaemolyticus* in oyster tissue, and that virulent sub-types of both *Vibrio* species may increase in percent abundance within oysters following a storm event.

Antimicrobial susceptibility testing showed that a large percentage of isolates from surface waters in the Chesapeake Bay displayed intermediate resistance to chloramphenicol. Most antimicrobial agents recommended for treatment of *Vibrio* illness by CDC were effective at controlling growth of *V. vulnificus* and *V. parahaemolyticus*. Results suggest treatment of pediatric illness with trimethoprim-sulfamethoxazole and the aminoglycoside, gentamicin, which was the only aminoglycoside 100% effective in controlling *Vibrio* growth in this study.

ECOLOGICAL AND EXPOSURE ASSESSMENT OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* IN AREAS OF IMPORTANCE FOR HUMAN USE IN CHESAPEAKE BAY

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

I dedicate this work to my children, Ruby Katherine and Oliver James. May you always follow your inner voice and find passion in the work that you pursue. Passion will give you the endurance to push forward when other resources are in short-supply.

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to be in respect to the more traditional dissertation projects that others have completed. Thank you to Dr. Raleigh Hood, who branched out from the field of Oceanography, to support my interest in this meld of Oceanography with Public Health. Thank you to Dr. Erin Lipp for offering your advice and time from several hundred miles away. Thank you to Dr. Amy Sapkota for your enthusiastic support and mentorship in applying Public Health principles to Marine Science problems. I greatly appreciate the time and guidance that Dr. John Jacobs has provided, and for answering my many, many perplexed phone calls. To both Amy and John, thank you for the generous use of your lab space, time and personnel. Without your guidance, I would not have been able to move these research questions forward to completed projects.

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Thank you to my children, who have been patient with my schedule and levels of stress, both in and out of utero. You have attended meetings, traveled internationally, and tempered physically demanding sampling trips and lab work, sometimes only days before your impending births. You have shared your needs for my time and attention with my work, and I know that was not easy for neither you, nor I. I promise to be patient and support your dreams, because I know the support it takes to achieve your goals. You are the most important creations of my time in this Doctoral program, and you have no doubt taught me more than anything I could have learned in a classroom or from a stack of journal articles.

Thank you lastly, and most importantly, to my ever-supportive husband, Bryan. You were by my side when I read "From Monsoons to Microbes" that summer day on the beach, celebrated my acceptance letter into a public health program two days before our wedding, listened to countless hours of research plans that no doubt mystified (and bored) you, and learned just enough microbiology to help me get home at night. You kayaked for hours during sample collections, carried lab supplies that were too heavy for my pregnant body and juggled the entertainment of two children so I could write my dissertation. You are truly my life partner and I am forever indebt to you for making my dreams, yours.

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CHAPTER 1: INTRODUCTION

Water Quality and Vibrio

Current microbial surveillance of water quality in marine and estuarine environments focuses primarily on fecal indicator concentrations to determine suitable conditions for swimming or fishing, including the commercial harvest of seafood products. Fecal indicator species have been chosen by regulatory agencies, such as the Environmental Protection Agency, as surrogates for harder-to-detect pathogenic bacteria, especially those arising from fecal pollution. However, there are many pathogens in our waters that occur in the absence of fecal pollution, such as *Vibrio vulnificus* and *V. parahaemolyticus*, and it remains unclear how well fecal indicator surveillance protects the public from infection (Harwood et al., 2005).

Reports of illness and death caused by virulent *Vibrio* species are increasing, but little is done to protect the public from deleterious health effects associated with these organisms, especially in recreational settings. Moreover, public health records of infection are limited to a small database of reported adverse health outcomes; possibly because most infections of healthy, immunocompetent individuals result in diarrheal disease, which tends to be self-limiting (i.e., not reported to a healthcare provider, and is resolved on its own). For this reason, monitoring reported health outcomes is an ineffective strategy for understanding risk of infection by these pathogens. The foci of this dissertation research was to study *V. vulnificus* and *V. parahaemolyticus* in select locations of the Chesapeake Bay region where human contact is likely, in order to quantify dermal transmission to humans based upon exposure time and surface water concentrations, describe the impact of adverse weather on the concentration of these pathogens in surface water, sediment, and oysters, and, determine the prevalence of

bacterial antimicrobial resistance.

Vibrio in the Environment

Vibrio parahaemolyticus and V. vulnificus are Gram-negative, heterotrophic members of natural marine bacterioplankton communities, and, like the other members of these communities, they are subject to natural environmental factors that control their abundance and distribution. Temperature and salinity are key determinants in the occurrence and growth of both *Vibrio* species although *V. parahaemolyticus* tolerates a broader range of salinities relative to *V. vulnificus* (Johnson et al. 2012). A normal salinity range for the two organisms has been reported between 5 and 25 ppt (Motes et al. 1998). For example, a recent drought in North Carolina from 2007-2009 caused an increase in Neuse River Estuary salinity and concurrent loss of detectable *V. vulnificus* (Froelich et al. 2012). Additionally, high-salinity exposure is considered a viable means to depurate *V. vulnificus* from retail oysters (Audemard et al. 2011). *Vibrio vulnificus* and *V. parahaemolyticus* have been isolated in water temperature ranges from *ca* 7-36°C (Motes et al. 1998, Parveen et al. 2008, Johnson et al. 2012), although optimal temperature for growth is typically above 17°C (Vezzulli et al. 2013).

Based on early studies by Kaneko and Colwell (1973), *Vibrio* spp. have long been thought to survive in sediments during the winter and move into the water column during the spring in response to warmer water temperatures and nutrient availability. *Vibrio* bacteria are believed to attach to zooplankton species (e.g., copepods), rich in chitin, which are used as a food source by the bacteria as they travel into surface waters (Kaneko & Colwell 1973). Once in the photic zone of surface waters, these heterotrophic bacteria are sustained by dissolved organic matter (DOM) from phytoplankton, supplied by the

processes of excretion, exudation and cellular death (Smith et al. 1995). *Vibrio* metabolism is broad, with the ability to utilize not only a wide variety of carbon sources, but nitrogen sources (Criminger et al. 2007) and even polycyclic aromatic hydrocarbons (Hedlund & Staley 2001). As a means to survive in nutrient limited conditions, such as often occurs in the water column, *Vibrio* have developed fitness-associated mechanisms of nutrient acquisition (Asplund et al. 2011). *Vibrio* may contribute to the aquatic cycling of organic matter, adding their biomass to the cycle after they are grazed upon by flagellates (Beardsley et al. 2003).

Vibrio are one of many bacterial genera capable of using a protective strategy, referred to as a "viable but nonculturable" (VBNC) state, against harsh conditions (Colwell 2000). The VBNC state allows cells to become metabolically dormant, limiting nutrition requirements, and then emerge from dormancy when environmental conditions improve and are favorable for bacterial growth (Nowakowska & Oliver 2012). Environmental triggers for the VBNC state among *Vibrio* have been recognized to include temperature, salinity, oxygen concentrations and nutrient deprivation (Colwell 2000, Oliver 2005). This strategy has become an accepted reason why *Vibrio* have historically not been detected during winter sampling efforts.

Virulence Factors of Vibrio

Vibrio vulnificus and *V. parahaemolyticus* are opportunistic pathogens of humans. The virulence mechanisms that they utilize are thought to have been developed to acquire nutrients in the environment (Johnson 2013), but are also deleterious to human health. For example, toxic hemolysins allow *Vibrio* to lyse host erythrocytes or access cell-bound nutrients such as iron (Johnson 2013). In *V. parahaemolyticus*, the genes

thermostable direct hemolysin (*tdh*), thermostable related hemolysin (*trh*) and thermolabile hemolysin (*tlh*) are responsible for three known virulence factors (Johnson 2013). While *tdh* and *trh* are commonly associated with clinical isolates, *tlh*, found in all strains of *V. parahaemolyticus*, has been found to upregulate in a mimicked human intestinal environment (Gotoh et al. 2010). The Type 3 Secretion Systems (T3SS1 and T3SS2) are also virulence factors found in *V. parahaemolyticus* that inhibit host's immune response by way of effectors that cause enterotoxicity and cytotoxicity (Broberg et al. 2011, Johnson 2013). Varying degrees of virulence are associated with *V. parahaemolyticus* depending on the combination of the virulence factors they carry in their genomes (Broberg et al. 2011). *Vibrio parahaemolyticus* also possess two flagellae that allow for swimming and swarming, siderophores to chelate iron from the host, and the ability to form an antiphagocytic capsule (Broberg et al. 2011).

Vibrio vulnificus is classified into three biotypes (Strom & Paranjpye 2000). Biotype 1 is associated with human infections, Biotype 2 is related to infections of eels and Biotype 3 was recently discovered in fish handlers in Israel (Bisharat et al. 1999). *Vibrio vulnificus* posseses many extracellular proteins, two of which are known hemolysin/cytolysin molecules (*HlyIII*, *VvhA)* similar to those in *V. parahaemolyticus* (Johnson 2013).

Vibrio vulnificus has a single flagellum and is able to encapsulate. Most important to the virulence of *V. vulnificus* is the lipopolysaccharide (LPS) in its outer cell membrane, which has been linked with human death (Bowdre et al. 1981, Oliver 2012). Estrogen has been shown to be a protective mechanism against the effects of LPS and it

has been deduced that this is the main reason why the epidemiology of *V. vulnificus* infections tends to be dominated by male patients (Merkel et al. 2001, Oliver 2012).

Vibrio epidemiology

Routes of infection associated with *V. parahaemolyticus and V. vulnificus* include consumption of seafood and contact with seawater and, in the case of *V. vulnificus*, animal to human transmission (CDC 1999a, 1999b, 2000). In the United States, illness associated with *Vibrio* exposure occurs most frequently during the warmer months of the year (United States Food and Drug Administration 1992a). While non-consumption infectious dose is largely unknown for both *V. vulnificus* and *V. parahaemolyticus* (FDA 2012), modeled risk assessment results from the United States Food and Drug Administration (FDA) suggest the 50% probability of illness' (ID_{50}) infective consumption dose for *V. parahaemolyticus* to be approximately 10^6 to 10^8 CFU g^{-1} (CFSAN (Center for Food Safety and Applied Nutrition 2005). Risk of illness modeled by Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) in a 2005 risk assessment approximated an infective consumption dose of 10^3 to 10^7 CFU g^{-1} oyster tissue (World Health Organization. & Food and Agriculture Organization of the United Nations. 2005).

Vibrio vulnificus is responsible for 95% of seafood-related deaths in the United States, with the highest rate of mortality among cases of food-borne illness, followed by wound infection, septicemia, and other presentations of infection (Lipp & Rose 1997, Mead et al. 1999), and a 50% mortality rate for individuals at increased risk (e.g., immunocompromised, liver disease) (Oliver 1995). There are approximately 93 serious (requiring hospitalization) cases of *V. vulnificus* reported in the U.S. annually (Scallan et

al. 2011). A study of nonfoodborne *Vibrio* infections (NFVI) from 1997-2006, before *Vibriosis* became a nationally notifiable disease, reported *V. vulnificus* was responsible for 35% of all NFVI illness and 78% of NFVI deaths in the United States (Dechet et al. 2008).

By contrast, *V. parahaemolyticus* is not as lethal as *V. vulnificus,* rarely progressing to septicemia (5%), but its clinical manifestation of wound infection (34%) is comparable to *V. vulnificus* (45%) in terms of proportion to other illness classifications (Dechet et al. 2008). While *V. vulnificus* is rarely reported as gastroenteritis (5%), a large percentage of *V. parahaemolyticus* infections are gastroenteritis (59%) (Dechet et al. 2008). *Vibrio parahaemolyticus* has been implicated in a number of recent outbreaks in the United States (CDC 1998, 1999b, United States Food and Drug Administration 1992), and was estimated to be responsible for 19% of all NFVI (Dechet et al. 2008).

In the Chesapeake Bay, the Centers for Disease Control and Prevention (CDC) reported 59 illnesses associated with *Vibrio* spp. infections in 2009 (Maryland and Virginia) (CDC 2011). Preliminary data from CDC shows a 115% increase in reported *Vibrio* infections in 2010 in relation to 1996-1998 FoodNet data (CDC 2012c).

Advances in Monitoring

Recent advances in efforts to monitor and predict *Vibrio* in the estuarine and marine environment will be useful tools in protecting human health. Modeling efforts have been made to predict the likelihood of occurrence of *Vibrio* in the Chesapeake Bay (Brown et al. 2012). Remote sensing technologies have been developed to link epidemiological and environmental data to predict the presence of *Vibrio* and associated illness (Ford et al. 2009, Baker-Austin et al. 2010, Baker-Austin et al. 2012). The ability

to pair these advances in predicting the occurrence and abundance of *V. vulnificus* and *V. parahaemolyticus* with risk assessment models (Dickinson et al. 2013) will move the science of monitoring forward to management implementation.

Vibrio in a Warming Chesapeake Bay

It is likely that *Vibrio* populations will spread geographically and increase in response to global climate change (Lipp et al. 2002, Vezzulli et al. 2013). Average global temperatures have increased by almost 1°C since the late nineteenth century and by approximately 0.2°C per decade for the last 25 years (IPCC 2007). Oceans are predicted to acidify, with an expected decrease in pH of 0.4 by the end of the century (Orr et al. 2005). It is not known how increased environmental temperatures will affect important *Vibrio* reservoirs or hosts, but a recent study suggests that plankton will be resilient to the predicted decrease in surface water pH (Nielsen et al. 2012).

Salinity is the primary determinant for the spatial distribution of *Vibrio* spp. (particularly *V. vulnificus*, Jacobs et al. 2010) in the Chesapeake (i.e., positioning up and down the Bay), while temperature increases influence when *Vibrio* spp. are present in significant quantities during the year. If salinity decreases due to projected increases in precipitation events, there will be a broader range of areas where *V. vulnificus* will be able to grow in the Chesapeake. Paired with a temperature increase, it is reasonable to assume that *V. vulnificus* and *V. parahaemolyticus* will have longer active seasons in the Chesapeake Bay as a result of climate change.

Additionally, storm events have been thought to be an important mechanism for distributing benthic *Vibrio* populations into the water column via resuspension of sediments caused by high winds and flushing due to large volumes of precipitation

(Randa et al. 2004, Fries et al. 2008, Wetz et al. 2008, Johnson et al. 2010). The frequency and intensity of storm events are predicted to escalate due to global climate change (Goldenberg et al. 2001), with increases in peak wind intensities and near-storm precipitation (Meehl et al. 2007) likely impacting mid-Atlantic areas such as the Chesapeake Bay. Therefore, relatively moderate wind speed and associated wave action in Chesapeake Bay storm events could increase the overall *Vibrio* density in surface waters.

Based on modeled projections of increased precipitation, temperature and sea-level rise, stratification in Chesapeake Bay is projected to increase (Boesch et al. 2007), allowing for more pronounced algal and bacterial blooms in the eutrophic, warm upper waters (Shiah & Ducklow 1994). This feature of global climate change is likely to increase *Vibrio* in surface waters of the Chesapeake. Benthic *Vibrio* concentrations will also likely increase, in response the increased organic material available for decomposition resulting from algal blooms.

It is conceivable that, as the climate warms, the human population will seek refuge in waterfront activities, including swimming and boating, to relieve themselves from the heat, possibly over a longer recreational season. Such a situation presents itself as a dangerous intersection of higher concentrations of human pathogens with a higher proportion of the public seeking out exposure to the water. Paired with the possibility that virulence may increase in response to global warming, clinical cases may be expected to increase (Oh et al. 2009, Mahoney et al. 2010).

Research Significance

This research describes the ecological and environmental conditions favoring the transmission of these pathogens to humans. This development of disease transmission theory (i.e., exposure assessment of humans recreating in a *Vibrio* dense environment, treatability of *Vibrio* infection, impact of storm events on the concentration of *Vibrio* in surface waters, sediment and oysters) will benefit public health and environmental management policy-making decisions. Moreover, given the magnitude of importance that climate change commands in making connections between ecological data and human exposure data related to thermophilic pathogens such as *Vibrio* spp., conducting a baseline exposure assessment of human interaction with waterborne pathogens is an important consideration in protecting public health in the future.

Specific Aim 1: Investigate the magnitude of exposure to V. vulnificus and V. parahaemolyticus in select locations of the Chesapeake Bay region where human contact is likely, in order to quantify dermal transmission to humans based on exposure time and surface water concentrations of these pathogens.

In order to protect human health in estuarine and marine communities, it is critical to quantify the numbers of microorganisms to which individuals are exposed during routine activities in the marine environment, such as swimming. Swimming exposure assessments were conducted to define and quantify important routes of exposure, to advance the understanding of how people become ill after environmental exposure, and to be included in risk assessments to protect the general public and important sub-populations (i.e., fishermen, boaters, swimmers). Benefits from this study include

understanding the relationship between exposure time and dermal acquisition of pathogens in the estuarine-marine environment in relation to the surface water concentrations of those organisms at the times of exposure. Such estimates are needed for constructing quantitative microbial risk assessments of the risk of infection to such pathogens following such exposure.

Specific Aim 2: Provide estimates of storm-related V. vulnificus and V. parahaemolyticus density changes in oyster tissues, sediment and surface water at an aquaculture facility in the Chesapeake Bay.

According to the Environmental Protection Agency, the Chesapeake Bay is home to 25% of the total shellfish harvesting waters in the United States (EPA 2011a). Recently, the Chesapeake Bay has become the site of interest for oyster aquaculture production to supplement the dwindling wild harvest of bottom dwelling oysters through on-bottom (submerged land) and off-bottom (water column) leases. Summer is generally considered to be a viable oyster-harvest season in Maryland, but summer is also when *Vibrio* populations reach their peak in the Bay (Wright et al. 1996, Parveen et al. 2008, Jacobs et al. 2010, Johnson et al. 2012). Oysters may concentrate *Vibrio* up to 100-fold higher than surrounding waters and it is expected that up to 100% of oysters may be contaminated with *V. vulnificus* and *V. parahaemolyticus* during summer months (Morris 2003). Thus, the harvest of oysters during seasons when surface water *Vibrio* populations are at high densities has the possibility to become a pressing issue for seafood safety.

Specific Aim 3: Evaluate the degree to which V. vulnificus and V. parahaemolyticus isolates from the Chesapeake Bay are susceptible to common antimicrobial treatments.

In the natural aquatic environment, environmental bacteria provide an unlimited source of resistance genes and determinants, and these genes and determinants can be passed to pathogenic bacteria sharing the aquatic environment, producing newly resistant pathogens (Baquero et al. 2008). In one study it was shown that more than 90% of seawater-derived bacterial strains were resistant to at least one antibiotic and 20% were resistant to at least five antibiotics (Martinez 2003). Human infection intensity and associated morbidity-mortality rates for *V. vulnificus* and *V. parahaemolyticus* would be greatly increased by resistance to antimicrobial drugs.

The spread of antimicrobial resistance in the microbial communities of our waterways is of concern for treatment of waterborne bacterial infections, especially as these infections can progress quickly and are likely lethal if not treated within a short period of time. A compelling study by Baker-Austin et al., (2009) indicated high levels of antimicrobial resistance in *V. vulnificus* and *V. parahaemolyticus* isolated from environmentally degraded sites in South Carolina. It was hypothesized that high levels of resistance may stem from antimicrobial compounds produced by naturally occurring environmental bacteria or horizontal transfer of resistance factors from anthropogenically introduced taxa (Baker-Austin et al., 2009). Widening the geographic scope of such studies will offer greater insight to the mechanisms of antibiotic resistance in these taxa.

CHAPTER 2: RECREATIONAL SWIMMERS' EXPOSURE TO *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* IN THE CHESAPEAKE BAY, MARYLAND, USA

(Formatted for submission to *Environmental Health Perspectives*)

Abstract

Background: *Vibrio vulnificus* and *Vibrio parahaemolyticus* are ubiquitous in the marine-estuarine environment, but the magnitude of human non-consumption exposure to these waterborne pathogens is largely unknown.

Objective: To evaluate the magnitude of exposure to *V. vulnificus* and *V. parahaemolyticus* among swimmers recreating in *Vibrio*-populated waters.

Methods: Swim studies were conducted at four individual swimming locations in the Chesapeake Bay in 2009 and 2011. Volunteers swam for set time periods (2-20 minutes (2009), 8 minutes (2011)), and surface water and handwash samples were collected. *Vibrio* concentrations were determined using quantitative PCR for each timed swim exposure. Descriptive statistics and regression analysis (linear and logistic) were used to evaluate factors associated with exposure.

Results: Mean surface water *V. vulnificus* (*Vv*) and *V. parahaemolyticus* (*Vp*) concentrations were 1128 (95% confidence interval (CI) 665.6, 1591.4) CFU mL^{-1} and 18 (95% CI: 9.8, 26.1) CFU mL^{-1}, respectively, across all sampling locations. *Vibrios* in handwash samples (*Vv* mean 180 (95% CI: 136.6, 222.5) CFU cm^{-2}; *Vp* mean 3 (95% CI:2.4, 3.7) CFU cm^{-2}) were significantly associated with *Vibrio* concentrations in surface water (*P*=<0.01 (*Vv*), *P*=<0.01 (*Vp*)), but not with salinity or temperature (adjusted R^2=0.067 (*Vv*), adjusted R^2=0.026 (*Vp*)). Handwashing reduced *Vibrios* on subjects' hands by 93.9% (*Vp*) and 89.4% (*Vv*).

Conclusions: During months when surface waters are host to an elevated abundance of *Vibrio* cells, a person recreating or working in those waters should expect a significant dermal exposure, highlighting the potential for illness associated with such an exposure.

Introduction

Vibrio parahaemolyticus and V. vulnificus are normal functioning members of natural bacterioplankton communities in estuarine and marine waters routinely used for recreation. Current microbial surveillance of water quality in these environments focuses primarily on fecal-indicator concentrations to determine suitable conditions for swimming (EPA 2011b). However, it remains unclear how well fecal-indicator surveillance protects the public from *Vibrio* infection (Harwood et al. 2005). There are approximately 93 serious (requiring hospitalization) cases of *V. vulnificus* reported in the U.S. annually (Scallan et al. 2011).

A study of non-foodborne *Vibrio* infections (NFVI) from 1997-2006, before Vibriosis became a nationally notifiable disease, reported *V. vulnificus* was responsible for 35% of all NFVI illness and 78% of NFVI deaths in the United States (Dechet et al. 2008). For individuals at increased risk (e.g., immunocompromised, liver disease), there is a 50% mortality rate (Oliver 1995). By contrast, *V. parahaemolyticus* is not as lethal as *V. vulnificus,* rarely progressing to septicemia (5%), but its clinical manifestation of wound infection (34%) is comparable to *V. vulnificus* (45%) (Dechet et al. 2008). While *V. vulnificus* is rarely reported as gastroenteritis (5%), a large percentage of *V. parahaemolyticus* infections are gastroenteritis (59%) (Dechet et al. 2008) *Vibrio parahaemolyticus* has been implicated in a number of recent outbreaks in the United States (CDC 1998, 1999b, United States Food and Drug Administration 1992), including

responsibility for 19% of all NFVI (Dechet et al. 2008). In the Chesapeake Bay, the Centers for Disease Control and Prevention (CDC) reported 59 illnesses associated with *Vibrio* spp. infections in 2009 (Maryland and Virginia) (CDC 2011). Preliminary data from CDC shows a 115% increase in *Vibrio* infections in 2010 in relation to 1996-1998 FoodNet data (CDC 2012c). Approximately 50% of all Maryland *V. parahaemolyticus* and *V. vulnificus* infections originate from non-foodborne exposures (Maryland Department of Health and Mental Hygiene (DHMH), personal communication).

Routes of infection associated with *V. parahaemolyticus* and *V. vulnificus* include consumption of seafood and contact with seawater and, in the case of *V. vulnificus*, animal (fish) to human transmission (CDC 1999a, 1999b, 2000). In the United States, illness associated with *Vibrio* exposure occurs most frequently during the warmer months of the year (United States Food and Drug Administration 1992a).

While non-consumption infectious dose is largely unknown for both *V. vulnificus* and *V. parahaemolyticus* (FDA 2012), modeled risk assessment results from the United States Food and Drug Administration suggest that the infective consumption dose producing 50% probability of illness (ID_{50}) for *V. parahaemolyticus* is approximately 10^6 to 10^8 CFU g^{-1} (CFSAN (Center for Food Safety and Applied Nutrition 2005)). Risk of illness modeled by the Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) in a 2005 risk assessment approximated a infective consumption dose of 10^3 to 10^7 CFU g^{-1} oyster tissue (World Health Organization & Food and Agriculture Organization of the United Nations 2005). It is conceivable that non-consumption dose, encountered from direct contact between an open wound and *Vibrio*-

populated media (e.g,, water, surfaces, seafood products), may require a fraction of the consumption-based infectious dose.

While reports of illness and death caused by virulent *Vibrio* species, including *V. vulnificus* and *V. parahaemolyticus,* are increasing (Scallan et al. 2011b), very little is known about the magnitude of human exposure to these environmental pathogens. In order to protect human health in estuarine and marine communities it is critical to quantify the numbers of organisms to which individuals are exposed during routine activities in the marine environment, such as swimming. This study investigates the magnitude of exposure to *V. vulnificus* and *V. parahaemolyticus* in select locations of the Chesapeake Bay region where human contact is likely, in order to quantify dermal transmission to humans based upon exposure time and surface water concentrations of these pathogens. It also assesses the efficacy of washing in clean water to remove *Vibrio* from the surface of skin following dermal exposure.

This study also describes the surface water conditions favoring the transmission of these pathogens to humans, including the study conditions under which virulent species were encountered. The estimates of exposure produced by this assessment will help quantify possible disease transmission from surface waters to humans in estuarine and marine environments.

Materials and Methods

Institutional Review Board

Study design and participant informed consent forms were reviewed and approved by the University of Maryland Institutional Review Board (Protocol: 11-0442).

Study population

The study population was a convenience sample of individuals recruited from a local academic institution. The initial 2009 swim (Sandy Point) included 19 participants, and subsequent 2011 swims (Choptank, Tred Avon, Chester) included four participants for each study, based upon a power analysis calculation for sample size performed using the 2009 data.

Randomization of volunteers

Subjects were assigned random letters from A to S, which were associated with each of their samples. Subject names were not associated with those sample letters and no identifiable information was recorded to associate samples with subjects.

Site selection

Swimming sites were selected based on differing salinities and geographic location to ensure a range of surface water *Vibrio* spp. concentrations in order to test the correlation of *Vibrio* spp. concentrations with overall degree of exposure. Swimming beaches on four different rivers in the Chesapeake Bay were chosen: Choptank River, Chester River, Tred Avon River and Chesapeake mid-Bay (Sandy Point State Park) (Fig. 1). All sites are regularly used by recreational swimmers and thus provide a realistic snapshot of exposure levels. Swims were conducted approximately 1-2 hours post high tide to standardize tidal cycle across swims.

Swim study times and description of activity

In 2009, a total of ten, independent-timed swims were conducted at each site for the same group of swimmers, ranging from 2 to 20 minutes, increasing incrementally. In 2011, a standardized swim time of 8 minutes per swim was chosen based upon the 2009

data. The average concentration of *Vibrio* in handwash samples stabilized at an approximate exposure duration of 8 minutes. Swimmers were requested to keep their hands submerged for the full time they were in the water. Other activity was not restricted. Swimmers were allowed to swim, wade, float, etc., to account for normal swimming behavior and thus, normal exposures. Time between handwash collection and the next subsequent timed swim was approximately 5 minutes.

Description of handwash stations and collection

Handwash stations were assembled on rectangular, plastic resin folding tables and shaded completely by a tent. Sterile phosphate buffered saline (PBS, pH 7.4, 500 mL) (FDA 1998) was aliquoted into Ziploc freezer bags (1 gallon size) and stored at 4°C until use (<24 h storage), including storage on ice until sample collection. Bags corresponding to each swim volunteer were clipped to a central holding apparatus and opened approximately 1 minute before each discrete swim time was completed. Each volunteer completely submerged their hands and rubbed them together in a vigorous manner for 60 seconds in the bag of PBS following the guidance of Larson et al. (1998), Brower et al. (2000) and Chen et al. (2001). During the Choptank 2011 swim study, an additional handwash sample was taken after each initial handwash to assess the efficiency of handwashing in the reduction of *Vibrio* concentrations on hands. All bags were immediately sealed upon handwash completion. Samples were either filtered in the field or frozen and filtered in the lab using 0.22 μm Sterivex-GP polyethersulfone filters (Millipore, Billerica, MA), wrapped in Parafilm M laboratory wrapping film (Bemis Flexible Packaging, Oshkosh, WI), sealed in a labeled 7 oz Whirlpak bag (Nasco, Fort Atkinson, WI) and stored at -20ºC until extraction.

Controls

Control handwash samples were collected (one per person) before individuals entered the water for the first time to account for any background *Vibrio* spp. on their hands. Also, control sample bags (n=2 at each time point) of sterile PBS were clipped onto the board and opened at the same time as each handwash collection bag to account for any potential airborne contamination. In 2011, swim studies at Tred Avon River and Chester River were conducted on the same day with the same study participants. Participants liberally applied Purell® brand ethyl alcohol antimicrobial hand sanitizer (GOJO Industries, Akron, OH) to their hands before the start of the second swim study to control for any cross-contamination between swim sites and handwash samples.

Surface water collection

Surface water samples were collected at each sampling location in sterile wide mouth polypropylene 1 L bottles (Nalgene Thermo Scientific, Waltham, MA). Bottles were rinsed three times with surface water and then dipped below the surface for final 1 L collection volume. Surface water (200 mL) was filtered through a 0.22 μm Sterivex-GP polyethersulfone filter (Millipore, Billerica, MA) using a 60 mL BD luer lock syringe (BD, Franklin Lakes, NJ). Air was pushed through the filter to remove as much water as possible, then wrapped in Parafilm M laboratory wrapping film (Bemis Flexible Packaging, Oshkosh, WI) and sealed in a labeled 7 oz Whirlpak bag (Nasco, Fort Atkinson, WI). Filters were stored on ice until return to the laboratory (approximately 1 h), where they were stored at -20ºC until extraction.

Fecal indicator measurements

Fecal indicator measurements were made following the standard methods for

enumerating *Enterococci* in Standard Methods for the Examination of Water and Wastewater (Eaton et al. 1998). Briefly, surface water samples were filtered in triplicate volumes onto sterile 0.45 um pore size, 47 mm diameter, gridded membrane filters, and plated onto Difco™ m *Enterococcus* (BD, Franklin Lakes, NJ) agar. They were incubated for 48 hours at 35ºC before inspection for isolate growth. All light to dark red colonies were recorded as presumptive *Enterococci*.

DNA extraction, detection and quantification

DNA was extracted following a modified MO BIO Powersoil extraction protocol (Jacobs et al. 2009) and stored at -80ºC until use. A Bio-rad CFX96 Touch™ Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA) was used to detect total *Vibrio vulnificus* (Panicker & Bej 2005) and total *Vibrio parahaemolyticus* (Nordstrom et al. 2007) in each sample using TaqMan chemistry. Samples testing positive for either species were subjected to further qPCR testing for virulence genes (*Vv*: virulence correlated gene *vcg* (Baker-Austin 2010); *Vp* thermostable direct hemolysin (*tdh*) and thermostable related hemolysin (*trh*) (Nordstrom et al. 2007)). Quantitative PCR was performed by using 2.50uL of 10X PCR Buffer (Qiagen, Valencia, CA), 1.25uL of 25 mM $MgCl_2$ (Qiagen), 0.50uL of 10 mM dNTP's solution (Qiagen), 5uL Q solution (Qiagen), 0.45 uL of 5U uL^{-1} TopTaq DNA polymerase (Qiagen), 0.188 uL of 10 uM internal control primers (each), 0.375 uL of 10uM internal control probe, 2uL internal control DNA, 0.50 uL of 10 uM primer (each), 0.188 uL of 10 uM probe and 3 uL DNA template per reaction, with the exception of the *Vv vcg* assay, in which 5uL of DNA template was used. DNase/RNase free water was added to bring the total reaction volume. Two-stage qPCR cycling parameters are presented in Table 2.

A unique internal control assay, including a primer set, probe with unique fluorochrome, and internal control DNA, was added to each tube, excluding the *vcg* analyses, to test for the presence and influence of inhibitors (Nordstrom et al., 2007). Positive controls were also run in a separate well of each qPCR assay plate. Strains used were: *V. parahaemolyticus* USFDA TX2103 and *V. vulnificus* ATCC 27562. Standard curves were constructed as reported in Jacobs et al. (2010) from spiked environmental matrices and used during each qPCR analysis with the appropriate qPCR parameters. Cycle threshold (Ct) value was plotted against the slope of the standard curve to determine PCR unit quantity of cell equivalents (CFU).

Physical and chemical measurements

Physical and chemical measurements were taken at each swim time point, including just before the first swim commenced. Measurements were taken with a YSI 556 Multiprobe System (YSI Incorporated, Yellow Springs, OH). Salinity measurements from July 10, 2011 at Choptank River, Cambridge were retrieved from the Maryland Department of Natural Resources monthly sampling on July 13, 2011, collected 1.23 nautical miles from the swim study site. According to almanac data records (http://www.wunderground.com/history/airport/KSBY/2011/7/10/DailyHistory.html), there was no precipitation between July 10 and July 13, so it can be deduced that the salinity was likely similar on July 10.

Sample size calculation for 2011 swim studies

Sample size was calculated for a desired power of 0.90, preferred detection level of 25 CFU and an alpha of 0.05, using standard deviation calculations from the 2009 swim study 4.89 (between swim), 10.5 (between swimmer) (*Vv*) and 3.31 (between

swim), 4.4 (between swimmer)(*Vp*) CFU mL^{-1}, respectively. It was determined that three swims were needed per site and three swimmers were needed for each swim. Based on these results, each 2011 study consisted of five swims per location, with four swimmers.

Data analysis

Quantitative PCR data was exported to Excel spreadsheet format using BioRad CFX Manager™ Software (Bio-Rad, Hercules, CA, USA). Statistical analysis was completed using Intercooled Stata 9.1 for Macintosh statistical software (StataCorp LP, College Station, TX). Descriptive statistics include means, standard deviations and range (min to max). A sample size calculation was conducted for 2011 swims based on the 2009 results (type I error rate=0.05; power=0.90). Linear regression was completed to determine degree of variance between handwash concentration by swim length, individual swimmer and surface water concentration. Handwash concentrations were then divided by the corresponding surface water concentration to normalize data before additional linear regression analysis for associations between exposure and salinity-temperature. Logistic regression analysis was conducted to evaluate the association of virulent strains occurrence in handwash samples in relation to surface water density and environmental conditions.

Conversion of handwash qPCR results to CFU cm^{-2}.

Previously calculated total body surface area (TBSA) averages for adults and children, including the ratio of hand and palm surface area in relation to the TBSA, were used to qualify dermal exposure from the data collected in this study. Measurements of patient hands are routinely employed by physicians to estimate the area of a burn injury (Amirsheybani et al. 2001). The average adult hand (distal wrist to finger tips) is ~1% of

total body surface area (TBSA) and palm (distal wrist to base of fingertips) is ~0.5% (Mosteller 1987). A rough, and likely conservative, estimate of the entire area of the hand (palm, fingertips and back of hand) would therefore be approximately double the average percentage of TBSA for a hand, equaling ~2% of TBSA for calculation purposes. The average TBSA for adult males and females is 1.9 m^{-2} and 1.6 m^{-2}, respectively, with a combined average of 1.73 m^{-2} (Mosteller 1987). If average handwash densities of each *Vibrio* species are interpreted as CFUs per hand area, an estimate of density for total body surface area can be calculated by dividing the PCR unit quantity by average hand area such that CFU cm^{-2} = CFU / (0.04 *17,300 cm^2).

Results

Environmental conditions

Average salinity and water temperature (+/-standard deviation) for each of the four swim sites was as follows: 9.9 ppt (+/-0.01), 27.7°C (+/-0.22) (Sandy Point); 6.1 ppt (+/-0.00), 31.4°C (+/-0.26) (Choptank); 7.5 ppt (+/-0.48), 31.0°C (+/-0.59) (Tred Avon); 5.5 ppt (+/-0.05), 30.9°C (+/-0.21) (Chester). Each site experienced small changes in salinity (0-1 ppt) and temperature (0.5-1°C) over the course of each swim study. Sandy Point mean salinity was calculated by averaging the first three swim salinities, as salinity values collected after those times were unreliable.

Enterococci counts

Enterococci counts confirmed that all swim study sites were appropriately open for recreational swimming according to Maryland's single sample maximum allowable density at a recreational beach (COMAR 2013), which is less than 104 CFU 100-mL. The geometric mean (+/-standard deviation) of the *Enterococci* counts (CFU 100 mL-1) for

each swim study site were as follows: 22.2 (+/-1.3) (Sandy Point); 9.9 (+/-13.7) (Choptank); 8.8 (+/-9.1) (Tred Avon); 22.5 (+/-7.9) (Chester).

Swim Study Results: Surface and Handwash Concentrations

Average concentrations (+/- standard deviation) of *Vibrio* CFU mL^{-1} in surface water and handwash samples are presented in Table 1. Mean surface water *V. vulnificus* and *V. parahaemolyticus* concentrations were 1128 (95% CI: 665.6, 1591.4) CFU mL^{-1} and 18 (95% CI: 9.8, 26.1) CFU mL^{-1}, respectively, across all sampling locations. Mean *Vibrio* in handwash samples were 180 (95% CI:136.6, 222.5) CFU cm^{-2} (V. vulnificus) and 3 (95% CI:2.4, 3.7) CFU cm^{-2} (*V. parahaemolyticus*).

During the Choptank swim sub-study of handwash efficiency in removal of *Vibrio* spp. there was an overall average reduction of 93.9% (95% CI: 86.5%, 101.3%) for *V. parahaemolyticus* and 89.4% (95% CI: 80.1%, 98.7%) for *V. vulnificus* concentrations in handwash samples.

Data were log transformed (log_{10}) to equalize variance before statistical testing with regression analyses. Linear regression analysis demonstrated a significant positive association between *V. parahaemolyticus* handwash CFU cm^{-2} and surface water CFU mL^{-1}, predicting the log handwash CFU cm^{-2} as y= 0.3563*(log surface water CFU mL^{-1}) – 0.0896 (adjusted R^2 = 0.3071; *P*=<0.002) (Figure 2A). When a similar regression model was fit for *V. vulnificus*, a significant positive association was also found (adjusted R^2 = 0.6139; *P*=<0.001) and log handwash CFU cm^{-2} were predicted as y= 0.808*(surface water cells CFU mL^{-1}) - 0.4192 (Figure 2B). Average proportion of CFU cm^{-2} in handwash samples, in relation to surface water CFU mL^{-1}, were 17.8% (95% CI: 8.5%, 27.2%) (*Vp)* and 13.1% (95% CI: 9.3%, 17.0%) (*Vv)* CFU cm^{-2}: CFU mL^{-1}.

Since estimated exposure (CFU cm^{-2}) was significantly associated with surface water concentrations, handwash concentrations (CFU cm^{-2}) were normalized to surface water concentrations (CFU mL^{-1}) associated with the time of testing. Once normalized, linear regression models incorporating the independent variables of salinity and temperature were performed to test for a relationship with handwash CFU cm^{-2}. The models accounted for less than 3% of the variability for exposure to *V. parahaemolyticus* (adjusted R^2=0.026;l *P*=0.0345) and only 6% for exposure to *V. vulnificus* (adjusted R^2=0.067; *P*=<0.001).

Handwash concentrations of *Vibrio* CFU tended to increase until approximately the third swim of the day and remained fairly constant (*V. vulnificus*) or decreased (*V. parahaemolyticus*) for subsequent, longer-timed swims during the 2009 swim study (Fig. 3). There was approximately 10 minutes between each swim interval. During each of the four swim studies (Fig. 4), there was an appreciable increase in surface water concentration of both *Vibrio* species at approximately the third swim. Time was not found to be a significant predictor of exposure when 2009 swim study data were analyzed with regression analysis. *Vibrio* CFU cm^{-2}, normalized to surface water CFU mL^{-1}, were plotted against time and demonstrated low to minimal regression coefficients for *V. vulnificus* (adjusted R^2=0.207, *P*=<0.001) and *V. parahaemolyticus* (adjusted R^2=0. 003, *P*=<0.01) (Fig. 5). ANOVA tests determined that individual swimmers did not contribute to the variance in the 2009 data (*P*=0.134(*Vp*), 0.282(*Vv*)).

Virulence genes

Thermostable direct hemolysin positive strains of *V. parahaemolyticus* were detected in 4.1% of handwash samples (10/243) and 7% of surface water samples (2/28).

No thermostable related hemolysin (*trh*) positive strains were detected. Of the samples positive for *tdh*, nine handwash samples were from Sandy Point and one was from Choptank River. Sandy Point and Choptank River each had one *tdh* positive surface water sample. *Vibrio vulnificus* virulence correlated gene (*vcg*) was not detected in any of the handwash samples or surface water samples

Vibrio parahaemolyticus virulence gene *tdh* presence was not statistically associated with salinity, temperature or surface water cell concentrations when tested in a logistic regression model (P=0.134).

Total body surface area exposures

Based on the range of *Vibrio* concentrations seen in handwash samples, the highest estimated exposure was 43 CFU cm^{-2} (*Vp*) and 3060 (*Vv*) CFU cm^{-2} and the average estimated exposure was 3 (95% CI: 2.4, 3.7) CFU cm^{-2} (*Vp*) and 180 (95% CI: 136.6, 222.5) CFU cm^{-2} (*Vv*) (Table 3).

Discussion

Handwash samples collected during this study suggest that the public is exposed to *Vibrio* while recreating in waters where such bacteria naturally occur. Secondary handwash studies conducted in 2011 at Choptank River confirm that the handwash methods employed in this study successfully removed a representative sample of *Vibrio* from swimmers' hands. The positive correlation between surface water concentrations and handwash samples provides a quantitative model to assess the degree of exposure and potential risk while recreating in waters harboring these bacteria. Moreover, virulent strains of *V. parahaemolyticus* were detected in surface waters and handwash samples, indicating that virulent species are present and the recreating public could be exposed.

While data regarding virulent strains was not quantitative, the presence of such strains raises concerns regarding the risk of infection from recreating in waters harboring *Vibrio*, especially given that the dose of non-virulent strains--let alone virulent strains--needed to cause illness is largely unknown for dermal exposure.

Predictive models of surface water *V. vulnificus* and *V. parahaemolyticus* concentrations have been developed for the Chesapeake, using the variables of salinity and temperature as the key determinants of surface water bacterial presence and abundance (Jacobs et al. 2010). Other studies have also determined that these are important environmental variables when modeling *V. vulnificus* and *V. parahaemolyticus* surface water concentrations in other geographical areas (Zimmerman et al. 2007, Johnson et al. 2010, Baker-Austin et al. 2012, Johnson et al. 2012). By coupling surface water predictive models with regression models of dermal exposure for these *Vibrio* species, it is possible to estimate an individual's level of dermal exposure when encountering water of known surface water concentration. These models may provide a powerful predictor of overall exposure for use by public health managers to protect public health.

Estimate of Exposure: Total Body

A key component to understanding the overall risk to an individual is to qualify the exposure in units of a predicted dose. The first step in translating the overall dermal exposure by an individual, based on these study findings, is to estimate an individual's total body exposure. The dose-response mechanism for *V. vulnificus* and *V. parahaemolyticus* is poorly understood and creates an obstacle in estimating true overall risk. Additionally, genetic virulence markers and overall mechanism of virulence for each

Vibrio species are debated within the scientific community, resulting in a level of uncertainty when depending only on virulence markers to estimate overall risk of illness (Staley & Harwood 2010, Thiaville et al. 2011, Jones et al. 2012).

By estimating the size of a typical wound for an adult or a child, one can begin to appreciate the relative exposure in terms of dose. For instance, if an adult experiences the average *V. vulnificus* handwash from this study of 180 CFU cm^{-2} and a wound is 2 cm^2, the person will be exposed to an estimated dose of 360 CFU. This initial estimate of dose is only a "snap-shot" of overall dose. Depending on the period of time that the person is immersed in *Vibrio*-laden surface waters, this dose might be multiplied many times as cells move from the environment into the wound and internal body.

It is unknown if this dose would cause an infection in an immuno-competent individual, much less someone with compromised immune function or a pre-existing condition known to increase susceptibility to *Vibrio* illness (e.g., liver cirrhosis). With immuno-compromised populations growing at a rapid pace, it is conceivable that a greater proportion of the population will be susceptible to illness at lower levels of exposure. While overtly immuno-compromised populations stand out (e.g., patients with HIV-AIDS, cancer, organ-transplant recipients), there are emerging populations in rising numbers that should be considered immuno-compromised, including diabetics (CDC 2012b) and those taking steroidal medications (e.g., to control asthma, rheumatoid arthritis, inflammatory bowel disease, etc.) (Myasoedova et al. 2010, Akinbami et al. 2012, Molodecky et al. 2012).

Given the increasing sub-population of children becoming ill with asthma and diabetes, it is prudent to consider the most sensitive populations when formulating

recommendations for recreational water use associated with *Vibrio* spp. Children do not have the same robust immune system of an adult and would therefore be susceptible to infection at lower dosages of an infectious organism. Children are also more likely to have skin abrasions from outdoor play, especially during the summer, when they would also be most likely to be exposed to recreational surface waters. While pediatric *Vibrio* case reports are limited, perhaps due to limited encounters between children and raw seafood, future wound infection and otitis (ear inflammation or infection) cases may be anticipated to increase as surveillance and detection improves for the two studied *Vibrio* species, as well as the emerging pathogen, *V. alginolyticus*.

Other routes of entry

Oral ingestion rates during swimming have been estimated by (Dufour et al. 2006) and are used in the Environmental Protection Agencies Exposure Factors Handbook (U.S. EPA, 2011). Based on these rates, the ingestion of surface water *V. vulnificus* and *V. parahaemolyticus* can be estimated using the average bacterial levels found in this study (Table 4). According to these estimates, a child (younger than 18 years of age) may ingest an average of 42,000 *V. vulnificus* CFU per swimming event or 55,296 CFU per hour. A dose at this level could lead to illness in a child or an immunocompromised adult, although ingestion rates are likely at the lower end of the dose-response continuum and symptoms, if any, may be limited to mild gastroenteritis.

Conclusions

This study of recreational water exposure to *V. vulnificus* and *V. parahaemolyticus* is the first of its kind to quantify the number of bacteria to which recreating swimmers are exposed and qualify that exposure in terms of dermal dose. Due

to a lack of information regarding non-consumption dose-response for *V. vulnificus* and *V. parahaemolyticus*, it is unknown if current levels of exposure in the Chesapeake Bay are likely to cause illness, but the public is being exposed to *V. vulnificus* and *V. parahaemolyticus* at rates for which illness is conceivable. It was confirmed in this study that washing ones hands following exposure to marine water is a useful practice to reduce the number of *Vibrio* on a person's skin by a large percentage. In order to better protect human health, estimates of non-consumption dose-response would be helpful in completing a quantitative microbial risk assessment to calculate relative risk of swimming in waters known to harbor *Vibrio* bacteria. Additionally, these data should be paired with models of surface water *Vibrio* concentration to predict exposure at local and regional scales. Finally, data should be incorporated into global climate change models to predict "tipping points" of sea-surface temperature and salinity that may result in an escalation of recreationally acquired illness.

Table 1: Results of all swim studies, including surface water CFU mL^{-1}, and handwash CFU cm^{-2} with standard deviation (+/-).

Site	Swim #	Swim time (min)	Surface water *Vp* CFU mL^{-1}	*Vp* CFU cm^{-2} handwash (standard deviation)	Surface water *Vv* CFU mL^{-1}	*Vv* CFU cm^{-2} handwash (standard deviation)
Sandy Point	1	2	15.86	3.90 (5.74)	631.85	18.68 (26.93)
Sandy Point	2	4	61.81	2.17 (1.84)	2411.39	86.90 (83.63)
Sandy Point	3	6	70.29	8.70 (10.11)	4699.56	159.40 (195.45)
Sandy Point	4	8	56.60	4.68 (4.04)	2544.68	170.94 (302.31)
Sandy Point	5	10	10.81	0.90 (0.70)	1546.65	161.44 (162.73)
Sandy Point	6	12	14.29	1.39 (1.96)	1792.27	218.45 (204.59)
Sandy Point	7	14	32.47	2.93 (8.06)	2373.90	529.57 (814.08)
Sandy Point	8	16	17.45	1.89 (4.31)	2839.74	406.41 (574.60)
Sandy Point	9	18	15.42	2.39 (3.74)	1742.38	216.01 (248.91)
Sandy Point	10	20	28.76	4.67 (3.15)	982.31	193.13 (196.50)
Choptank	1	8	19.44	2.32 (1.34)	644.40	88.21 (69.62)
Choptank	2	8	29.31	1.37 (1.58)	153.18	17.89 (12.20)
Choptank	3	8	26.11	2.88 (2.94)	703.10	79.17 (63.83)
Choptank	4	8	12.25	1.30 (1.01)	297.63	26.82 (21.94)
Choptank	5	8	10.51	0.60 (1.19)	477.23	19.53 (26.21)
Tred Avon	1	8	7.08	3.74 (3.23)	316.39	51.90 (49.43)
Tred Avon	2	8	3.00	1.09 (1.55)	164.69	13.21 (14.62)
Tred Avon	3	8	10.12	8.17 (10.90)	988.06	133.45 (41.37)
Tred Avon	4	8	6.70	0.41 (0.81)	330.67	104.39 (196.15)
Tred Avon	5	8	0.00	0.82 (0.84)	123.12	15.28 (7.16)
Chester	1	8	0.00	1.45 (2.13)	874.13	70.57 (72.54)
Chester	2	8	0.00	2.06 (3.20)	621.47	62.56 (38.87)
Chester	3	8	0.00	1.71 (3.42)	239.45	109.54 (104.24)
Chester	4	8	0.00	0.28 (0.56)	327.58	52.22 (34.96)
Chester	5	8	0.00	0.49 (0.99)	387.00	39.96 (26.57)

Table 2: PCR conditions for detecting *V. vulnificus* and *V. parahaemolyticus* virulence genes.

Primer	Primer (forward & reverse)/Probe Concentrations (nM)	PCR conditions
Vibrio vulnificus/*vvh*	400/240	1x: 95 °C for 60 s; 41x: 95 °C for 5 s, 59 C for 45 s
Vibrio vulnficus/*vcg*	250/180	1x: 95°C for 10 m; 40x: 95ºC for 15 s, 60°C for 90 s
Vibrio parahaemolyticus/*tlh*	200/150	1x: 95°C for 10 m; 45x: 95ºC for 5 s, 66°C for 45 s
Vibrio parahaemoylticus/*tdh trh*	200/75	1x: 95°C for 60 s; 50x: 95ºC for 5 s, 59°C for 45 s

Table 3. Estimated CFU cm^{-2} of body surface area, by swim site, for handwash (HW), Total Body Surface Area (TBSA) and per cm^2 body surface area.

Swim site	Cells CFU per:	Overall *Vp* average	overall *Vp* std dev	Highest HW *Vp*	Overall *Vv* average	overall *Vv* std dev	Highest HW *Vv*
Choptank	HW	1,315	480	4,844	32,843	34,957	116,795
	TBSA	32,881	11,988	121,104	823,914	575,401	2,919,865
	cm^2	1.9	0.7	7.0	47.5	50.5	169
Tred Avon	HW	2,286	1,902	16,909	44,044	66,393	275,770
	TBSA	57,152	47,552	422,736	1,032,290	828,803	6,894,238
	cm^2	3.3	2.8	24.4	59.7	47.9	398.5
Chester		2,346	1,541	4,731.56	46,343	41,962	179,643
	TBSA	58,649	38,514	118,289	1,158,582	457,061	4,491,080
	cm^2	3.4	2.2	6.8	67.0	60.6	259.6
Sandy Point	HW	2,633	1,590	29,816	125,290	176,781	1,675,186
	TBSA	65,818	39,754	745,403	1,474,215	1,585,427	52,943,859
	cm^2	3.8	2.30	43.1	218.5	382.6	3,060.3

Table 4: Mean and 97% upper percentile (UP; in parentheses) of estimated oral ingestion of surface water and Vibrio CFU during swimming activity (EPA 2011).

	Surface water ingestion		*V. vulnificus* ingestion		*V. parahaemolyticus* ingestion	
	mL event^{-1}	mL hour^{-1}	CFU event^{-1}	CFU hour^{-1}	CFU event^{-1}	CFU hour^{-1}
Children	37 (90)	49 (120)	41,754 (101,565)	55,296 (135,421)	663 (1,613)	878 (2,151)
Adult	16 (53)	21 (71)	18,056 (59,811)	23,698 (80,124)	286 (950)	376 (1,273)

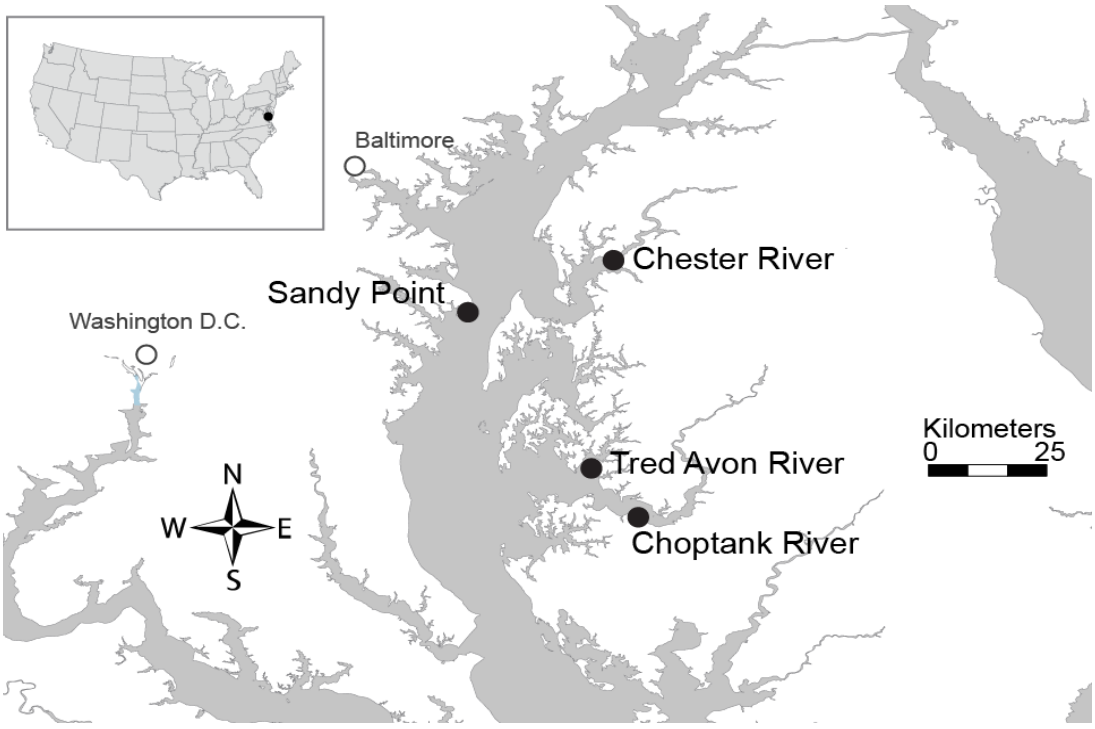


Figure 1. Map of swim study locations in Chesapeake Bay. From: Tracey Saxby, Kate Boicourt, Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/displayimage-127-5815.html)

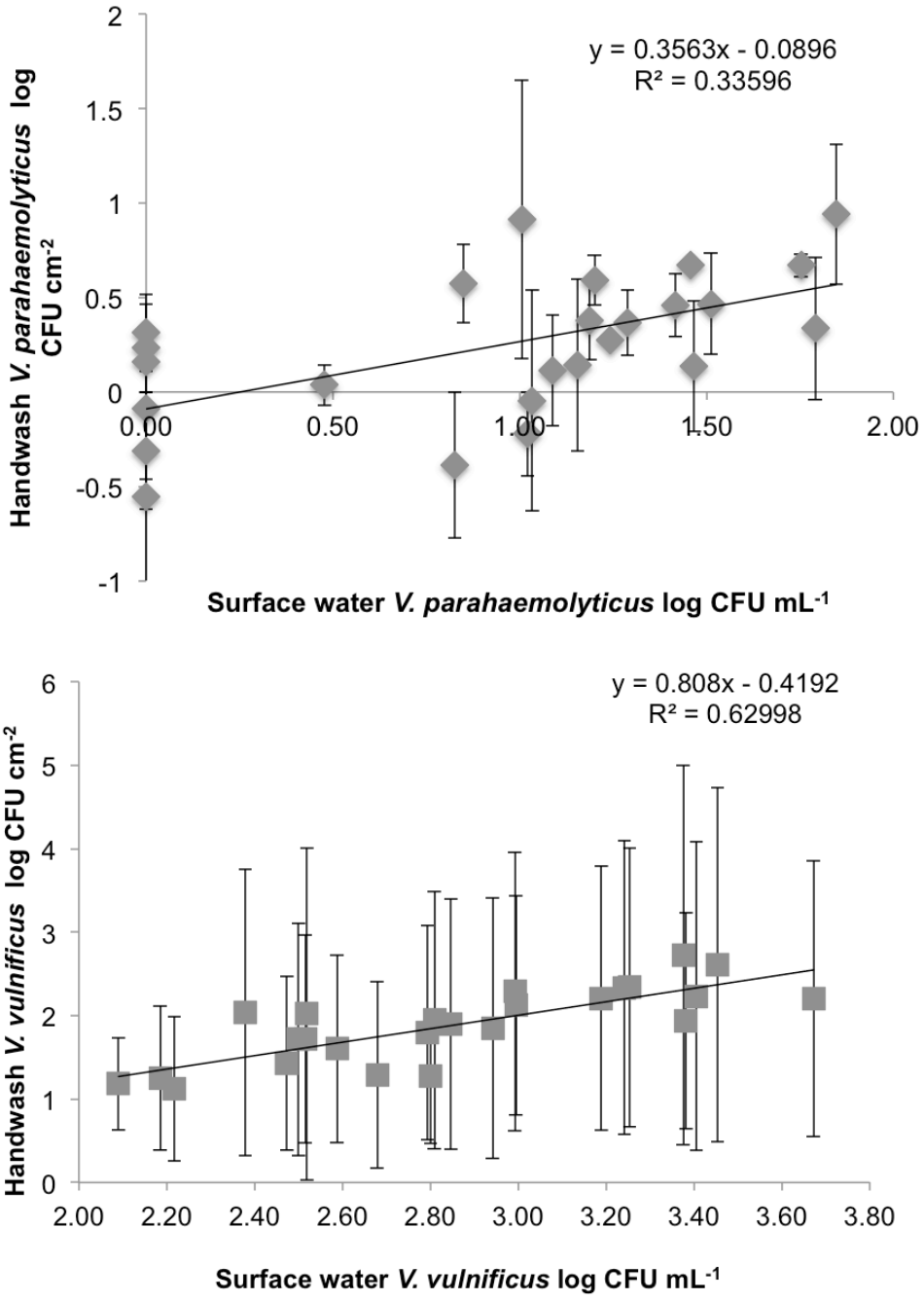


Figure 2A, 2B: *Vibrio parahaemolyticus* and *V. vulnificus* average handwash CFU cm^{-2} in relation to surface water concentrations for all swim studies.

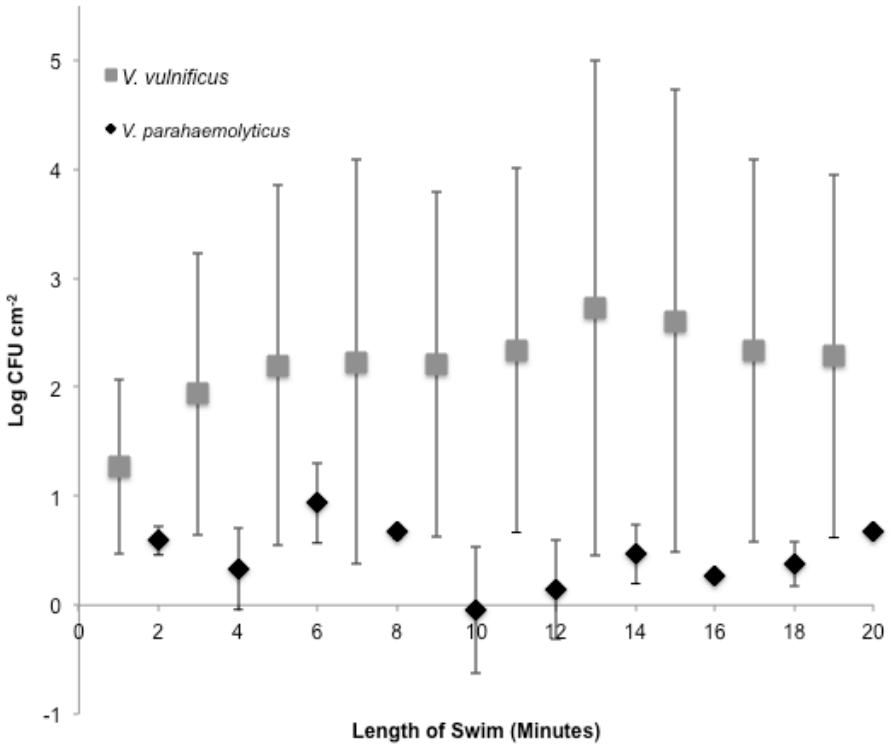
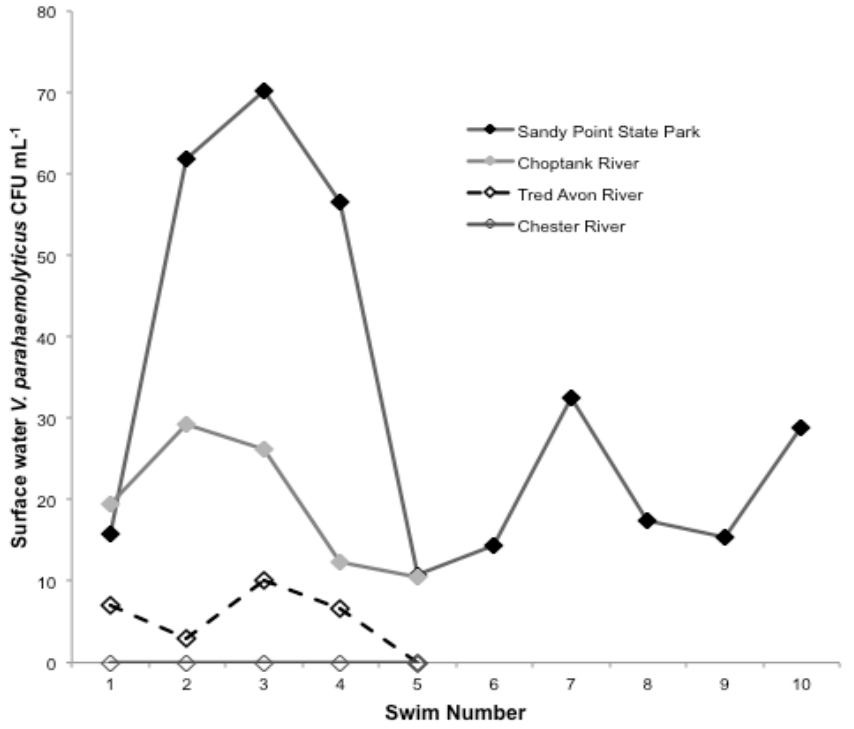


Figure 3. *Vibrio parahaemolyticus* and *V. vulnificus* log handwash CFU cm^{-2} for each swim time point during 2009 swim study.



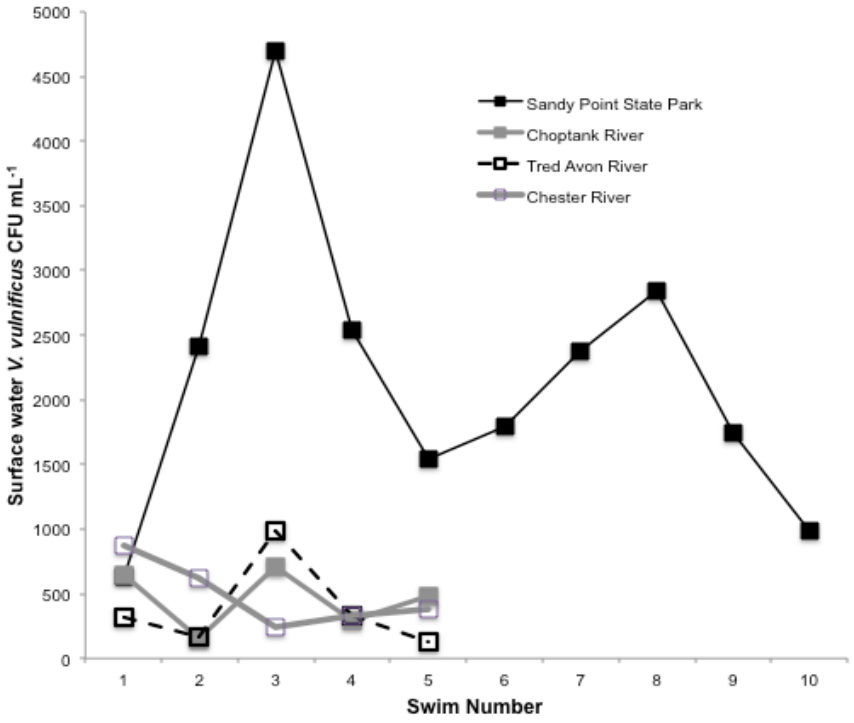


Figure 4A, 4B: Surface water CFU mL^{-1} of *V. parahaemolyticus* and *V. vulnificus* for each swim study. Most CFU mL^{-1} peaked at approximately the third swim. Individual swim times were not statistically significant (Fig. 5A, 5B).

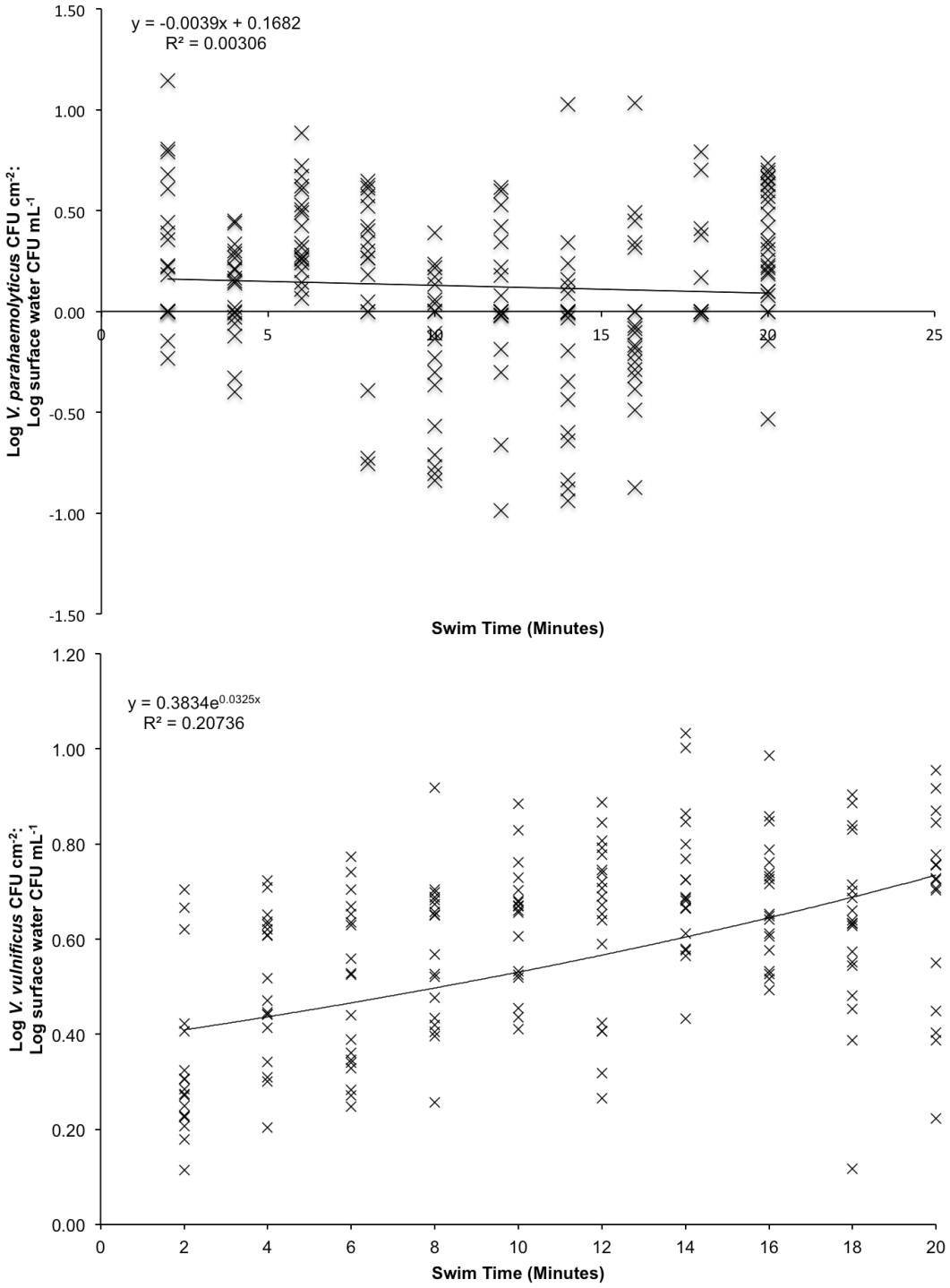


Figure 5A, 5B. *Vibrio parahaemolyticus* (5A) and *V. vulnificus* (5B) CFU cm^{-2}, normalized to surface water concentration, in relation to swim time during 2009 swim study.

CHAPTER 3: IMPACT OF STORM EVENT, HURRICANE IRENE, ON *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* CONCENTRATIONS IN SURFACE WATER, SEDIMENT AND OYSTERS AT AN AQUACULTURE FACILITY IN THE CHESAPEAKE BAY, MARYLAND, USA

(Formatted for submission to *Applied Environmental Microbiology*)

Abstract

To determine if storm events, (i.e., high winds, large volumes of precipitation) increase surface water, sediment and oyster concentrations of *Vibrio vulnificus* and *Vibrio parahaemolyticus*, this study followed a sampling timeline before and after Hurricane Irene impacted the Chesapeake Bay in late August 2011. Oysters were sampled from two levels in the water column (surface water defined as the upper 0.3 m of the water column, and just above sediment layer) to determine if there was a difference in uptake of either *Vibrio* species based on location of the oyster. Sediment and surface water samples were directly extracted and tested with real-time PCR, while oyster samples were tested by a combination of real-time PCR and most probable number enrichment methods. Results indicated no difference in *Vibrio* uptake between surface water and near bottom oyster samples, but did show a difference in species uptake, with *V. parahaemolyticus* increasing 1 day post-Irene, unlike *V. vulnificus*. *Vibrio* concentrations in surface water samples decreased at 1 day post-Irene, but increased at 4 days post-Irene, at the same time oyster concentrations decreased. At 8 days post-Irene, surface water and sediment concentrations were only a fraction of their initial values before the hurricane impacted the area, but oyster concentrations were similar (*V. vulnificus*), if not higher (*V. parahaemolyticus*), in comparison to their pre-storm concentrations. This study suggests that storm events may cause a temporally limited increase in *V. parahaemolyticus* in oyster tissue and that virulent sub-types of both *Vibrio* species may increase in percent abundance within oysters following a storm event.

Introduction

Storm events are thought to be important mechanisms for the distribution of benthic *Vibrio* populations into the water column via resuspension of sediments associated with high winds, and flushing due to large volumes of precipitation (Randa et al. 2004, Fries et al. 2008, Wetz et al. 2008, Johnson et al. 2010). Frequent storm events in the Chesapeake Bay are associated with the summer season, when *V. vulnificus* and *V. parahaemolyticus*, autochthonous bacteria known to cause human illness, are at their highest densities in surface waters (Wright et al. 1996, Parveen et al. 2008, Jacobs et al. 2010, Johnson et al. 2012). The frequency and intensity of storm events are predicted to escalate in response to global climate change (Goldenberg et al. 2001), with increases in peak wind intensities and near-storm precipitation (Meehl et al. 2007) likely impacting mid-Atlantic areas such as the Chesapeake Bay. In the Chesapeake Bay, a shallow, partially mixed estuary prone to tidal circulation (average depth 6.5 m), storm events may increase the overall *Vibrio* density in surface waters with relatively moderate wind speed and associated wave action.

According to the U.S. Environmental Protection Agency, the Chesapeake Bay is home to 25% of the total shellfish harvesting waters in the United States (EPA 2011a). Recently, the Chesapeake Bay has become a site of interest for oyster (*Crassostrea virginica*) aquaculture production to supplement the dwindling wild harvest of bottom dwelling oysters, both through on-bottom (submerged land) and off-bottom (water column) leases (Maryland Department of Natural Resources, Shellfish Aquaculture Program). As of September 2010, 169 aquaculture operation permit applications (~4000

acres) were submitted to Maryland Department of Natural Resources for water-column and submerged-land leases (Donald Webster, University of Maryland Extension, personal communication), and a total of 300 submerged-land leases (~3500 acres) and 23 water-column leases (~94 acres) permitted. A small number of new aquaculture operations are in year-round production of retail oysters, with the supposition that many new operations will soon be joining their ranks.

Summer is generally considered to be a viable oyster harvest season in Maryland, but summer is also when *Vibrio* populations reach their peak in the Bay (Wright et al. 1996, Parveen et al. 2008, Jacobs et al. 2010, Johnson et al. 2012). Studies are currently being conducted to determine ways to reduce *Vibrio* concentrations in oysters (e.g., high salinity relay), but factors influencing the accumulation of high numbers or virulent strains of *Vibrio* in oysters are not completely understood (Warner 2008, Johnson et al. 2010). Thus, the harvest of oysters during seasons when surface water *Vibrio* populations are at high densities could become a pressing issue for seafood safety. If *Vibrio* density in oysters increases after storm events, shellfish managers may need to institute shellfish harvest closure periods to allow for oyster depuration. At present, shellfish harvest areas are closed after heavy rainfall to account for increased fecal coliform levels, and while a recent study found a weak, but significant positive correlation between total *Vibrio* counts and the occurrence of rainfall two days prior, a negative relationship was found between fecal coliforms and total *Vibrio* counts (Yamazaki & Esiobu 2012). Thus, shellfish harvest area closures based upon fecal coliform levels are likely not protective against increased *Vibrio* concentrations in shellfish.

This study was conducted to test the hypothesis that a storm event, generating enough wave energy to cause resuspension of sediment, would cause an increase in oyster-tissue density of *V. vulnificus* and *V. parahaemolyticus*. Oysters were tested in Taylor-style surface-water floats (Luckenbach et al. 1999) and in on-bottom cages, to determine if there was an accumulation difference based on water column position. Results from this study provide the first estimates of storm-related *Vibrio* density changes in oyster tissues, sediment and surface water at an aquaculture facility in the Chesapeake Bay.

Materials and Methods

Sampling site

The study was conducted at an oyster aquaculture facility in a mesohaline tributary of the Chesapeake Bay. The oyster facility was approximately 250,000 m^2 (6 acres) with a water depth of approximately 1.2 m (4 ft) at low tide and 2.1 m (7 ft) at high tide. The oyster farm had sediment types ranging from predominantly sand to predominantly silt. The sampling location within the oyster farm was chosen for the predominance of silty sediment (20.4% sand: 66.6% silt: 13.0% clay)(Micheal Owens, Jeffrey Cornwell, University of Maryland Center for Environmental Science, personal communication). Three sampling sub-locations were chosen along the outermost matrix of oyster floats, which covered approximately 1 acre, both for the sediment composition and the likelihood of the area being unprotected from wind events and resultant resuspension activity. Estimates of wind speeds and resultant wave height were made using equations from Young and Verhagen (1996). Calculations of maximum bottom-sheer stress were made according to Sanford (1994) incorporating an approximate bottom

depth of 1m and sand grain roughness of 0.0005 m. Erosion rate was calculated using the equation E (g m^{-2} hr^{-1}) = Mo (kg m^{-2} s^{-1} Pa^{-1}) * 3600 sec hr^{-1} * 1000 gm kg^{-1} * (tau_b – tau_c) (Pa), with site-specific estimates of tau_c = 0.025 Pa and Mo = 0.000315 kg m^{-2} s^{-1} Pa^{-1} (Tau_b: bottom-related sheer stress; Tau_c: current-related shear stress; Pascal (Pa); Mo is erosion rate constant) (Sanford, Kwon, University of Maryland Center for Environmental Science, personal communication). These calculations do not acknowledge the potential for a wave-dampening effect by the large array of oyster floats tied together at the aquaculture site, although a physical oceanographer conducting experiments at the same site shares that long period waves at the bottom of the water column are damped out by perhaps as much as 50% by the floats, but not so much that resuspension would be negated (Lawrence P. Sanford, University of Maryland, personal communication).

Environmental sample collection

Baseline surface water, oyster and sediment samples were collected from the field location on 26 August 2011, the day before Hurricane Irene, and any associated storm impacts, was forecast to be present along the Maryland coastline. Subsequent samples were taken at time points 1, 4 and 8 d after Hurricane Irene. All samples were collected at approximately 10:00 h to approximate a uniform water and air temperature at the time of sampling due to solar irradiation.

Surface-water samples were collected at each sampling location in sterile wide mouth polypropylene 1 L bottles (Nalgene Thermo Scientific 2105-0032) following the methods described by Jacobs et al. (2009). Briefly, surface water (200 mL) was filtered through a 0.22 μm Sterivex-GP polyethersulfone filter (Millipore, Billerica, MA) using a

60 mL BD luer lock syringe (BD, Franklin Lakes, NJ), wrapped in Parafilm M laboratory wrapping film (Bemis Flexible Packaging, Oshkosh, WI) and sealed in a labeled 7 oz. Whirlpak bag (Nasco, Fort Atkinson, WI). Filters were stored on ice until return to the laboratory (approximately 1 h), where they were stored at -20ºC until extraction.

Oyster sample collection

Oyster samples (a composite of six oysters (Kaufman et al. 2003)) were collected from the top (n=3) and bottom (n=3) of the water column on each of the four sampling dates. Oysters had shell heights (oyster hinge to opposite edge periphery) of approximately 8 cm (3.1 in). Surface water oyster samples were collected from Taylor-style floats and bottom-water oyster samples were enclosed in 1.3 cm mesh bags deployed inside of crab pots to keep the oysters at the bottom of the water column, but out of the sediment layer. Upon removal, oysters were immediately placed in a refrigerated cooler (ice covered by insulation material) and transported to the lab within an hour, where they were immediately processed.

Crab pots consistently had a coating of top layer sediment on the bottom of the pot from being deployed in the sediment. That sediment was collected at each of the three sites by filling a 50 mL Falcon sterile polypropylene conical centrifuge tube (BD Vacutainer Labware Medical 352070). Sediment samples were placed on ice and transported back to the laboratory within 1 h, where they were frozen at -20ºC until defrosted and extracted following the PowerSoil extraction method.

Physical/chemical measurements

Temperature, salinity, conductivity, and dissolved oxygen were sampled using a YSI Model 85 (YSI, Yellow Springs, OH). Secchi depth was recorded to the nearest 0.05

meter. Total suspended solids (TSS) measurements were completed using 250-400 mL of surface water, filtered onto pre-weighed 47mm glass fiber-filter membranes.

Sample size

Based on standard deviations reported in Johnson et al. (2010), sample size needed was calculated for a statistical power of 0.8, significance criterion of 0.05, and preferred detection difference of 500 CFU $gram^{-1}$. Based upon this calculation, three samples were required for each depth (top and bottom), per sampling period.

Oyster processing

Six oysters (Kaufman et al. 2003), collected from each sampling location (top (n=3), bottom (n=3)), were homogenized following the three-tube MPN method as described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) methods (DePaola & Kaysner 2004) with slight modifications. Briefly, oysters were scrubbed, shucked with a sterile knife into a sterile blender, diluted with an equal weight of sterile phosphate-buffered-saline (FDA 1998) and blended for 90 s to create a 1:1 (wt:wt) shellfish:diluent homogenate. A 1:20 dilution of oyster homogenate was prepared in triplicate by adding 1 mL of the 1:1 diluted homogenate to 9 mL alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.5 ±0.2). Additional 10-fold dilutions to 5 $x10^{-7}$ were prepared volumetrically by transferring 3 x 1 mL portions into 10 mL APW. Following overnight incubation at 35 ± 2°C, the top 1 mL of tubes showing growth were collected and frozen at -20°C.

DNA extraction, detection and quantification

Extraction of surface water DNA was completed following a modified MO BIO Powersoil extraction protocol (Jacobs et al. 2009). The standard MO BIO Powersoil

extraction protocol was used for sediment samples. Extracted DNA was stored at -80°C until use. Quantitative PCR was used to quantify CFU mL^{-1} and CFU g^{-1} from each environmental matrix, respectively.

DNA template was obtained from MPN cultures by producing crude cell lysates by boiling 1 mL aliquots of APW cultures in 2 mL micro-centrifuge tubes for 10 min. Following boiling, tubes were plunged into ice until cool and then centrifuged at 14,000 xg for 2 min. Supernatant template was added to real-time PCR reactions at concentrations of 3-5 uL (see PCR methods) to determine presence or absence of *V. vulnificus* and *V. parahaemolyticus* in cultured samples. Bio-rad CFX96 Touch™ Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA) was used to confirm the species with primers designed to detect *V. vulnificus* (Panicker & Bej 2005) or *V. parahaemolyticus* (Nordstrom et al. 2007). Following initial detection, samples testing positive for either species were subjected to further PCR testing for virulence genes (*V. vulnificus*: virulence correlated gene (*vcg*) (Baker-Austin 2010); *V. parahaemolyticus*: thermostable direct hemolysin (*tdh*), thermostable related hemolysin (*trh*) genes (Nordstrom et al. 2007).

Quantitative PCR was performed on surface water and sediment sample extracts by using 2.50uL of 10X PCR Buffer (Qiagen, Valencia, CA), 1.25uL of 25mM $MgCl_2$ (Qiagen), 0.50uL of 10mM dNTP's solution (Qiagen), 5uL Q solution (Qiagen), 0.45uL of 5U/uL TopTaq DNA polymerase (Qiagen), 0.188 uL of 10uM internal control primers (each), 0.375 uL of 10uM internal control probe, 2uL internal control DNA, 0.50 uL of 10uM primer (each), 0.188 uL of 10 uM probe and 3uL DNA template per reaction, with the exception of the *V.vulnificus vcg* assay, in which 5uL of DNA template was used.

DNase-RNase free water was added in a quantity sufficient for a 25uL total reaction volume. Two-stage qPCR cycling parameters were optimized to the conditions presented in Table 3. A unique internal control, including a primer set, probe and internal control DNA, was incorporated simultaneously into each assay, excluding *V. vulnificus vcg*, to test for the presence and influence of inhibitors (Nordstrom et al., 2007). Positive controls used for each qPCR were *V. parahaemolyticus* USFDA TX2103 and *V. vulnificus* ATCC 27562. Standard curves were constructed as reported in Jacobs et al. (2010) from spiked environmental matrices and used during each qPCR analysis with appropriate parameters. Cycle threshold (Ct) value was plotted against the slope of the standard curve to determine PCR unit quantity.

Most Probable Number (MPN) calculation using qPCR results

Corresponding qPCR-MPN values were derived using the U.S. Food and Drug Administration MPN calculator, downloaded from the online publication "Bacteriological Analytical Manual, Appendix 2: Most Probable Number from Serial Dilutions" (http://www.fda.gov/Food/scienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm).

Statistical analysis

Oyster MPN g^{-1}, sediment and surface water data (CFU mL^{-1}) were log transformed (log_{10}) to equalize variances. Multivariate analysis of variance (MANOVA) was conducted to test for differences in sampling location (top vs. bottom oyster concentrations), sampling date (oyster, surface water, sediment) and the interaction effect of sampling location and date for each species of *Vibrio*. Pearson pairwise correlation

analysis was conducted for the experimental variables of oyster MPN g^{-1}, surface water CFU mL^{-1}, sediment CFU g^{-1}, virulence genes (*tdh* and *vcg*) MPN g^{-1}, salinity, temperature, total suspended solids, dissolved oxygen, tidal height and secchi depth.

Results

Hurricane details

During the early morning hours of 28 August 2011, Hurricane Irene was just off the Delmarva coastline and the associated winds and rain impacted the Chesapeake Bay region (Figure 1). At the study site, there were approximately 18.4 cm (7.23 inches) of rainfall (NOAA 2011). Barometric pressure over the area reached a minimum of 976.2 (mb) at approximately 18:40 h on 28 August 2011. Wind gusts were recorded in excess of 26 m s^{-1} (58 MPH). Highest sustained winds were measured at 19.5 m s^{-1} (44 MPH) at 23:30 h on 27 August 2011 (Avila & Cangialosi 2011) (Figure 2A). Tidal height did not deviate from the predicted normal height on the first day of sampling, so there was no Hurricane related tidal forcing at the first sampling time point.

Physical and chemical conditions

After Hurricane Irene, salinity at the study site dropped from 10.6 to 8.0, and by Day 8 increased to 9.9. Dissolved oxygen increased from 5.01 mg L^{-1} to 6.37 mg L^{-1} after the storm, and remaining above 6 mg L^{-1}. Water temperature decreased from 25.6ºC to 24.1ºC after the storm, and by Day 8 increased to 25.7ºC. Secchi depth increased from 0.4 m to 0.45 m on the day after the storm, returned to 0.4 m on Day 4, and increased to 0.55 m on day 8 (Figure 3). Total suspended solids (TSS) started at 25.1 mg L^{-1} and decreased over the course of the study to 19.5 mg L^{-1} (Day 1), 14.7 mg L^{-1} (Day 4) and 14.9 mg L^{-1} (Day 8). Tidal height ranged from low tide during initial sampling efforts

(pre-storm: 0.20 m above mean lower low water (MLLW), Day 1: 0.15 m MLLW) to close to high tide (Days 4: 0.38 m MLLW; Day 8: 0.55 m MLLW). While changes in temperature, salinity, dissolved oxygen, secchi depth and TSS were small, tidal height was significantly correlated with temperature (P=0.001), TSS (P<0.001), and secchi depth (P<0.001).

Resuspension calculations

Rates of erosion were calculated based on highest wind gusts (26.9 and 22.6 m s^{-1}) and highest sustained wind speeds (9-9.8 m s^{-1}). Most winds during the storm were moving in a NNE or NE direction. Erosion rates were predicted to range from 2,343 to 3,616 g m^{-2} hr^{-1} during periods of wind gusts and 487 to 730 g m^{-2} hr^{-1} during highest sustained winds. Given the lowest wind speed (m s^{-1}) during the height of the storm, the oyster farm would have expected an erosion rate of approximately $3x10^5$ g sediment hr^{-1}.

Vibrio vulnificus

Oyster MPN

Average *V. vulnificus* in oysters (MPN g^{-1}) changed little between the first sampling pre-storm (26 August 2011) and 1 day after the storm (29 August 2011), with an average 6% increase (Table 1, Figure 4B). Average *V. vulnificus* decreased approximately 83% between Day 1 and Day 4 post-storm, but increased again between Day 4 and Day 8. This pattern of average *V. vulnificus* in oysters was driven by samples collected from the top of the water column. Oysters collected from the bottom of the water column had approximately the same average number of *V. vulnificus* MPN g^{-1} over the time series, with a small increase 1d post-storm (~9%). Overall, *V. vulnificus* in oysters decreased by only 4% during the study period. A multivariate analysis of

variance found no statistical difference between the sampling locations, sampling dates or an interaction effect of sampling date and location for *V. vulnificus* MPN values of oysters (*P*=0.7960). Correlation analysis of oyster *V. vulnificus* MPN g^{-1} showed no significant associations with any of the sampled environmental variables (Table 2).

Surface water and sediment

Vibrio vulnificus decreased in surface waters and sediment on Day 1 post-storm, increased markedly on Day 4, and decreased again to very low concentrations on Day 8 (Table 1). One-way ANOVA analysis of sediment and surface water CFU mL^{-1} determined no statistically significant difference between dates for either variable (sediment, *P*=0.1261; surface water, *P*=0.8219). Correlation analysis of sediment *V. vulnificus* revealed significant negative relationships with the environmental variables of salinity (*P*=0.0224, R= -0.4641), secchi depth (*P*=0.0000, R= -0.9343) and tidal height (*P*=0.0256, R= -0.4548). Correlation analysis of surface water *V. vulnificus* found significant associations with sediment *V. vulnificus* concentrations (*P*=0.0000, R= 0.9882) and secchi depth (*P*=0.0000, R= -0.8917) (Table 2).

Vibrio vulnificus virulence correlated gene (vcg)

Vibrio vulnificus' vcg was detected in oysters during each of the sampling dates, but concentrations were reduced during the Day 1 and 4 sampling time points (393 and 105 MPN g^{-1}, respectively) relative to concentrations pre-storm (789 MPN g^{-1}) and on Day 8 (622 MPN g^{-1}) (Table 1, Fig. 5). Interestingly, while concentrations of *vcg* decreased, the presence of *vcg* increased from 50% of sampled oysters with detectable virulence (pre-storm and Day 1) to 83% at Day 4 post-Irene. Overall percentage of sampled oysters (2/6) positive for *vcg* was at its lowest percentage on Day 8 (33%). The

percentage virulent *V. vulnificus* MPN g^{-1} of overall *V. vulnificus* MPN g^{-1} was highest on Day 4 (0.6%). *Vibrio vulnificus vcg* was detected in both surface and bottom sampled oysters, but not in sediment or surface waters during this study. No statistically significant correlations were found associated with *V. vulnificus vcg* concentrations.

Vibrio parahaemolyticus

Oyster MPN

Average overall *V. parahaemolyticus* MPN g^{-1} approximately doubled between pre-storm and 1 d post-storm (+54% top oysters, +862% bottom oysters), and then decreased by 88% 4 d post-storm (-84% top, -92% bottom) amounting to a 64% reduction from the initial MPN g^{-1} by day 4. By 8 d post-storm, *V. parahaemolyticus* MPN g^{-1} increased by 86% from 4 d post-storm (+508% top, +790% bottom), amounting to a 162% increase from the pre-storm measurements. Highest oyster *V. parahaemolyticus* MPN g^{-1} were approximately 54% greater at the end of the study and bottom oyster *V. parahaemolyticus* MPN g^{-1} were over six times greater than pre-storm values. Analysis using multivariate analysis of variance found no statistical difference between the sampling locations, sampling dates or the interaction of sampling location and date for *V. parahaemolyticus* MPN values of oysters (P=0.5415). Oyster *V. parahaemolyticus* MPN g^{-1} did not correlate significantly with any of the environmental variables tested (Table 2).

Surface water and sediment

Vibrio parahaemolyticus decreased in surface waters, but increased in sediment, 1 day after the storm. Surface water *V. parahaemolyticus* then increased on Day 4 post-

storm and decreased on Day 8 post-storm. Conversely, sediment *V. parahaemolyticus* decreased on Day 4 and decreased further on Day 8 (Table 1).

Sediment and surface water *Vibrio* abundances (CFU mL^{-1}) were log transformed (log_{10}) to equalize variances. One-way ANOVA analysis of difference among sampling dates for sediment and surface water CFU mL^{-1} showed no statistically significant difference between dates for either variable (sediment, P=0.8080; surface water, P=0.6978). Correlation analysis of sediment *V. parahaemolyticus* CFU g^{-1} revealed significant associations with the environmental variables of temperature (P=0.0124, R= -0.5019), total suspended solids (P=0.0000, R=0.8569), dissolved oxygen (P=0.0094, R=-0.5187), secchi depth (P=0.0161, R=-0.4856) and tidal height (P=0.0000, R=-0.9592). Correlation analysis of surface water *V. parahaemolyticus* CFU mL^{-1} found a significant negative relationship with salinity (P=0.0414, R= -0.4193), secchi depth (P=0.0000, R= -0.9727) and tidal height (P=0.0024, R=-0.5903). Conversely, a strong positive association was found between surface water *V. parahaemolyticus* CFU mL^{-1} and surface water and sediment *V. vulnificus* (P<0.0001, R=0.9595, R=0.9866, respectively)(Table 2).

Vibrio parahaemolyticus tdh/trh

The *trh* gene was not detected in any of the oyster MPN cultures, nor the sediment or surface water samples. The *tdh* gene was detected in oyster MPN cultures at all time points except on Day 8. Two samples were positive for *tdh* during pre-storm sampling (average 658 MPN g^{-1}), and three samples were positive post-storm (Day 1, 1239 MPN g^{-1}; Day 8, 294 MPN g^{-1}). Concentrations of *tdh* decreased over the sampling period (Figure 5), although overall percent *V. parahaemolyticus tdh* MPN g^{-1}, when compared to

total *V. parahaemolyticus* MPN g^{-1}, was greatest at Day 4 (2.9%). The percent of sampled oysters positive for *tdh* was lowest on Day 8 ((2/6) = 33%). *Vibrio parahaemolyticus tdh* MPN g^{-1} correlated significantly with surface water *V. vulnificus* (*P*=0.0093, R=-0.0097), sediment *V. vulnificus* (*P*=0.0400, R=-0.9600), surface water *V. parahaemolyticus* (*P*=0.0152, R=-0.9648) and tidal height (*P*=0.0463, R=-0.9537) (Table 2).

Discussion

Hurricane Irene produced a significant wind event for the Chesapeake Bay region and wave action was sufficient to cause sediment resuspension at the aquaculture study facility, according to estimates of erosion based on wind speed and direction. Additionally, there was a large amount of precipitation (18 cm) during the storm event. Although our data lacks a sampling time point during the storm, *in situ* continuous monitoring data archives of turbidity (accessed at Maryland Department of Natural Resources "Eyes on the Bay;" http://mddnr.chesapeakebay.net/eyesonthebay/index.cfm) depict sharp spikes in nephelometric turbidity units (NTU) during the peak of the storm winds and a rapid subsequent decrease of NTU, most likely due to the large amount of rainfall experienced during the storm and a resultant flushing effect (Figure 6). This flushing affect may be the cause of reduced turbidity and lowered surface water CFU mL^{-1} for both *Vibrio* species 1 d after the storm.

Many concentrations of *V. vulnificus* and *V. parahaemolyticus* detected during this study were greater than similar studies documenting the detection of these species in the same sampled matrices in the Chesapeake Bay. Maximum concentrations of *Vibrio* detected in previous studies of oyster tissue were considerably lower (*V.*

parahaemolyticus: 6.0 x 10^2 CFU g^{-1} (Parveen et al. 2008), 1.0 x 10^4 CFU g^{-1}(Johnson et al. 2012, a); *V. vulnificus*: 1.2 x 10^4 CFU g^{-1} (Johnson et al. 2012)) than the findings of this study (*V. parahaemolyticus*: 5.0 x 10^4 MPN g^{-1}; *V. vulnificus*: 2.5x10^5 MPN g^{-1}). Johnson et al. (2012) detected lower surface water and sediment *V. vulnificus* concentrations (surface water: 150 CFU mL^{-1} vs. 1.2 x10^3 CFU mL^{-1} (this study)*;* sediment: 3.5 x 10^4 CFU g^{-1} vs. 3.6 x10^5 MPN g^{-1} (this study)), while *V. parahaemolyticus* concentrations found in Johnson et al. (2012) were approximately double the concentrations detected in this study (surface water: 60 CFU mL^{-1} vs. 17.5 CFU mL^{-1} (this study); sediment: 1.5 x 10^4 CFU g^{-1} vs. 6.0 x 10^3 MPN g^{-1}(this study). The lower oyster MPN g^{-1} and surface water/sediment *V. vulnificus* values from previous studies may be due to a difference in sampling depth for oysters (i.e., natural oyster bar depth and open water versus near shore shallows) or a difference in recovery efficiencies of methodologies used in either study, such as under-detection (culture-based methods, previous studies) or detection of non-viable cells by qPCR (direct detection, this study) in sampled surface water and sediment matrices.

While there were changes in the average *V. vulnificus* and *V. parahaemolyticus* cell densities in oysters, surface water, and sediment, the values quantified in each of these substrates was not significantly different over the course of the study. There was a species difference in oyster tissue absorption immediately after the storm, with *V. parahaemolyticus* increasing substantially, but *V. vulnificus* increasing only slightly. Unlike oyster *V. vulnificus* MPN counts, *V. parahaemolyticus* MPN counts were similar at each time point for oysters sampled from the top and the bottom of the water column, with levels increasing 2 to 9 times, respectively, from pre-storm MPN concentrations.

Previously, it has been shown that *V. vulnificus* outnumbers *V. parahaemolyticus* in sediment, oyster tissue and the water column (Johnson et al. 2010). During this study, *V. parahaemolyticus* cell g^{-1} was approximately 5% of the total *V. vulnificus* cell g^{-1} in sediment, which supports the Johnson et al. findings (Johnson et al. 2010). However, despite the relative dominance of *V. vulnificus* in sediments, post-storm increases in *Vibrio* were dominated by *V. parahaemolyticus,* suggesting species-specific variation in the degree to which these bacteria are resuspended from sediments or are retained in oyster tissues, perhaps differing from *V. vulnificus* in properties of adhesion to marine aggregates, which may have been subsequently filtered by oysters.

Interestingly, on Day 4 post-storm, oyster tissue *Vibrio* MPN g^{-1} decreased precipitously from pre-storm concentrations (-82%, *V. vulnificus*; -64% *V. parahaemolyticus*), while surface water CFU mL^{-1} and sediment CFU g^{-1} increased substantially (+337% and +84%, respectively). On Day 8, oyster tissue *V. vulnificus* concentrations returned to pre-storm concentrations, while *V. parahaemolyticus* MPN g^{-1} concentrations approximately tripled. Conversely, surface water and sediment concentrations decreased to a fraction of their original concentrations at Day 8 post-storm (-92%, -66% *V. vulnificus*, respectively; -100% for both sediment and surface water, *V. parahaemolyticus*). One possible explanation for this observation is a bacterial response to the flushing effect from the wind and rain at the study site, but the most likely is changes in filtration rates of the oysters over the course of this study.

Oysters have been shown to reduce or halt filtration during periods of high suspended solids (Loosanoff & Tommers 1948), which was probable during the height of the storm (Figure 6). Oysters may have responded to the increase in suspended solids

during the storm by reducing filtration until some time between Day 1 and Day 4 post-storm. During the period between Day 1 and Day 4, the dramatic decrease of both *Vibrio* species in the oysters may have been due to an increase in their filtration rate, possibly depurating the *Vibrio* from their tissues. Approximately 4.5 million oysters are present at the aquaculture site, in various stages of maturity, and it is conceivable that their associated filtration would produce detectable changes in water concentrations of *Vibrio*. This depuration may have increased turbidity in the water column (i.e., high TSS, low Secchi depth) and increased surface water concentrations of both *Vibrio* species. On Day 8 post-storm, the oysters may have re-filtered the *Vibrio* from the surface water back into their tissues, reducing surface water and sediment concentrations by 1 to 2 orders of magnitude and decreasing turbidity to the lowest level seen during the study. Similar to Fries et al. (2008), who noted an increase in sediment concentrations of total *Vibrio* when Hurricane Ophelia impacted the Neuse River Estuary, NC, there was also an increase in the sediment concentrations of both *Vibrio* species during the first four days post-storm. However, this pattern then reversed with an overall decrease in sediment CFU g^{-1} (-100%, *V. parahaemolyticus;* -66%, *V. vulnificus*). Whether this was due to a change in oyster filtration or a difference in how each *Vibrio* species was introduced into the water column as a function of resuspension, and associated particle adhesion, remains to be understood. In contrast to other studies (Fries et al. 2008, Hsieh et al. 2008, Wetz et al. 2008, Johnson et al. 2010), surface water CFU mL^{-1} decreased following the storm, possibly due to oyster filtration.

Notably, virulent *V. vulnificus and V. parahaemolyticus* were not detected in surface waters or sediment during the course of this study. This is counter to other study

findings, such as Johnson et al. (2010), which reported virulent *V. parahaemolyticus* at similar frequencies in sediment, surface water and oysters (Johnson et al. 2010). Virulent *V. vulnificus* was found routinely in oyster tissues, especially on Day 4 when virulence genes were detected in 5 of the 6 oyster samples (Figure 5). The incidence of *V. vulnificus vcg* in oyster samples increased by approximately 30% and the MPN g^{-1} of *vcg* doubled from pre-storm concentrations on Day 4. This finding is counter to previous, laboratory-based studies, examining the relationship between *V. vulnificus'* virulence in oysters. These previous studies found no change in *V. vulnificus* virulence during the passage through the oyster (Groubert & Oliver 1994, Staley et al. 2011). Similarly, the percentage of *V. parahaemolyticus tdh* MPN g^{-1} increased to 2.9% of total *V. parahaemolyticus* MPN g^{-1} at Day 4, although the percent of oyster samples positive for *tdh* was lower than pre-storm samples (16.7%). Incidence and concentration of virulent *V. vulnificus* and *V. parahaemolyticus* were at their lowest points at Day 8.

Movement towards increased aquaculture production of oysters in the Chesapeake Bay, in combination with forecasted environmental responses to global climate change (e.g., warmer surface waters, increased frequency and/or intensity of storm events), may create a situation of higher *Vibrio* density in oysters, especially during the summer harvest season. Because oysters are routinely consumed raw, understanding how oyster concentrations of *Vibrio* might be impacted is vital. One relevant question posed by the aquaculture community is whether *Vibrio* concentrations in oysters differ based on their position in the water column. This study found no difference in *Vibrio* concentrations between oysters collected from the bottom and the top of the water column. The sampling location may have been too shallow to see a true difference in surface versus

bottom culture, but since much of the Chesapeake is shallow, and most aquaculture operations will likely be near-shore, this was probably a good indicator site.

Our data shows that a temporally limited increase in *V. parahaemolyticus* may be expected after a storm, although impacts on *V. vulnificus* are not as clear. Post-storm, it can be anticipated that detectability of virulence genes may be increased in oysters for both *Vibrio* species. At the end of the study, in-oyster concentrations were approximately similar to the pre-storm concentrations, suggesting a possible "ambient" concentration of summer *Vibrio* density. Further testing should be conducted to determine if these levels vary based on site, position in the water column, and after storm impacts. Ideally, further research would have the opportunity to sample before and after separate wind and precipitation events.

Given that *V. parahaemolyticus* and *V. vulnificus* appeared to respond differently during post-storm in oyster samples, further research is needed to determine if patterns of adherence to oyster tissues is different between *V. parahaemolyticus* and *V. vulnificus*, as well as among virulent subsets of each species. On the spectrum of storm intensity, this study fell on the high end of impacts with Hurricane Irene. As Hurricane Irene consisted of both high winds and large amounts of precipitation, it would be useful to examine storm events with a range of wind speeds and precipitation to account for the individual response variables of resuspension and surface water flushing. Such information would help managers of shellfish harvest decide if there should be a cessation of harvest post-storm, what winds or rainfall would be significant for a given aquaculture site, and how long that suspension of harvest should be recommended. At this time, it is inconclusive whether a storm event should trigger closure of a shellfish fishery. However,

concentrations of *Vibrio* in oysters were very high throughout the study and the percentage of samples that were positive for virulent *V. vulnificus* and *V. parahaemolyticus* increased after the storm. From the results of this study, it can be concluded that sampling sediment and surface water for general concentrations of *Vibrio,* or pathogenic subspecies, may not be enough to predict the concentrations in oysters. Additionally, climate change estimates of increases in surface water temperature, changes in salinity and intensity or frequency of storm events may also drive changes to shellfish management practices.

Table 1. *Vibrio vulnificus (Vv) and V. parahaemolyticus (Vp) (n=3)* concentrations.

Date	*Vv* average MPN	+/- std. err.	*Vv* top average MPN	+/- std. err.	*Vv* bottom average MPN	+/- std. err.	*Vv* *vcg*[1] average MPN	+/- std. err.	*Vv* SW CFU mL^{-1}	+/- std. err.	*Vv* sediment CFU g^{-1}	+/- std. err.
26-Aug-11	313,320	170,001	206,819	0	419,820	258,030	789	353	827	108	363,767	172,175
29-Aug-11	332,936	165,909	206,819	0	459,053	246,702	393	321	318	76	296,857	106,683
1-Sep-11	56,913	30,280	30,394	6,121	419,820	43,624	105	39	3,616	1,216	669,908	431,266
5-Sep-11	302,089	173,302	145,126	43,624	419,820	246,702	662	52	68	9	122,769	91,153

Date	*Vp* average MPN	+/- std. err.	*Vp* top average MPN	+/- std. err.	*Vp* bottom average MPN	+/- std. err.	*Vp* *tdh*[2] average MPN	+/- std. err.	*Vp* SW CFU mL^{-1}	+/- std. err.	*Vp* sediment CFU g^{-1}	+/- std. err.
26-Aug-11	28,426	20,281	46,032	2,901	10,820	9,484	658	56	14	1	9,754	6,204
29-Aug-11	87,604	124,368	71,093	117,551	104,116	155,024	1,239	0	7	0.5	14,791	5,555
1-Sep-11	10,235	9,285	11,694	8,898	8,777	11,401	293	0	49	28	20	7
5-Sep-11	74,641	102,537	71,149	117,503	78,133	111,539	0	0	0	0.3	7	5

Table 2. Correlation table of environmental parameters and *Vibrio* concentrations in oysters, sediment and surface water.

	Oyster Vp (log MPN g^{-1})	Oyster Vp tdh (log MPN g^{-1})	Oyster Vv (log MPN g^{-1})	Oyster Vv vcg (MPN g^{-1})	Surface Water Vp (log CFU mL^{-1})	Surface Water Vv (log CFU mL^{-1})	Sediment Vp (log CFU g^{-1})	Sediment Vv (log CFU g^{-1})
Oyster Vp (log MPN g-1)	1.000							
Oyster Vp tdh (MPN g-1)	0.9356 0.0644	1.000						
Oyster Vv (log MPN g-1)	0.2249 0.2908	0.8547 0.1453	1.000					
Oyster Vv vcg (MPN g-1)	0.2341 0.4414	0.6990 0.5072	0.6679 0.0126 (n=13)	1.000				
Water Vp (log CFU mL-1)	-0.2282 0.2834	-0.9848 0.0152 (n=4)	-0.2072 0.3312	-0.3562 0.2322	1.000			
Surface Water Vv (log CFU mL-1)	-0.2900 0.1693	-0.9907 0.0093 (n=4)	00.2817 0.1823	-0.3685 0.2153	0.9595 0.0000 (n=24)	1.000		
Sediment Vp (log CFU mL-1)	0.1776 0.4064	0.8843 0.1157	0.2432 0.2522	0.1721 0.5740	0.3671 0.0776	0.1056 0.6235	1.000	
Sediment Vv (log CFU mL-1)	-0.2714 0.1996	-0.9600 0.0400 (n=4)	-0.2615 0.2171	-0.3909 0.1867	0.9866 0.0000 (n=24)	0.9882 0.0000 (n=24)	0.2113 0.3215	1.000
Salinity (ppt)	0.1919 0.3691	0.0805 0.9195	0.2400 0.2586	0.4677 0.1071	-0.4193 0.0414 (n=24)	-0.3787 0.0680	0.0551 0.7982	-0.4641 0.0224 (n=24)
Temperature ©	-0.0055 0.9796	-0.5057 0.4943	0.0036 0.9867	0.2575 0.3958	-0.3369 0.1074	-0.1351 0.5292	-0.5019 0.0124 (n=24)	-0.2799 0.1853
TSS (mg L-1)	0.1829 0.3924	0.4607 0.5393	0.2643 0.2121	0.3127 0.2982	0.2811 0.1834	0.1034 0.6306	0.8569 0.0000 (n=24)	0.1377 0.5210
DO (mg L-1)	-0.2321 0.2752	-0.3009 0.6991	-0.3050 0.1468	-0.4420 0.1305	0.1360 0.5264	0.2189 0.3042	-0.5187 0.0094 (n=24)	0.2456 0.2473
Secchi (m)	0.1762 0.4103	0.7617 0.2383	0.1380 0.5201	0.2468 0.4162	-0.9727 0.0000 (n=24)	-0.9143 0.0000 (n=24)	-0.4856 0.0161 (n=24)	-0.9343 0.0000 (n=24)
Tidal Height (m)	-0.0851 0.6925	-0.9537 0.0463 (n=4)	-0.1413 0.5101	-0.0205 0.9470	-0.5903 0.0024 (n=24)	-0.3434 0.1005	-0.9592 0.0000 (n=24)	-0.4548 0.0256 (n=24)

indicates significance of P=0.05

Table 3: PCR conditions for the detection of *V. vulnificus* and *V. parahaemolyticus* virulence genes.

Primer	Primer (forward & reverse)/Probe Concentrations (nM)	PCR conditions
Vibrio vulnificus/vvh	400/240	1x: 95 °C for 60 s; 41x: 95 °C for 5 s, 59 C for 45 s
Vibrio vulnficus/*vcg*	250/180	1x: 95°C for 10 m; 40x: 95ºC for 15 s, 60°C for 90 s
Vibrio parahaemolyticus/tlh	200/150	1x: 95°C for 10 m; 45x: 95ºC for 5 s, 66°C for 45 s
Vibrio parahaemoylticus/*tdh trh*	200/75	1x: 95°C for 60 s; 50x: 95ºC for 5 s, 59°C for 45 s

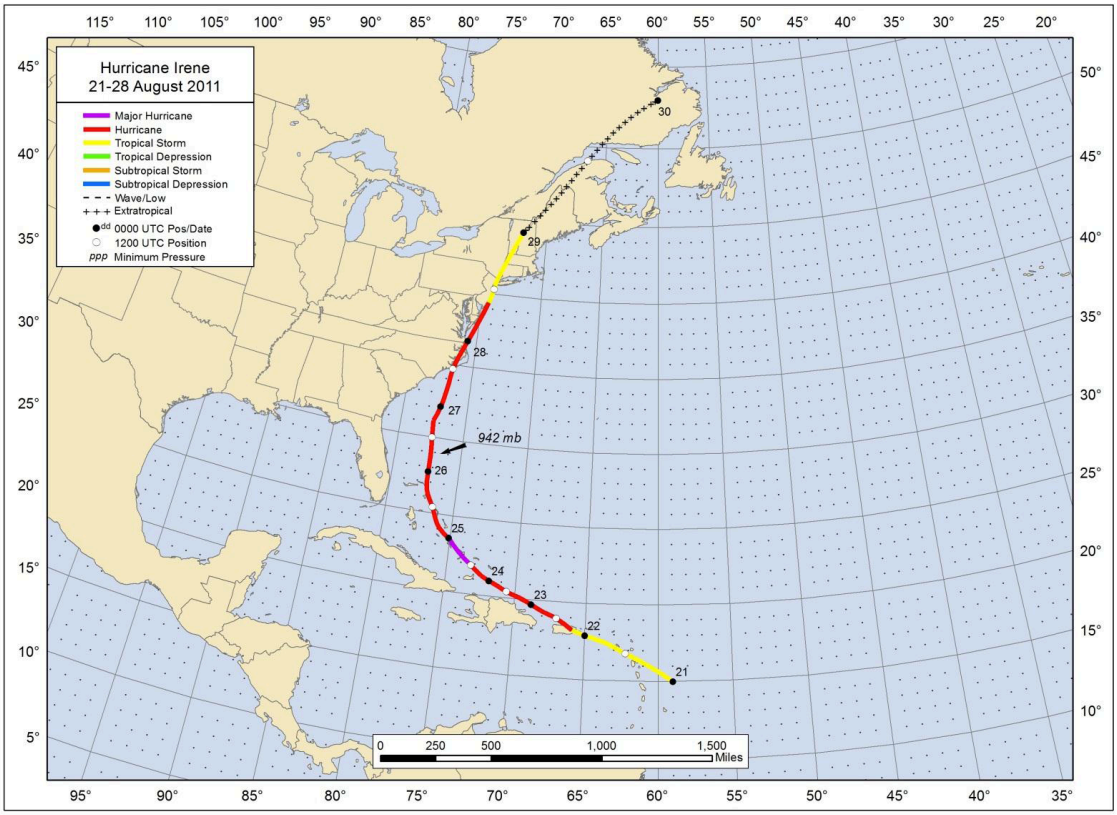


Figure 1. Best track positions for Hurricane Irene, 21 -28 August 2011. Track during the extratropical stage is based on analyses from the NOAA Hydrometeorological Prediction Center. (Avila & Cangialosi 2011)

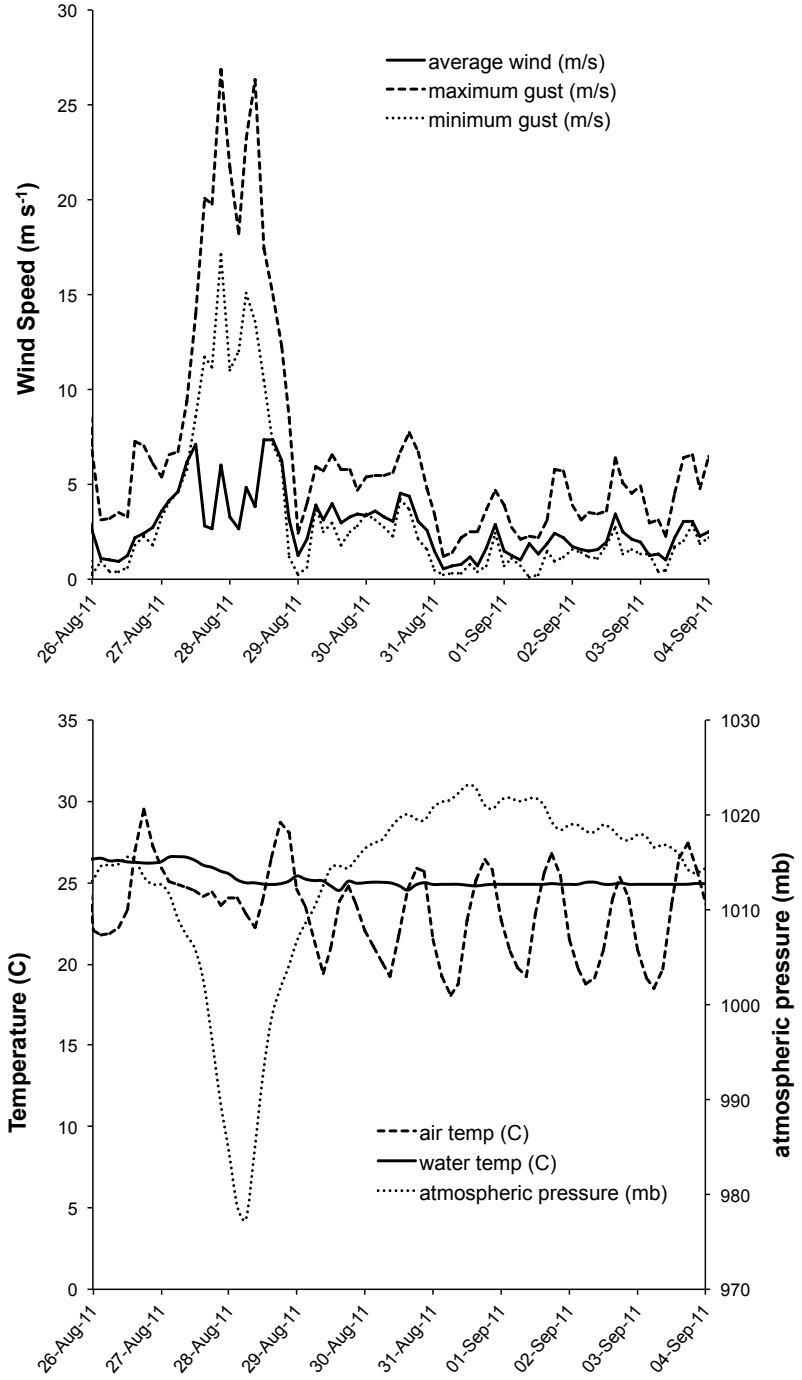


Figure 2A, 2B. Wind speed and direction at study site during Hurricane Irene Data from NOAA station CAMM2.

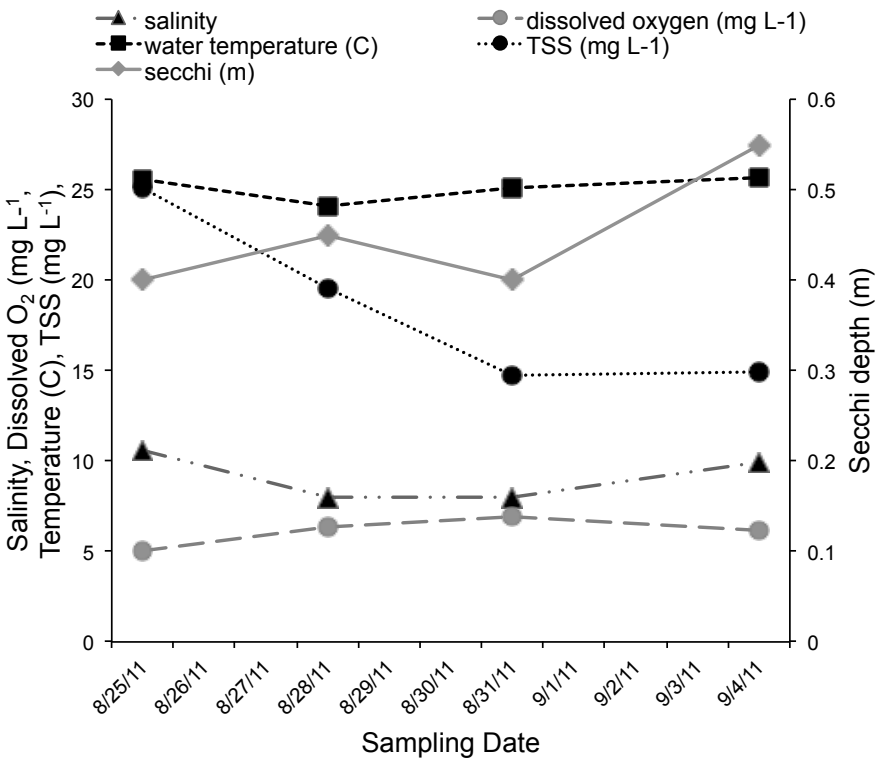


Figure 3. Physical and chemical measurements of the environment.

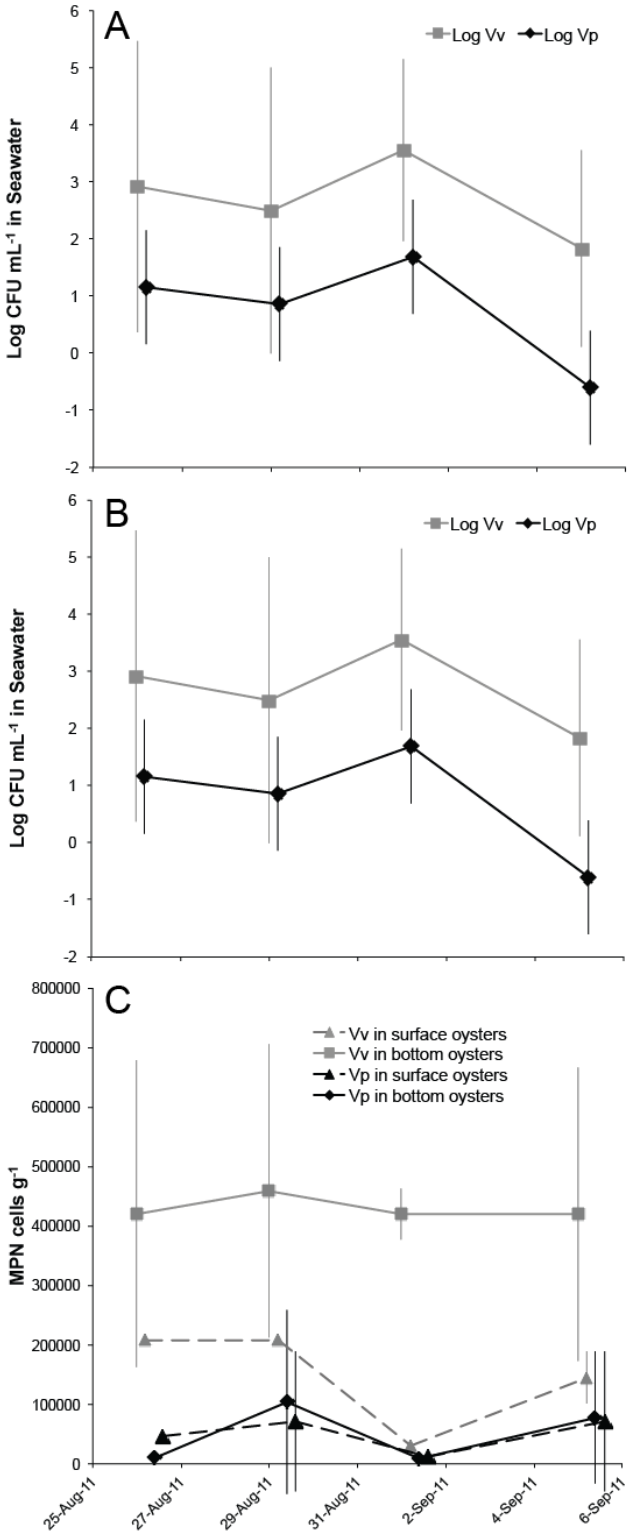
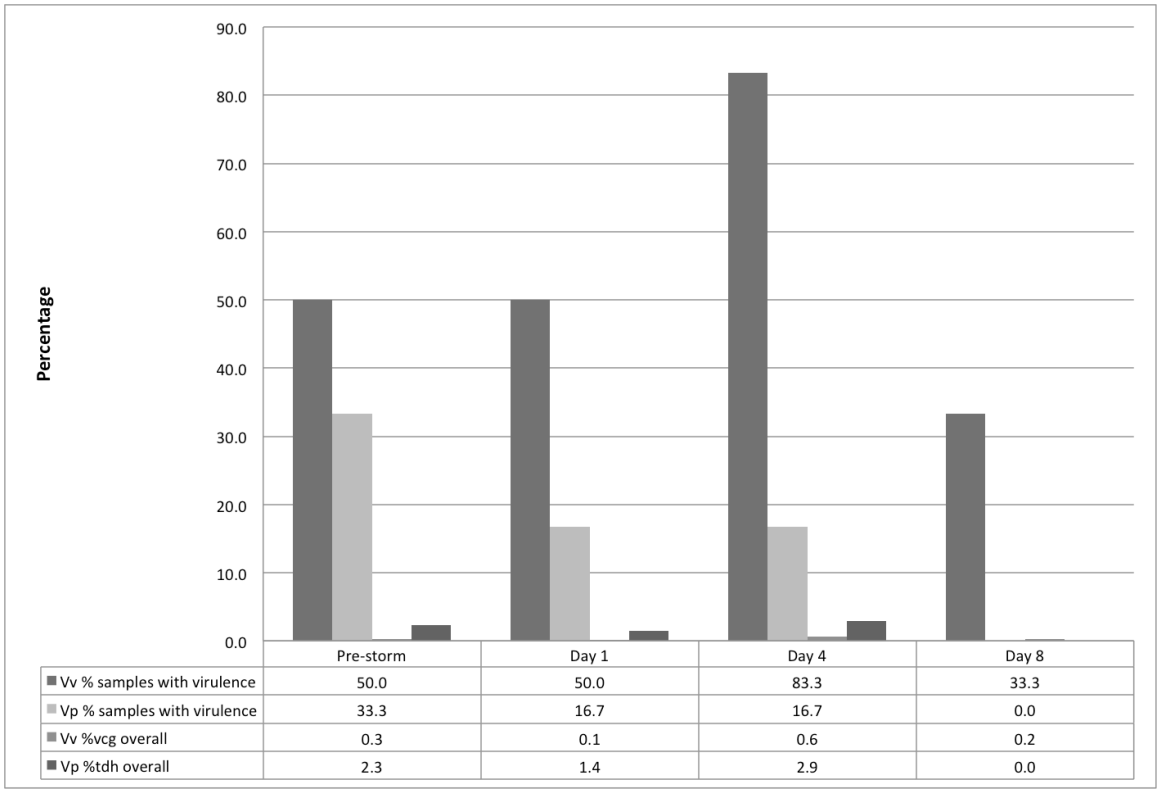


Figure 4. (A) Changes over time of log-transformed CFU mL^{-1} *V. vulnificus* and *V. parahaemolyticus* in surface waters (+/- standard error); (B) Log-transformed MPN g^{-1} *V. vulnificus* and *V. parahaemolyticus* in oysters (+/- standard error);; (C) Changes over time of MPN g^{-1} *V. vulnificus* and *V. parahaemolyticus* in oysters based on position in water column and overall averages (+/- standard error);.



	Pre-storm	Day 1	Day 4	Day 8
Vv % samples with virulence	50.0	50.0	83.3	33.3
Vp % samples with virulence	33.3	16.7	16.7	0.0
Vv %vcg overall	0.3	0.1	0.6	0.2
Vp %tdh overall	2.3	1.4	2.9	0.0

Figure 5. Changes in *Vibrio* virulence during study.

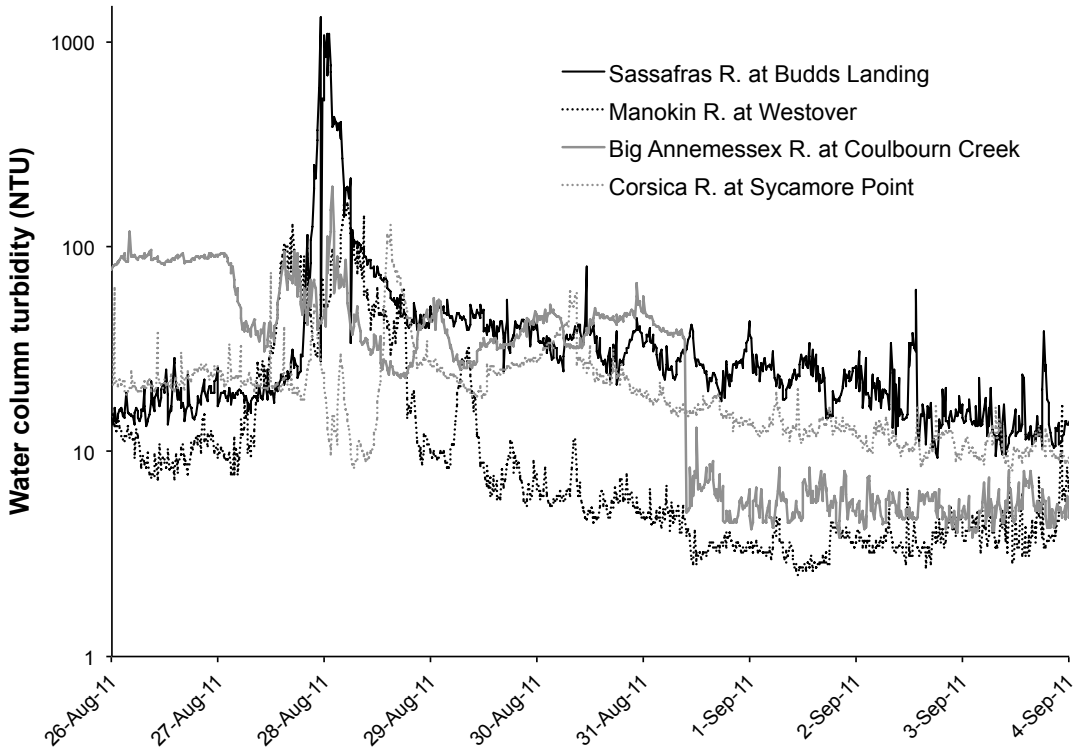


Figure 6. Turbidity in Chesapeake Bay during Hurricane Irene.

CHAPTER 4: ANTIMICROBIAL SUSCEPTIBILITY OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* RECOVERED FROM RECREATIONAL AND COMMERCIAL AREAS OF THE CHESAPEAKE BAY AND COASTAL BAYS

(Formatted for submission to *Water Research*)

Abstract

Vibrio vulnificus and *V. parahaemolyticus* in the estuarine-marine environment are of human health significance and may be increasing in pathogenicity and abundance. *Vibrio* illness originating from contact with waters of the Chesapeake Bay or through seafood originating from the Chesapeake, can cause deleterious health effects, particularly if the strains involved are resistant to clinically-important antibiotics. To our knowledge, little data exists regarding antimicrobial susceptibility patterns of *V. vulnificus* and *V. parahaemolyticus* isolated from the Chesapeake Bay. The purpose of this study was to evaluate antimicrobial susceptibility among these pathogens. Surface-water samples were collected from three sites, of recreational and commercial importance, from July to September 2009. Samples were plated onto species-specific media and resulting *V. vulnificus* and *V. parahaemolyticus* strains were confirmed using polymerase chain reaction assays and tested for antimicrobial susceptibility using the Sensititre® microbroth dilution system. Descriptive statistics, Friedman two-way Analysis of Variance (ANOVA) and Kruskal-Wallis one-way ANOVA were used to analyze the data. *Vibrio vulnificus* (n=120) and *V. parahaemolyticus* (n=77) were isolated from all sampling sites. Most isolates were susceptible to antibiotics recommended for treating *Vibrio* infections, although some isolates expressed intermediate resistance to chloramphenicol (78% *V. vulnificus*, 96% *V. parahaemolyticus*). *Vibrio parahaemolyticus* also demonstrated resistance to penicillin (68%). No location or month differences were detected in *V. parahaemolyticus* resistance patterns, but *V. vulnificus* isolates from St. Martin's River had lower intermediate resistance than the other two sampling sites during the month of July (P=0.0166). Antibiotics recommended to treat

adult *Vibrio* infections were effective in suppressing bacterial growth, while some antibiotics recommended for pediatric treatment displayed intermediate resistance and resistance.

Introduction

Bacterial antimicrobial resistance is an important public health consideration with regard to coastal microbiology. Pathogenic bacteria and antimicrobial resistance genes are often released with wastewater discharges into aquatic environments (Baquero et al. 2008). Naturally occurring bacteria produce antibiotics in the environment for signaling and regulatory roles in microbial communities (Martinez 2008). Bacteria protect themselves from the toxicity of the compounds they generate by evolving antibiotic resistance elements (Wright 2007). Because of this resistance, naturally-occurring bacteria are also capable of serving as reservoirs of resistance genes and, coupled with the introduction and accumulation of antimicrobial agents, detergents, disinfectants, and residues from industrial processes, these bacteria may play an important role in the evolution and spread of antibiotic resistance in aquatic environments (Baquero et al. 2008).

Vibrio bacteria in the estuarine-marine environment are of particular concern to human health and may be increasing in pathogenicity and abundance (Baker-Austin et al. 2010). In order to protect recreational and commercial users of estuarine-marine environments, and ensure the safety of locally-harvested seafood, the antibiotic resistance patterns among these pathogens need to be better understood. Identifying antibiotic resistance patterns among *Vibrio* will highlight potential treatment obstacles that the public may experience upon exposure to and infection with these microorganisms. In the

United States, previous studies exploring antimicrobial susceptibility of *Vibrio vulnificus* and *V. parahaemolyticus* have been conducted in South Carolina and the Gulf region (Han et al. 2007, Baker-Austin et al. 2008, Baker-Austin et al. 2009). However, to our knowledge, no studies have been completed in the Chesapeake Bay, which lies in a watershed where 17 million people work, live and play.

Previous work has demonstrated that human recreational exposures to *V. vulnificus* and *V. parahaemolyticus* in the Chesapeake Bay are at significant enough concentrations to potentially elicit deleterious health effects, particularly among immunocompromised recreationists (Shaw et al. 2011). Moreover, current models predict that total tissue loading of shellfish and finfish with *V. vulnificus* and *V. parahaemolyticus* is associated not only with surface water concentrations but also with the risk of illness for those consuming contaminated seafood products (CFSAN (Center for Food Safety and Applied Nutrition) 2005, World Health Organization. & Food and Agriculture Organization of the United Nations 2005, 2011). Given these data, along with the knowledge that environmental conditions may be increasingly more favorable for *Vibrio* growth (Baker-Austin et al. 2012), it is not surprising that rates of *Vibrio* infections are increasing in Maryland and other U.S. states (Scallan et al. 2011a). In this context, it is critical to gain a better understanding of the antimicrobial susceptibility patterns of *V. vulnificus* and *V. parahaemolyticus* originating from estuarine-marine environments.

This study evaluated the degree to which *V. vulnificus* and *V. parahaemolyticus* isolates from the Chesapeake Bay were susceptible to a broad range of antimicrobial treatments, and our findings provide the first data on antimicrobial resistance patterns

among *Vibrio* bacteria isolated from this region. These data will be helpful in short- and long-term predictions of human health risks associated with exposures to *Vibrio* populations within the Chesapeake Bay.

Materials and Methods

Sampling sites

Sampling sites were selected based on their importance for human use in the Chesapeake Bay, Maryland Coastal Bays region. Two sites, Sandy Point State Park and St. Martin's River, were characterized by frequent recreational use, and one site, the Pocomoke Sound, was characterized by heavy commercial fishing use (Figure 1). Sandy Point State Park is an artificial beach on the western shore of the Chesapeake mid-Bay region, at the base of the Chesapeake Bay Bridge. It is open year round and frequented by approximately 768,000 visitors annually, with many users frequenting park beaches during the summer (Sandy Point Park staff, Maryland Department of Natural Resources, personal communication). St. Martin's River is a tributary of the Maryland Coastal Bays with approximately 10,000 residents. Land-use in the St. Martin's River watershed is ~10% residential, ~48% agricultural, and ~34% forested (Thomas et al. 2009). The Pocomoke Sound is a major embayment of the Chesapeake Bay's Eastern Shore. It is influenced by agricultural practices, including high-density concentrated poultry feeding operations, and is a popular destination for commercial and recreational fishing.

Sample collection

Sampling dates were chosen to coincide with times of high recreational and/or commercial use. Surface water samples (n=9) were collected during summer 2009, once a month, for three consecutive months (July, August, September) within two hours of

high tide and on approximately the same date each month. Water samples were collected just below the surface in sterile wide mouth polyproylene 1 L environmental sampling bottles (Nalgene Thermo Scientific, Waltham, MA). Bottles were rinsed three times with surface water and then dipped below the surface for a final 1 L collection volume. Samples collected for *Vibrio* culture were kept in insulated coolers, while water samples for *Enterococci* were stored in an insulated container on ice (4°C) upon collection and returned to the laboratory within four hours.

Physical and chemical water quality measurements

Water-column depth and surface-water salinity, temperature, dissolved oxygen, conductivity, and pH were measured on every sampling date and location with a YSI 556 Multi-probe system (YSI Incorporated, Yellow Springs, OH) in accordance with the manufacturer's instructions.

Fecal indicator measurements

Fecal-indicator measurements were conducted following the standard methods as described for *Enterococci* in Standard Methods for the Examination of Water and Wastewater (Eaton et al. 1998). Briefly, surface-water samples were filtered in triplicate onto sterile 0.45 um pore size, 47 mm diameter, gridded membrane filters, and plated onto Difco™ m *Enterococcus* (BD, Franklin Lakes, NJ) agar. Plates were incubated for 48 hours at 35ºC. All light to dark red colonies were recorded as presumptive *Enterococci*.

Vibrio isolation

Surface water samples (100uL) were spread plated in triplicate onto Chromagar *Vibrio* media (DRG International, Mountainside, NJ) and incubated for 24 hours at 37°C.

After incubation, each plate was observed for characteristically colored bacterial colonies associated with *V. vulnificus* (turquoise) or *V. parahaemolyticus* (mauve). As *V. vulnificus* and *V. cholerae* both appear as turquoise colonies on Chromagar *Vibrio* media, all turquoise colonies were replated onto cellobiose-collistin (CC) agar (FDA 2004) media to confirm *V. vulnificus* species. The CC agar cultures were incubated for 24 hours at 37°C and yellow-colored colonies were considered presumptive *V. vulnificus.* Tryptic soy broth (TSB), supplemented with 5% sodium chloride, was then inoculated with individual colonies of *V. vulnificus* or *V. parahaemolyticus* and incubated at 37°C for 24 hours and stored in 30% glycerol stock at -80°C.

Vibrio species confirmation

A DNA template was obtained by producing crude cell lysates by boiling 1 mL aliquots of TSB cultures in 2 mL micro-centrifuge tubes at 100°C for 10 min. A Bio-rad CFX96 Touch™ Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA) was used to confirm the species of isolates with primers designed to detect *Vibrio vulnificus* (Panicker & Bej 2005) or *Vibrio parahaemolyticus* (Nordstrom et al. 2007). Following initial detection, samples testing positive for either species were subjected to further PCR testing for virulence genes (*V. vulnificus*: virulence correlated gene (*vcg*) (Baker-Austin 2010); *V. parahaemolyticus*: thermostable direct hemolysin (*tdh*), and thermostable related hemolysin (*trh*) genes (Nordstrom et al. 2007)).

Real-time PCR was performed by using 1X PCR Buffer (Qiagen, Valencia, CA), (Qiagen), 0.2mM dNTP's solution (Qiagen), 1X Q solution (Qiagen), 2.25U TopTaq DNA polymerase (Qiagen), 75nM internal control primers (each), 150nM internal control probe, 2uL internal control DNA, target primer and probe concentrations as detailed in

Table 1, and 3 uL DNA template per reaction, with the exception of the Vv *vcg* assay, where 5uL of DNA template was used and the internal control components were absent. DNase-RNase free water was added in a quantity sufficient for a 25uL total reaction volume. Two-stage qPCR cycling parameters are presented in Table 1. A linear synthetic exogenous DNA internal control, including a primer set, probe and internal control DNA, was incorporated simultaneously into each assay, excluding *V. vulnificus vcg*, to test for the presence and influence of inhibitors (Nordstrom et al., 2007). The following positive controls were used in each qPCR: *Vibrio parahaemolyticus* USFDA TX2103 *and Vibrio vulnificus* ATCC 27562.

A randomly chosen subset of bacterial isolates were taxonomically identified with 16S rRNA gene sequences. DNA extracted from cultures was PCR amplified with bacteria-specific primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 907r (5'-CCGTCAATTCCTTTRAGTTT-3') using the following conditions: 94°C for 2 min, followed by 25 cycles of 55°C for 30 s, 72°C for 30 s, and 94°C for 2 min, followed by 72°C for 5 min. The PCR products were sequenced bi-directionally using the same primers on an ABI 3730 XL Genetic Analyzer in the BioAnalytical Services Laboratory at the University of Maryland Center for Environmental Science. Paired reads for each organism were analyzed and assembled with Phred and Phrap (Ewing & Green 1998, Ewing et al. 1998), manually edited with Consed (Gordon et al. 1998), and aligned and analyzed with the ARB sequence alignment program (Ludwig et al. 2004).

Clinical isolates

Vibrio parahaemolyticus (n=8) were graciously provided by the State of Maryland's Department of Health and Mental Hygiene for comparison purposes with our environmental isolates. Sample type and source of infection are presented in Table 2.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Sensititre® microbroth dilution system (Trek Diagnostic Systems, Westlake, Ohio) in accordance with the manufacturer's instructions on all PCR-confirmed *V. vulnificus* (n=120 (3 *vcg*+)) and *V. parahaemolyticus* (n=77 (1 *tdh*+/ 1 *trh*+)). Cultures were grown overnight on tryptic soy agar (TSA) + 2.5% NaCl plates at 37°C. *Vibrio* cultures were transferred to sterile demineralized 2.5% saline solution to achieve a 0.5 McFarland standard. Then, 100 µL of each suspension was transferred to sterile cation-adjusted Mueller Hinton broth (Trek Diagnostic Systems, Westlake, Ohio), and 50 µL of the broth solution was dispensed into CML1FMAR custom minimal inhibitory concentration (MIC) plates (Trek Diagnostic Systems Inc.) with the following twenty-six antibiotics (range of concentrations in µg/mL): amikacin (AMI; 8-64), ampicillin (AMP; 4-32), ampicillin-sulbactam 2:1 (A/S2; 8/4-32/16), apramycin (APR; 8-32), cefoxitin (FOX; 8-32), ceftriaxone (AXO; 8-64), cephalothin (CEP; 8-128), chloramphenicol (CHL; 8-32), ciprofloxacin (CIP; 1-4), oflaxacin (OFL; 1-8), ceftazidime (TAZ; 8-32), cefepime (FEP; 8-32), cefotaxime (FOT; 8-64), meropenem (MERO; 2-16), doxycycline (DOX; 2-16), imipenem (IMI; 2-16), levofloxacin (LEVO; 2-8), cefuroxime (FUR; 8-32), trimethoprim-sulfamethoxazole (SXT; 2/38-4/76), penicillin (PEN; 16-128), piperacillin (PIP; 16-128); piperacillin-tazobactam (P/T4; 16/4-128/4), streptomycin (STR; 8-128),

tetracycline (TET; 4-32), gentamicin (GEN; 2-16), amox/clav 2:1(AUG2; 8/4-32/16). *Escherichia coli* ATCC 25922 and *E.coli* ATCC 35218 were used as quality control strains. Next, MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth (CLSI 2010). Resistance breakpoints published by the Clinical and Laboratory Standards Institute were used (CLSI 2010a,b). Breakpoints not available from CLSI (streptomycin, apramycin, penicillin) were derived from ranges used in similar studies (Chiew et al. 1998, Baker-Austin et al. 2008, Baker-Austin et al. 2009, Vizcaino et al. 2010). Multidrug resistance (MDR) was defined as resistance to two or more antibiotics.

Statistical analyses

Descriptive and inferential statistics were used to compare the percentage of isolates demonstrating intermediate resistance or resistance to tested antibiotics at each sampling site and sampled month, as well as the average number of antibiotics that *V. vulnificus* and *V. parahaemolyticus* isolates were resistant to at each sampling location and each month. *P*-values of ≤ 0.05 were defined as statistically significant. Due to the violation of normality assumptions, nonparametric Friedman two-way Analysis of Variance (ANOVA) was used to determine effects related to sampling site and month sampled. For samples in which there was a significant month effect, stratified Kruskal-Wallis one-way ANOVA and pairwise post-hoc tests were conducted for each month separately. Kruskal-Wallis analysis was conducted to evaluate differences in the occurrence of antimicrobial susceptibility in non-virulent and virulent bacteria. All statistical analyses were performed using StataIC 12 (StatCorp LP, College Station, TX).

Results

Physical, chemical and bacterial water quality

Water temperature, pH, and dissolved oxygen (DO) were uniform across the three sampling locations (Table 3). Average salinity in St. Martin's River (24.5 ppt) was approximately double that of the Pocomoke Sound (10.5 ppt) and Sandy Point State Park (9.4 ppt) sampling site. Water depth at the Pocomoke Sound was approximately double that of Sandy Point State Park and three to four-fold deeper than St. Martin's River.

Enterococci counts (colony forming units (CFU)) per 100 mL^{-1} were uniformly low at Sandy Point during each sampling time point and below the single sample regulatory closure level of 104 CFU per 100 mL^{-1} (COMAR 2013). On one sampling occasion (Table 3), St. Martin's River (August) *Enterococci* counts exceeded closure levels.

Presumptive *Vibrio* colonies isolated during the culture portion of this study indicated that *Vibrio vulnificus* and *V. parahaemolyticus* were present in all tested water samples (Table 3). 120 *V. vulnificus* and 77 *V. parahaemolyticus* were purified, confirmed via PCR and tested for antimicrobial susceptibility.

Species and virulence identification

Sequence analysis (16S rRNA) of a selected subset of tested *Vibrio* isolates confirmed all isolates (Figure 2), except in the instance of two isolates where sequences were more similar to *Photobacterium damselae*. Due to the fact that *P. damselae* (Daniels & Shafaie 2000) has been indicated in human illness much akin to the infections caused by *V. vulnificus* and *V. parahaemolyticus* (Daniels & Shafaie 2000), these isolates were kept in the study. Virulence testing of all isolates identified three *V. vulnificus* isolates as positive for *vcg*, one *V. parahaemolyticus* isolate for *tdh*, and one isolate for

trh.

Prevalence of antimicrobial resistance in V. vulnificus.

All tested *Vibrio vulnificus* isolates (n=120) were susceptible to 14 of the 26 antibiotics tested, including the following drug classes that are important for the treatment of *Vibrio* infections and antimicrobials recommended by CDC (Centers for Disease Control and Prevention) for the treatment of *V. vulnificus* infections (denoted in Table 4, Figure 3): tetracyclines, quinolones, and folate pathway inhibitors. In regard to CDC recommended antimicrobial agents, 2% of the tested isolates exhibited intermediate resistance against ceftazidime, a 3rd generation cephalosporin. Within the aminoglycoside class of antibiotics, isolates exhibited resistance to apramycin (1%) and streptomycin (4%). Intermediate resistance was expressed against amikacin (1%), apramycin (5%) and streptomycin (8%). Gentamicin was the only tested aminoglycoside to which all *V. vulnificus* isolates were completely susceptible. The aminoglycoside, streptomycin, was associated with the highest percentage of resistance (7% of all tested isolates) and second highest percentage of intermediate resistance (17% of all tested isolates) out of all of the antimicrobials tested. Isolates displayed the highest percentage of intermediate resistance (78% of all isolates) to chloramphenicol.

Antimicrobial resistance in vcg+ V. vulnificus

Of the three isolates positive for the virulence correlated gene (*vcg*), none displayed resistance to any of the tested antibiotics, but all three expressed intermediate resistance (100%) to chloramphenicol.

Prevalence of antimicrobial resistance in V. parahaemolyticus

All tested *Vibrio parahaemolyticus* isolates were susceptible to 11 of the 26 tested

antibiotics and four (carbapenems, tetracyclines, quinolones folate pathway inhibitors) of the eight tested antimicrobial classes (Table 4, Figure 3). Conversely, 96% of isolates had intermediate resistance to chloramphenicol, followed by ampicillin (25%), cephalothin (17%), penicillin (16%) and cefuroxime sodium (14%). A high percentage of isolate resistance was seen within the penicillin class (penicillin (68%); ampicillin (53%)), while a low percentage of resistance was detected in piperacillin (4%) and streptomycin (4%).

Antimicrobial resistance in tdh/trh+ V. parahaemolyticus

One isolate was *tdh*+ and one isolate was *trh*+. Both virulent isolates exhibited multiple resistance and intermediate resistance patterns. The *trh*+ *V. parahaemolyticus* isolate was resistant to ampicillin and penicillin and expressed intermediate resistance to chloramphenicol. The *tdh*+ *V. parahaemolyticus* was resistant to ampicillin, ampicillin-sulbactam, penicillin, piperacillin-tazobactam, and amoxicillin-clavulanic acid and expressed intermediate resistance to chloramphenicol.

Impact of sampling site and month on antimicrobial resistance

Friedman two-way ANOVA

Based on the Friedman two-way ANOVA, there were significant month effects for *V. parahaemolyticus* expressing antibiotic resistance ($P<0.0001$) and intermediate resistance ($P<0.0001$); and for *V. vulnificus* expressing resistance ($P=0.0008$) and intermediate resistance ($P=0.0098$). After adjusting for the repeated measures over time (month), site effects were significant for *V. vulnificus* expressing antibiotic resistance ($P=0.0321$) and intermediate resistance ($P=0.0029$), but not significant for *V. parahaemolyticus* expressing resistance ($P=0.6133$) and intermediate resistance

(*P*=0.7660).

Kruskal-Wallis one-way ANOVA

As there was a significant month effect in the Friedman two-way ANOVA for both *V. vulnificus* and *V. parahaemolyticus* isolates expressing antibiotic resistance and intermediate resistance, stratified Kruskal-Wallis one-way ANOVA and pairwise post-hoc tests were conducted on the site difference for each month separately. Analysis with Kruskal-Wallis showed no significant difference between sites by month for *V. vulnificus* or *V. parahaemolyticus* expressing resistance (July, August, September; (*P*=0.5340, 0.2801, 0.4966); (*P*=0.7246, 0.9448, 0.6809), respectively) or *V. parahaemolyticus* expressing intermediate resistance (*P*=0.5959, 0.8046, 0.2135). After testing *V. vulnificus* expressing intermediate resistance for site differences by each month separately, it was determined that there was a significant site effect only in July (*P*=0.035). Post-hoc testing clarified that the site, St. Martin's River, was different from Sandy Point during the month of July, with reduced intermediate resistance for each *V. vulnificus* isolate recovered from St. Martin's River (*P*=0.0166).

Kruskal-Wallis one-way ANOVA further elucidated that there was no significant difference in the median intermediate resistance or resistance patterns during the sampling period when St. Martin's River (August) (*P*=0.44) had higher levels of bacterial-indicator species.

Clinical V. parahaemolyticus

Clinical isolates tested displayed comparable resistance profiles to environmental isolates tested (Table 4). Environmental isolates demonstrated intermediate resistance and resistance to a greater range of antibiotics (15 antibiotics in 4 classes) when

compared to clinical isolates (5 antibiotics in 3 classes). However, based on analyses with two-sample proportion tests, the overall percentage of resistance and intermediate resistance (%= number of antimicrobials demonstrating resistance/ number of total antimicrobials tested) in clinical isolates was not statistically different (P=0.511, 0.430; respectively) from environmental isolates.

Discussion

Treatability of Chesapeake Bay related Vibrio illness in Maryland

Vibrio vulnificus and *V. parahaemolyticus* are the causative agents for wound infections, primary septicemia, and gastroenteritis related to seafood and seawater exposure (CDC 2012a). While gastroenteritis does not typically necessitate antibiotic treatment, it is required for wound infection and primary septicemia caused by both *Vibrio* species tested in this study. Most isolates tested in this study were susceptible to the antimicrobial agents recommended by the CDC. Treatment recommendations for such infections include tetracyclines (doxycycline, tetracycline), flouroquinolones (ciprofloxacin, levofloxacin), third-generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone), aminoglycosides (amikacin, apramycin, gentamicin, streptomycin) and folate pathway inhibitors (trimethoprim-sulfamethoxazole) (Daniels & Shafaie 2000, CDC 2009). The CDC recommends a treatment course of doxycycline (100 mg PO/IV twice a day for 7-14 days) and a third-generation cephalosporin (e.g.,ceftazidime 1-2 g IV/IM every eight hours), although they state that single agent regimens employing a fluoroquinolone has been reported to be at least as effective in an animal model as combination drug regimens with doxycycline and a cephalosporin (CDC 2009).

Most isolates tested in this study were susceptible to the antimicrobial agents recommended by the CDC. All tested *V. vulnificus* isolates were susceptible to third and fourth generation cephalosporins, although two *V. parahaemolyticus* isolates (3%) demonstrated intermediate resistance to cefotaxime, a 3rd generation cephalosporin and two isolates demonstrated a degree of resistance to cefepime, a 4th generation cephalosporin. While isolate intermediate resistance and resistance was relatively low for

the newer generation cephalosporins, these antibiotics are considered to be some of the best defenses against the dangerous infections that these organisms can elicit (CDC 2009).

Due to the contraindication of doxycycline and fluoroquinolones in children, a combination of trimethoprim-sulfamethoxazole and an aminoglycoside antibiotic is recommended (CDC 2009). Given that three of the four tested aminoglycosides (amikacin, apramycin, streptomycin) were associated with intermediate resistance or resistance (e.g., streptomycin intermediate resistance and resistance in *V. vulnificus*: 17%, 7%, respectively; *V. parahaemolyticus*: 8%, 4%, respectively) in a subset of isolates, this may be a resistance pattern of concern. Conversely, for the aminoglycoside, gentamicin, all tested isolates were fully susceptible. Based on these data, it is feasible that only one aminoglycoside, gentamicin, could be administered with full confidence in its ability to fight *Vibrio* infections contracted by children recreating in the Chesapeake Bay.

While the detection of virulence genes was very low, each gene was present in at least one tested isolate (Table 6A). Resistance patterns among these virulent isolates were similar to the patterns seen in non-virulent isolates. Due to the very limited number of environmental isolates possessing virulence markers, it is not possible to say that most virulent strains would behave as the non-virulent environmental isolates, but the susceptibilities of the clinical and virulent isolates was similar enough to the non-virulent isolates in this study that expecting similar patterns would not be unfounded.

Antimicrobial susceptibility as compared to fecal indicator measurements

A range of *Enterococci* counts were observed over the course of this study, although most studied locations were within the range of acceptable water quality for

recreation on each sampling date. For the sake of this study, it can be gleaned that indicator bacterial water quality was not a major determinant of levels of antibiotic resistance in the studied environments. During the one instance that the geometric mean of *Enterococci* was higher than regulation limits, there was no discernable difference in antimicrobial susceptibility patterns of isolates originating from that site (St. Martin's – August). This is counter to patterns seen in similar studies, during which antimicrobial resistance was elevated at sites contaminated with fecal waste of humans (de Oliveira & Pinhata 2008) and animals (Sapkota et al. 2007).

Comparison to other U.S. studies of V. vulnificus and V. parahaemolyticus antimicrobial susceptibility

Results of this study were comparable to a similar study conducted on *Vibrio* isolated from Gulf Coast oysters in Louisiana (Han et al. 2007). The previous study, conducted in 2005-2006, also found a higher resistance profile in *V. parahaemolyticus* than *V. vulnificus*. In addition, ampicillin was the only tested antimicrobial in the Gulf Coast study to which a large percentage of *V. parahaemolyticus* isolates demonstrated intermediate resistance to resistance (~81% of all tested isolates). This trend was seen as early as the 1970s in a study that tested resistance of *V. parahaemolyticus* to ampicillin and β-lactamase inhibitors (Joseph et al. 1978), where over 90% of isolates were found to be resistant to ampicillin. In contrast to the present study, the Gulf Coast study (Han et al. 2007) found no resistance in either *Vibrio* species to chloramphenicol, cefotaxime, or ceftazidime, while we observed intermediate resistance among a subset of *V. vulnificus* and *V. parahaemolyticus* against these antimicrobial agents (78/96%, 0/3%, 2/0%, (*Vv*/*Vp*), respectively).

Our findings are also in partial agreement with two large studies of *V. vulnificus* and *V. parahaemolyticus* isolates originating from the Georgia and South Carolina coastline of the United States (Baker-Austin et al. 2008, Baker-Austin et al. 2009). While the Chesapeake Bay isolates did not show the same high degree of prevalence of antimicrobial resistance, the antimicrobial agents to which isolates showed resistance were similar (i.e., amoxicillin, apramycin, penicillin and streptomycin for *V. parahaemolyticus*). *Vibrio vulnificus* isolates demonstrated similar resistance profiles, especially in regard to percent intermediate resistant and resistant to the penicillin class and cefoxitin. Baker-Austin et al. (2009) reported much higher percent intermediate resistance and resistance in *V. vulnificus* to apramycin and streptomycin as compared to this study. In addition, key antimicrobials to which *V. parahaemolyticus* isolates from Georgia and South Carolina displayed full susceptibility were also found to have identical susceptibilities in this study (i.e., ceftriaxone, ciprofloxacin, imipenem, ofloxacin, meropenem, tetracycline), except in the case of chloramphenicol, for which no or low (*V. vulnificus*) resistance was observed in the Georgia and South Carolina study. In contrast to this study, Baker-Austin et al. (2009) found only one *V. vulnificus* isolate to be completely susceptible to all antimicrobials tested, while this study found 15 (12.5%) isolates fully susceptible.

Study sites and influences of pollution

Each studied site has a history of water pollution. Sandy Point State Park has historically been the site of low bacteriological water quality and is adjacent to the Magothy River, a site where there have been numerous wastewater treatment overflows. The Pocomoke River is located adjacent to many farming operations, including poultry

concentrated animal feeding operations (CAFOs), which may increase the introduction of antimicrobial residues to the waterway due to runoff of fecal mater contaminated with antimicrobials used in animal husbandry (Campagnolo et al. 2002). Finally, St. Martin's River is adjacent to many homes on septic systems, notorious for leakage (Jones et al. 2004). While each of the studied sites has a history of contamination that may increase the incidence of antimicrobial residues and associated changes in resident bacteria in the estuarine environment, this study only detected a small difference with regard to levels of antibiotic resistance between St. Martin's River and Sandy Point during the month of July in *V. vulnificus* expressing intermediate resistance. These results are unlike the high levels of resistance seen in other studies (Baker-Austin et al. 2008, Baker-Austin et al. 2009), perhaps due to differences in contamination sources (i.e., heavy metal pollution in Baker-Austin et al. (2009)), although a significant difference in antibiotic resistance was not detected between pristine sites and those with heavy metal contamination in that study.

Conclusions

This study represents the first investigation of antimicrobial susceptibility of *Vibrio* species recovered from the Chesapeake Bay and provides a baseline against which future studies can be compared to determine whether susceptibilities change over time. Isolates tested in this study displayed high intermediate resistance to chloramphenicol, when compared to similar studies. Isolate intermediate resistance and resistance to aminoglycosides is of note concerning the treatment of pediatric *Vibrio* illness originating from the Chesapeake waters or seafood. Low-level intermediate resistance and resistance to third and fourth generation cephalosporins is also of interest with regard to treatment

effectiveness and should be monitored. Consensus with previous studies was reached in terms of the prevalence of intermediate resistance and resistance to the penicillin class of antimicrobials. As most of the antimicrobial agents recommended for treatment of *Vibrio* illness by CDC were fully effective against *V. vulnificus* and *V. parahaemolyticus* isolated from the Chesapeake Bay, treating infections contracted from the Bay, at least in adults, should not be problematic. Treatment of pediatric illnesses should gravitate towards the use of trimethoprim-sulfamethoxazole and the aminoglycoside, gentamicin, which was the only aminoglycoside 100% effective against *Vibrios* recovered in this study.

Table 1. PCR conditions for the detection of *V. vulnificus* and *V. parahaemolyticus* virulence genes.

Primer	Primer (forward & reverse)/Probe Concentrations (nM)	PCR conditions
Vibrio vulnificus/vvh	400/240	1x: 95 °C for 60 s; 41x: 95 °C for 5 s, 59 C for 45 s
Vibrio vulnficus/*vcg*	250/180	1x: 95°C for 10 m; 40x: 95ºC for 15 s, 60°C for 90 s
Vibrio parahaemolyticus/tlh	200/150	1x: 95°C for 10 m; 45x: 95ºC for 5 s, 66°C for 45 s
Vibrio parahaemoylticus/*tdh trh*	200/75	1x: 95°C for 60 s; 50x: 95ºC for 5 s, 59°C for 45 s

Table 2. Clinical isolates provided by Maryland Department of Health and Mental Hygiene. Sample type, infection source and associated, if any, antimicrobial resistance.

Clinical isolate	Sample source	Infection source	AST results (# antibiotics) Intermediate resistance; Resistance
1	Stool	Undercooked seafood	ampicillin,penicillin; 0
2	Stool	Undercooked seafood	chloramphenicol; ampicillin, penicillin
3	Stool	No data available	Chloramphenicol, penicillin; 0
4	Stool	Undercooked seafood	Chloramphenicol, apramycin, streptomycin; ampicillin, penicillin
5	Stool	Undercooked seafood	chloramphenicol ; 0
6	Stool	No data available	Chloramphenicol, ampicillin; penicillin
7	No data available	Beach, unknown location	Chloramphenicol, ampicillin, penicillin; 0
8	Wound	No data available	chloramphenicol ; ampicillin, penicillin

Table 3. Physical, chemical and bacterial water quality.

Site	Date	Salinity	Temp. (°C)	pH	Dissolved oxygen (mg L^{-1})	Depth (feet)	Average *Enterococcus* geometric mean CFU 100mL^{-1} (+/- std. dev.)	Average *Vibrio vulnificus* CFU mL^{-1} (+/- std. dev.)	Average *Vibrio parahaemolyticus* CFU mL^{-1} (+/- std. dev.)
Pocomoke	16-Jul 09	10.5	26.1	7.6	n/a	15.6	24 (8)	51 (41)	13 (9)
Pocomoke	18-Aug-09	10.0	28.8	7.4	4.9	14.4	15 (10)	35 (29)	8 (9)
Pocomoke	21-Sep-09	11.1	22.6	7.3	6.3	13.8	38 (6)	52 (40)	9 (10)
Sandy Point	9-Jul-09	8.6	24.5	8.3	7.4	7.6	2 (3)	204 (137)	11 (23)
Sandy Point	3-Aug-09	10.0	26.5	8.0	7.0	7.6	5 (4)	234 (76)	19 (15)
Sandy Point	3-Sep-09	9.6	24.6	7.8	7.1	7.6	2 (3)	294 (71)	18 (11)
St. Martin's	6-Jul-09	24.5	25.9	7.9	6.6	4.4	3 (7)	28 (46)	17 (20)
St. Martin's	9-Aug-09	23.4	26.5	7.8	5.6	4.8	365 (6)	122 (47)	48 (40)
St. Martin's	6-Sep-09	25.5	23.1	7.5	2.9	4.9	3 (5)	32 (24)	12 (12)

Table 4. Antimicrobial intermediate resistance and resistance, for respective number and percent, denoted for antibiotic class and specific antibiotic.

	Penicillins and β-Lactam/β-Lactamase Inhibitor Combinations						Cephems							Carba-penems		Aminoglycosides				Tetra-cyclines		Quinolones			Other	Folate Pathway Inhibitors
	Ampicillin (≥32)	**Amoxicillin-clavulanic acid (≥32/16)**	**Ampicillin-sulbactam (≥32/64)**	**Penicillin (≥64)**	**Piperacillin (≥128)**	**Piperacillin-tazobactam (≥128/4)**	**Cefepime (≥32)**	**Cefotaxime** (≥64)**	**Cefoxitin (≥32)**	**Ceftazidime** (≥32)**	**Ceftriaxone** (≥64)**	**Cefuroxime sodium (≥32)**	**Cephalothin (≥32)**	**Imipenem (≥16)**	**Meropenem (≥16)**	**Amikacin (≥64)****	**Apramycin (≥64)****	**Gentamicin (≥16)****	**Streptomycin (≥64)****	**Doxycycline** (≥16)**	**Tetracycline** (≥16)**	**Ciprofloxacin** (≥4)**	**Levofloxacin** (≥8)**	**Oflaxacin (≥8)**	**Chloramphenicol (≥32)**	**Trimethoprim-sulfamethoxazole** (≥4/76)**
V. vulnificus																										
# SUSCEPTIBLE	115	118	120	116	119	118	120	120	108	118	119	120	114	118	120	117	106	120	90	120	119	120	120	120	26	120
# INTERMEDIATE	3	0	0	0	1	0	0	0	6	2	0	0	4	0	0	3	9	0	20	0	0	0	0	0	94	0
# RESISTANT	1	0	0	4	0	1	0	0	5	0	0	0	2	1	0	0	5	0	8	0	0	0	0	0	0	0
% SUSCEPTIBLE	97	100	100	97	99	99	100	100	91	98	100	100	95	99	100	98	88	100	76	100	100	100	100	100	22	100
% INTERMEDIATE	**3**	0	0	0	**1**	0	0	0	**5**	**2**	0	0	**3**	0	0	**3**	**8**	0	**17**	0	0	0	0	0	**78**	0
% RESISTANT	**1**	0	0	**3**	0	**1**	0	0	**4**	0	0	0	**2**	**1**	0	0	**4**	0	**7**	0	0	0	0	0	0	0
V. parahaemolyticus																										
# SUSCEPTIBLE	17	76	76	13	71	76	73	75	74	77	77	65	63	77	77	76	72	76	68	77	77	77	77	77	3	77
# INTERMEDIATE	19	1	0	12	1	0	1	2	3	0	0	11	13	0	0	1	4	0	6	0	0	0	0	0	74	0
# RESISTANT	40	0	1	52	3	1	1	0	0	0	0	1	1	0	0	0	1	0	3	0	0	0	0	0	0	0
% SUSCEPTIBLE	22	99	99	17	95	99	97	97	96	100	100	84	82	100	100	99	94	100	88	100	100	100	100	100	4	100
% INTERMEDIATE	**25**	**1**	0	**16**	**1**	0	**1**	**3**	**4**	0	0	**14**	**17**	0	0	**1**	**5**	0	**8**	0	0	0	0	0	**96**	0
% RESISTANT	**53**	0	**1**	**68**	**4**	**1**	**1**	0	0	0	0	**1**	**1**	0	0	0	**1**	0	**4**	0	0	0	0	0	**0**	0

**CDC recommended

Table 5. Comparison of environmental and clinical isolates and their respective associated antimicrobial resistance to a subset of antibiotics to which highest resistance within tested isolates was displayed.

	Environmental Isolates		Clinical Isolates	
Antibiotic	Intermediate n (%)	Resistant n (%)	Intermediate n (%)	Resistant n (%)
Ampicillin	19 (25)	40 (53)	1 (12.5)	2 (25)
Apramycin	4 (5)	1 (1)	1 (12.5)	0 (0)
Streptomycin	6 (8)	3 (4)	1 (12.5)	0 (0)
Chloramphenicol	74 (96)	0 (0)	7 (87.5)	0 (0)
Penicillin	12 (16)	52 (68)	3 (37.5)	4 (50)

Table 6. Antibiotic resistance (AR) and multiple antibiotic resistance (MAR) by virulence factors (6A), site (6B) and month (6C).

6A.

	Vv *vcg*+ (n=3)		Vv *vcg* – (n=117)		Vp *tdh*+ (n=1)		Vp *trh*+ (n=1)		Vp *tdh*/*trh*- (n=75)	
	AR	MAR	AR	MAR	AR	MAR	AR	MAR	AR	MAR
Resistant	0 (0%)	0 (0%)	21 (18%)	5 (4%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	51 (68%)	39 (52%)
Intermediate resistance	3 (100%)	0 (0%)	101 (86%)	29 (25%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	74 (99%)	44 (59%)

6B.

	Pocomoke (n=44)		St. Martin's (n=11)		Sandy Point (n=65)	
V. vulnificus	AR	MAR	AR	MAR	AR	MAR
Resistant	10 (23%)	3 (7%)	0 (0%)	0 (0%)	12 (18%)	2 (3%)
Intermediate resistance	42 (95%)	11 (25%)	6 (55%)	0 (0%)	58 (89%)	18 (28%)
	(n=14)		(n=29)		(n=34)	
V. parahaemolyticus	AR	MAR	AR	MAR	AR	MAR
Resistant	10 (71%)	7 (50%)	22 (76%)	17 (59%)	22 (65%)	17 (50%)
Intermediate resistance	14 (100%)	8 (57%)	28 (97%)	15 (52%)	34 (100%)	23 (68%)

6C.

	July (n=40)		August (n=47)		September (n=33)	
V. vulnificus	AR	MAR	AR	MAR	AR	MAR
Resistant	3 (8%)	0 (0%)	13 (28%)	4 (9%)	6 (18%)	1 (3%)
Intermediate resistance	32 (80%)	4 (10%)	42 (89%)	16 (34%)	30 (91%)	9 (27%)
	(n=11)		(n=40)		(n=26)	
V. parahaemolyticus	AR	MAR	AR	MAR	AR	MAR
Resistant	9 (82%)	8 (73%)	31 (78%)	22 (55%)	14 (54%)	10 (38%)
Intermediate resistance	11 (100%)	7 (64%)	39 (98%)	24 (60%)	26 (100%)	14 (54%)

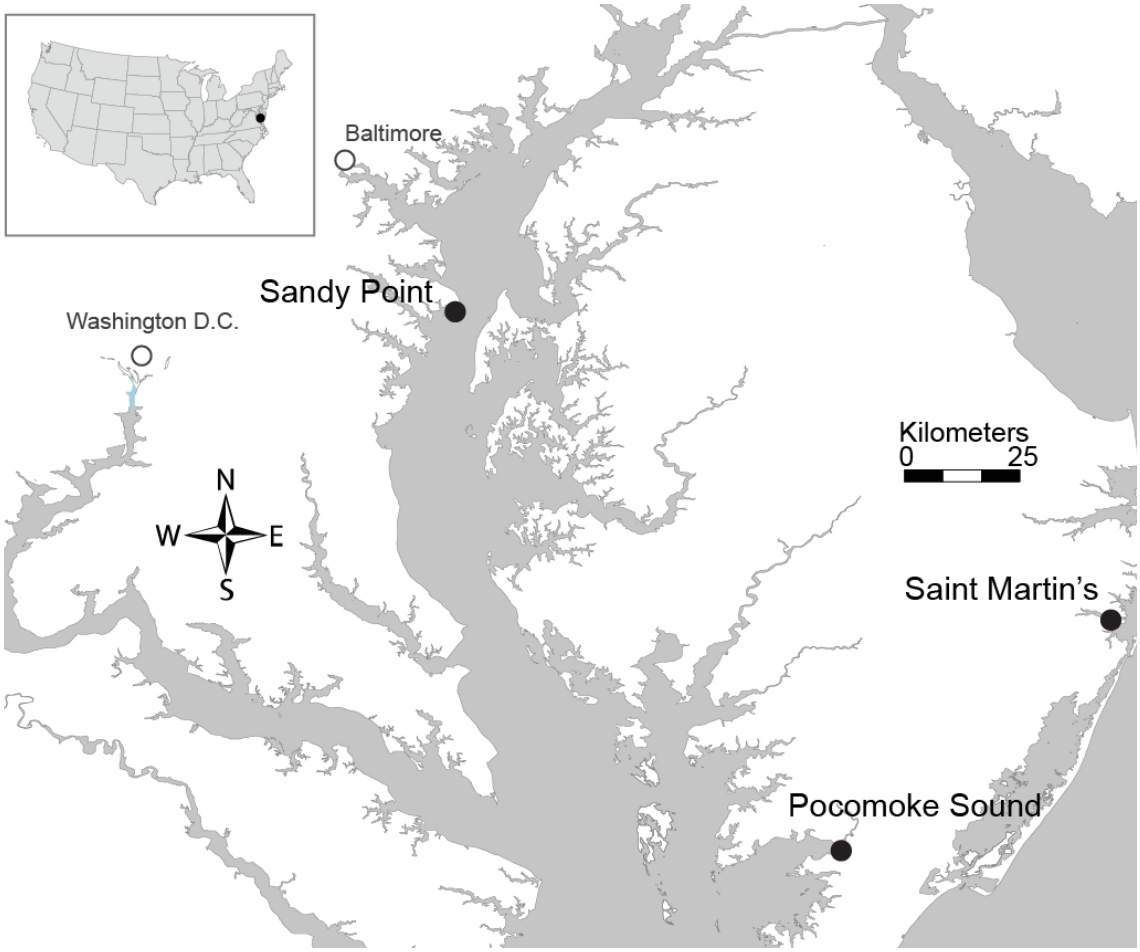


Figure 1. Sampling sites. (Tracey Saxby, Kate Boicourt, Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/ displayimage-127-5815.html)

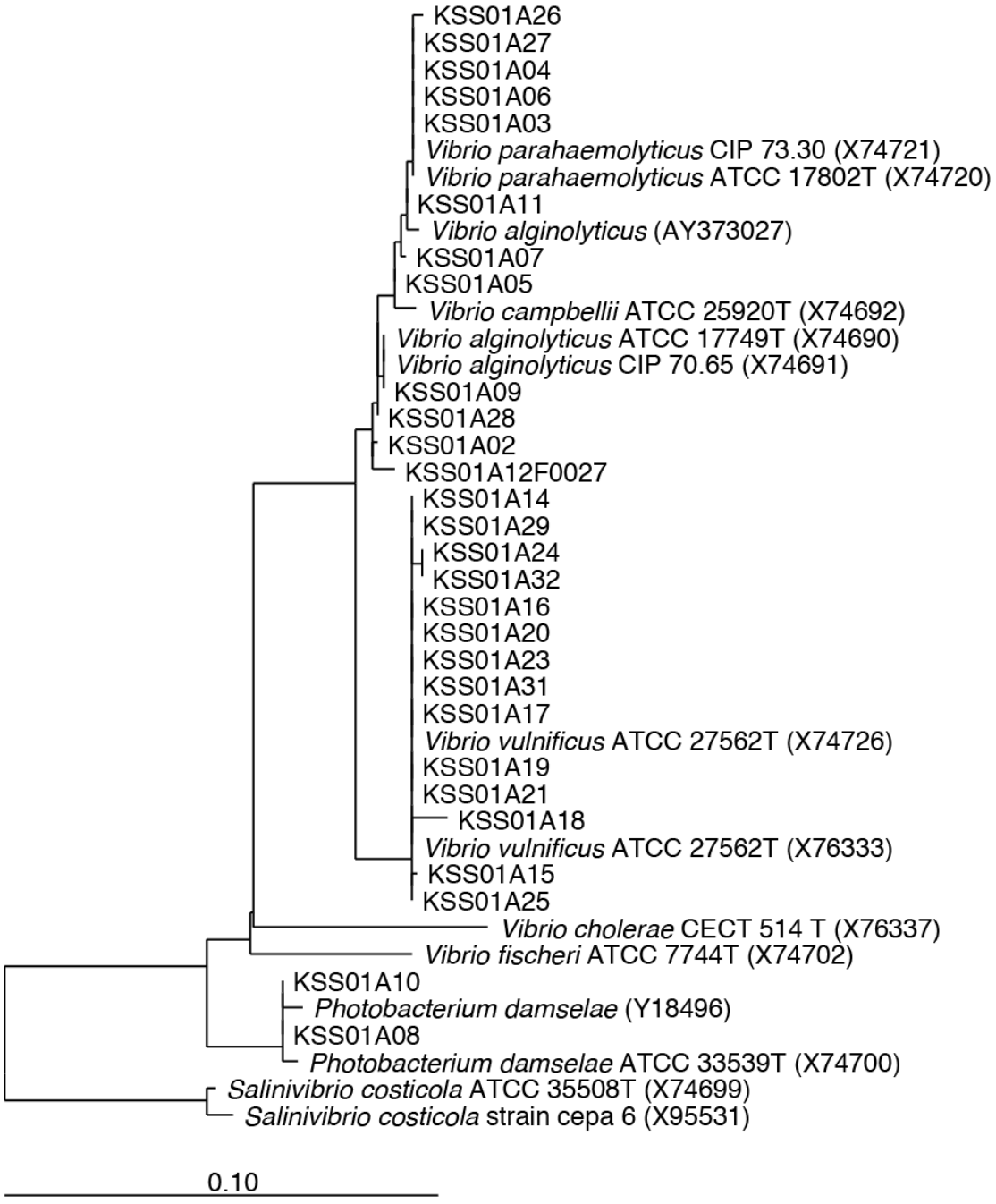


Figure 2. 16S rRNA sequencing analysis of a subset of *Vibrio* isolates tested.

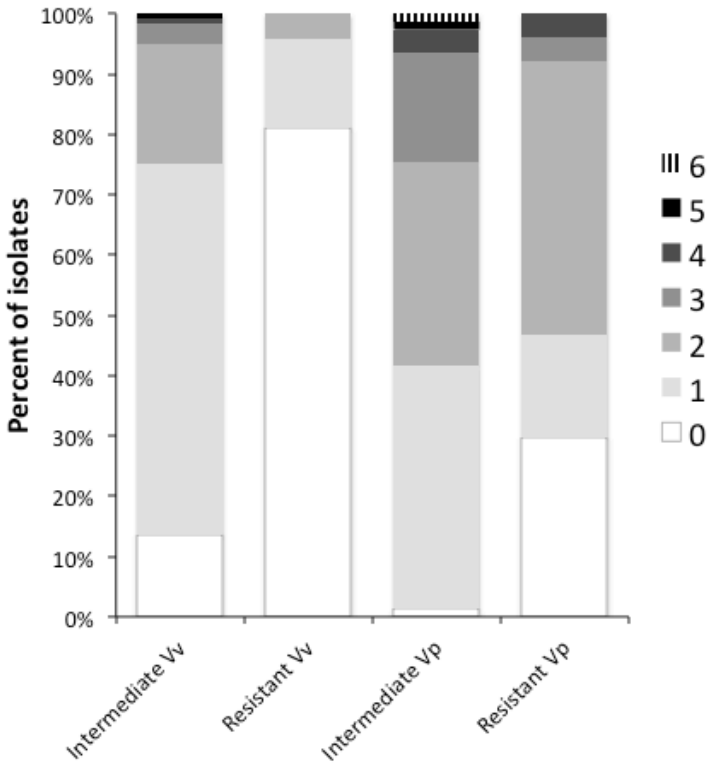


Figure 3. Number of antibiotics against which *Vibrio* isolates expressed resistance or intermediate resistance.

CHAPTER 5: CONCLUSIONS

This dissertation work was designed to answer pressing questions concerning potentially pathogenic *Vibrio* species of bacteria in the Chesapeake Bay. Recently, public health managers in the Chesapeake Bay region have been armed with models for predicting surface water concentrations of *V. vulnificus* and *V. parahaemolyticus*, but it is difficult to know how, or if, that information should translate to policy changes in closures of beaches or shellfish harvest areas in the Chesapeake Bay. Additionally, it has been proposed to make these predictions available to the general public, to interpret the data based on their own depth of knowledge. These interpretations carry great risk not only for the public's perception of the safety of their environment but also for the associated economic risk if people improperly interpret predictions to mean that water and seafood products are unsafe. It would be irresponsible to share these data without the appropriate framework in which to approximate relative risk of infection. As similar predictive models are developed for other estuaries and coastal regions, it will not be surprising to find regional managers and scientists faced with this same problem regarding translation of predictive models. Portions of this dissertation research were undertaken to begin to answer the basic question: "What do the predictions of *Vibrio* abundance mean for human health?"

The first goal of this dissertation was to quantify exposure of humans to potentially pathogenic *Vibrio* cells when swimming. Exposure assessment swim studies were undertaken to calculate the first estimates of how many *Vibrio* bacterial cells a person may come into contact with and subsequently contract while recreating in the Chesapeake. Results were clear that during the months when surface water is host to an elevated abundance of *Vibrio* cells, a person recreating or working in those waters could

expect a significant dermal exposure. If that user has any abrasions or wounds, it is more possible that they are exposed to a number of potentially pathogenic cells. The dose of *V. vulnificus* or *V. parahaemolyticus* needed to cause an infection in a healthy, moreover an immunocompromised, individual is poorly understood, particularly for non-consumption exposure. Future research will aim to take these first exposure estimates and interpret them in terms of a quantitative microbial risk assessment. Additionally, these estimations will be paired with calculations of climate change related increases in surface water concentrations of *Vibrio*.

The second goal of this dissertation was to determine if storm events, especially those causing wind-driven resuspension of sediments, are important mechanisms of *Vibrio* introduction to the water column and to aquacultured oysters. Because the increase in aquacultured oyster operations will likely result in a larger summer harvest of oysters, which are typically consumed raw on the half shell, understanding how oyster concentrations of *Vibrio* might be impacted is vital. One relevant question posed by the aquaculture community is whether *Vibrio* concentrations in oysters differ based on their position in the water column. This study found no difference in *Vibrio* concentrations between oysters collected from the bottom and the top of the water column. The sampling location may have been too shallow to see a true difference in surface versus bottom culture, but since much of the Chesapeake is shallow, and most aquaculture operations will likely be near-shore, this was probably a good indicator site.

Our study identified two storm-induced events – wind-driven resuspension and flushing due to heavy precipitation. While the wind likely drove *Vibrio* into the water column from the sediment, and into contact with oysters, the subsequent rain event likely

caused a flushing effect, which may have diluted *Vibrio* cell concentrations at the aquaculture site. An inverse relationship between in-oyster concentrations and surface-water concentrations of *Vibrio* was observed, suggesting a dynamic relationship between oysters and *Vibrio* in the water column of an aquaculture facility in which rates of oyster filtration alter the water column concentration of *Vibrio*. The pattern observed was potentially caused by changes in the rate of oyster filtration during and directly after the storm, when suspended solids were likely high and oysters likely slowed or ceased filtration for a period of time. This reaction to high concentrations of suspended solids may prevent increases of in-oyster *Vibrio*, but this research should be repeated during more storm events to verify that prediction.

Although not statistically significant due to the Most Probable Number variability between samples, an increase was detected in in-oyster concentrations of *V. parahaemolyticus* directly after the storm, but not of *V. vulnificus*. At the end of the study, in-oyster concentrations were approximately similar to the pre-storm concentrations, suggesting a possible "ambient" concentration of summer *Vibrio* density. Further testing should be conducted to determine if these levels vary based on site, position in the water column, and after storm impacts. Ideally, further research would have the opportunity to sample before and after separate wind and precipitation events. Initially, this work was proposed for a full summer storm-period, with multiple events, but 2011 was a very quiet year in term of summer squalls. On the spectrum of storm intensity, the study fell on the high end of impacts with Hurricane Irene. Further inquiries should be repeated with measurements along a spectrum of storm intensity, not just the most intense.

At this time, it is inconclusive whether a storm event should trigger closure of a shellfish fishery. However, concentrations of *Vibrio* in oysters were very high throughout the study and the percentage of samples that were positive for virulent *V. vulnificus* and *V. parahaemolyticus* increased after the storm. Changes of in-oyster virulence is another area where further research would be beneficial in determining the public health risk of oyster consumption after a storm event. From the results of this study, it can be concluded that sampling sediment and surface water for general concentrations of *Vibrio,* or pathogenic subspecies, may not be enough to predict the concentrations in oysters. Additionally, climate change estimates of increases in surface water temperature, changes in salinity and intensity or frequency of storm events may also drive changes to shellfish management practices.

Finally, this research addressed the ability to treat *Vibrio* infections contracted from exposure with Chesapeake Bay waters or seafood products. Environmental isolates from three areas known for use by recreationists and commercial fishermen were tested for their susceptibility to a wide range of antibiotic agents. Antibiotics were chosen not just for their clinical importance (i.e., CDC recommended antibiotics), but also to compare resistance patterns in relation to other studies conducted on *Vibrio* in other geographical areas of the United States. Overall, *V. parahaemolyticus* isolates from the Chesapeake Bay displayed more intermediate resistance and resistance to tested antimicrobial agents than *V. vulnificus*. Since approximately 34% of *V. parahaemolyticus* are wound infections (Daniels et al. 2000), this may be more troubling than if cases simply resulted in self-resolving gastroenteritis, due to the deleterious nature of such infections. Luckily, most CDC recommended antibiotic treatments for *Vibrio*

*i*llness were effective in controlling growth of these bacteria, although there was some low-level intermediate resistance to 3rd and 4th generation cephems and moderate intermediate resistance and resistance to three of the four tested aminoglycosides. Chesapeake Bay isolates expressed high-level intermediate resistance to chloramphenicol, unlike the findings from other sites within the United States. Resistance patterns were not related to site contamination, as measured by fecal indicator bacteria, and only one site, St. Martin's, had lower *V. vulnificus* intermediate resistance, as compared to Pocomoke Sound and Sandy Point, during the month of July. Overall, most antibiotics recommended by CDC would be expected to control *Vibrio* infection, but clinicians may need to consider gentamicin as the only aminoglycoside that was 100% effective against controlling *Vibrio* growth. Such information should be taken into consideration when treating pediatric patients, for whom a combination treatment of trimethoprim-sulfamethoxzaole and an aminoglycoside is recommended.

In summary, the culmination of this work begins to answer some of the questions that have recently been asked by clinicians, research scientists and public health managers in the Chesapeake Bay region concerning pathogenic *Vibrio*. When exposed to typical summer season surface water concentrations of *V. vulnificus* and *V. parahaemolyticus*, an exposure should be expected. Storm-related changes in the aquatic environment will change the density of *Vibrio* in surface waters, but possibly not in simple increases and decreases of *Vibrio* concentrations. Finally, if a patient contracts a *Vibrio* illness from the Chesapeake Bay, it is likely that it can be controlled with recommended antimicrobial treatment regimes, if the clinician is properly informed and diagnosis is accurate. Clinicians treating patients in the Chesapeake Bay region should

be well-informed about the symptoms, proper diagnosis and treatment of *Vibrio* infections. Although these results are not the end-all conclusions needed to inform managers about decisions to take preventative action to control *Vibrio* illness, these data sets serve as useful starting points to direct the fine-tuning of questions and future research projects.

APPENDIX

Appendix: Microcosm Sediment Resuspension Experiment

Specific Aims

Laboratory based microcosm experiments were conducted to mimic a resuspension of sediment event, with two levels of resuspension (low, high) used to determine the approximate increase of *V. vulnificus* and *V. parahaemolyticus* in oysters following exposure to suspended sediment.

Methods

Three treatment levels of control (no sediment resuspension), low, and high resuspension were used to mimic the field environment. Three tanks (10 gallon) were used for each treatment. On October 10, 2011, a surface layer sediment sample was retrieved from the same area of the aquaculture site that was used for the environmental resuspension experiment. Sediment (550 g) was spread evenly on the bottom of each tank and ambient Choptank River water was slowly poured over sediment to minimize resuspension into water. Tanks were allowed to settle for 24 h in a climate controlled chamber heated to 32°C. Oxygen air lines were run to each tank, with an air stone anchoring the lines in the tank, above the sediment layer, and air was not turned on until the experiments were commenced to allow for full particle settlement in tanks prior to experiments. Chamber lights were used only during sample collection.

On the morning of October 11, 2011 oysters were collected from the aquaculture site and placed on the bottom of the tank (36 oysters per tank) after 24 h. An initial representative oyster sample (homogenate of 6 oysters, as described in Chapter 3 Methods) was taken at this time to determine background *Vibrio* level in oysters.

Tank sediment was resuspended for 2 hours per treatment, excluding control tanks. Resuspension was achieved by placing tanks on wooden frames custom built to support tanks above stir plates. A large magnetic stir bar was added to each tank and stirring activity was set to achieve the desired level of relative resuspension (low and high).

Water, total suspended solids (TSS), and oyster samples were taken at each sampling time point (before sediment suspension (T0), directly after suspension (T1), 24h (T2), 72h (T3), 7d (T4)) from each tank. Water samples were collected by filtering 180 mL of tank water and analyzed using qPCR as described in Chapter 3. Water for TSS (50 mL) was collected. Oysters were sampled (6 oysters per sample, per tank) and analyzed using MPN-PCR methods as described in Chapter 3.

Results

Average (± standard error) TSS for treatments were 227 (±5.43) mg L^{-1} (high) and 57 (± 10.6) mg L^{-1} (low) directly following resuspension treatments (Table 1). Treatments, including controls, had appreciably the same TSS averages at subsequent sampling time points.

Oyster MPN g^{-1} *V. parahaemolyticus* increased from pre-treatment average of 46,798 to 466,140 (high),105,533 (low) and 496,597 (control) at T1 (Table 2). At T2, *V. parahaemolyticus* decreased to 77,373 (high), 22, 766 (low) and 147,588 (control) MPN g^{-1}. High and control treatments reduced further at T3 (6,533 and 72,333, respectively), while the low treatment increased to 47,733 MPN g^{-1}. At T4, all treatments had reduced to 3,000-5,000 MPN g^{-1}. Water concentrations of V. parahaemolyticus were highest in control tanks (160 CFU mL^{-1}) at T1, while the resuspension treatments averaged 111 (high) and 47 (low) CFU mL^{-1}. All tanks had approximately the same CFU mL^{-1} *V.*

parahaemolyticus at T2-T4. Due to control *V. parahaemolyticus* MPN g^{-1} and CFU mL^{-1} being appreciably higher in concentration than the "high" treatment, this experiment was deemed unsuccessful.

Oyster MPN g^{-1} *V. vulnificus* increased from pre-treatment average of 459,052 to 1,002,621 (high),1,148,601 (low) and 834,673 (control) at T1 (Table 3). At T2, *V. vulnificus* decreased to 834,674 (high and low) and increased in the control treatment to 1,148,600 MPN g^{-1}. High treatments reduced further at T3 (459,053), while the low and control treatments increased (low) or plateaued (control)to 1,148,600 MPN g^{-1}. At T4, high treatments had increased to 1,148,600 MPN g^{-1}, but decreased in low treatments (834,674 MPN g^{-1}) . Water concentrations of V. *vulnificus* were highest in low treatment tanks (32,210 CFU mL^{-1}) at T1, followed by the control and high resuspension treatments (6,843 (control), 1,310 (high) CFU mL^{-1}). All tanks had approximately the same CFU mL^{-1} *V. vulnificus* at T2-T3, with a slight increase at T4 (5,000-8,000 CFU mL^{-1}. Due to control *V. vulnificus* MPN g^{-1} and CFU mL^{-1} being appreciably the same concentration as high and low treatments, this experiment was deemed unsuccessful.

Table 1. Average Total Suspended Solids (mg L^{-1})

Sample Date	Treatment 1 - High	Treatment 2 - Low	Control
T0 (11 OCT 11)	10	10	10
T1 (11 OCT 11)	227	57	19
T2 (12 OCT 11)	10	6	6
T3 (14 OCT 11)	6	5	4
T4 (17 OCT 11)	4	4	5

Table 2. Oyster MPN and water CFU mL^{-1} *V. parahaemolyticus.*

	Oysters			Water		
Sample Date	**Treatment 1 - High (MPN g^{-1})**	**Treatment 2 - Low (MPN g^{-1})**	**Control (MPN g^{-1})**	**Treatment 1 - High (CFU mL^{-1})**	**Treatment 2 - Low (CFU mL^{-1})**	**Control (CFU mL^{-1})**
T0 (11 OCT 11)	-	-	46,798	-	-	-
T1 (11 OCT 11)	466,140	105,533	496,597	111	47	160
T2 (12 OCT 11)	77,373	22,766	147,588	35	39	17
T3 (14 OCT 11)	6,533	47,733	72,333	6	5	7
T4 (17 OCT 11)	3,000	5,066	5,066	5	4	2

Table 3. Oyster MPN and water CFU mL^{-1} *V. vulnificus.*

	Oysters			Water		
Sample Date	**Treatment 1 - High (MPN g^{-1})**	**Treatment 2 - Low (MPN g^{-1})**	**Control (MPN g^{-1})**	**Treatment 1 - High (CFU mL^{-1})**	**Treatment 2 - Low (CFU mL^{-1})**	**Control (CFU mL^{-1})**
T0 (11 OCT 11)	-	-	459,052	-	-	-
T1 (11 OCT 11)	1,002,621	1,148,601	834,673	1,310	32,210	6,843
T2 (12 OCT 11)	834,674	834,674	1,148,600	1,774	2,474	1,488
T3 (14 OCT 11)	459,053	1,148,600	1,148,600	2,082	1,401	704
T4 (17 OCT 11)	1,148,600	834,674	1,148,600	5,164	8,473	7,042

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