ABSTRACT

Title of Dissertation:	**INVESTIGATING LONG-TERM TRENDS IN VIBRIOSIS SEVERITY AND RISK ASSOCIATED WITH WATER EXPOSURE AND CLIMATE-INDUCED STRESSORS IN THE CHESAPEAKE BAY: A MIXED METHODS STUDY**
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The number of outbreaks and sporadic illnesses associated with non-cholera *Vibrio* spp. contaminated seafood and coastal water sources have been increasingly reported across the United States and many other nations, and may continue to rise as a result of ocean warming, adversely impacting public health. However, there are limited data concerning the trends in virulence and antibiotic resistance of these bacterial pathogens outside of Southern U.S. waters, including critical estuarine systems such as the Chesapeake Bay. Moreover, there are few studies that have evaluated longitudinal trends in *Vibrio* illness (vibriosis) among water users (recreational and commercial) and shellfish consumers in more northern states such as Maryland. To address these knowledge gaps my dissertation research involved 1) a retrospective longitudinal molecular analysis, as well as antimicrobial susceptibility testing, to evaluate changes in *V. vulnificus* and *V. parahaemolyticus* antibiotic resistance patterns and virulence factors; and 2) an epidemiological study using publicly-available data to evaluate vibriosis

trends. My specific aims were as follows: 1) To evaluate changes in virulence factors among *V. vulnificus* and *V. parahaemolyticus* isolates recovered from the Chesapeake Bay between 2009-2022; 2) To evaluate changes to antibiotic susceptibility among *V. vulnificus* and *V. parahaemolyticus* isolates recovered from the Chesapeake Bay between 2009-2022; and 3) To evaluate long-term trends in the incidence of severe vibriosis among water users (recreational and commercial) and shellfish consumers in Maryland between 2006-2019. Overall, my results indicate that potentially pathogenic *V. vulnificus* and *V. parahaemolyticus* occur across the Chesapeake Bay throughout every season, including at frequencies that may still lead to an increased risk of infection in the fall and possibly early winter. Moreover, the mid and lower sections of the Bay, which include many commercial and recreationally important areas, may harbor a greater risk of severe vibriosis from wound-associated water exposures, given the greater presence of *V. vulnificus* isolates characterized by clinically-relevant virulence factors. Interestingly, I also found that antibiotic resistance patterns among *V. vulnificus* and *V. parahaemolyticus* isolates collected from Tangier Sound in the lower Chesapeake Bay have remained relatively stable since 2009. Notwithstanding, recovered *Vibrio* spp. isolates exhibited varying levels of resistance and intermediate-resistance to antibiotics used to treat severe vibriosis, underscoring the need for prompt diagnosis and treatment with effective first line antibiotic agents. Finally, my epidemiological analysis revealed that long-term increases in *Vibrio* infections, notably *V. vulnificus* wound infections, are occurring in Maryland. This trend, along with increased rates in hospitalizations and average hospital durations, underscore the need to improve public awareness, water monitoring, post-harvest seafood interventions, and environmental forecasting, particularly as our climate warms and creates environmental conditions that support the growth of estuarine vibrios.

INVESTIGATING LONG-TERM TRENDS IN VIBRIOSIS SEVERITY AND RISK ASSOCIATED WITH WATER EXPOSURE AND CLIMATE-INDUCED STRESSORS IN THE CHESAPEAKE BAY: A MIXED METHODS STUDY

by

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Dedication

I dedicate this dissertation to my curious, talented, and amazing daughter Miriam Eliana M. Zarikian.

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List of Abbreviations

AK30	Amikacin, 30µg
AMP10	Ampicillin, 10µg
ANOVA	Analysis of Variance
CAZ30	Ceftazidime, 30µg
CDC	Centers for Disease Control and Prevention
CFR	Case Fatality Rate
CI	Confidence Interval
CIP5	Ciprofloxacin, 5µg
COVIS	Cholera and Other Vibrio Illness Surveillance
CTX30	Cefotaxime, 30µg
DNR	Maryland Department of Natural Resources
FDA	U.S. Food and Drug Administration
FoodNet	Foodborne Diseases Active Surveillance Network
IPM10	Imipenem, 10µg
MD	Maryland
MDE	Maryland Department of the Environment
MDH	Maryland Department of Health
NCCOS	National Centers for Coastal Ocean Science
NSSP	National Shellfish Sanitation Program
SST	Sea Surface Temperature
SXT25	Sulfamethoxazole-trimethoprim, 25µg
TE30	Tetracycline, 30µg

U.S.	United States
VBNC	Viable but Nonculturable

Chapter 1: Introduction

Outbreaks associated with non-cholera *Vibrio* spp. contaminated seafood have been increasingly reported across, with increases greater than 30%, the United States (Baker-Austin et al., 2018; CDC, 2011; Potasman et al., 2002) and may continue to grow under a warming ocean, taxing our health care system. *Vibrio* spp. bacteria are especially concerning since they are not always associated with fecal contamination but occur naturally in marine and estuarine environments, flourishing in warm coastal waters (Hlady, 1997; Iwamoto et al., 2010; Kaspar & Tamplin, 1993; Kelly, 1982). Furthermore, recent studies have described an alarming trend in cases of *Vibrio* related illness (vibriosis) during the past two decades, with increases greater than 30% in annual incidence per 100,000 (A. Newton et al., 2012; Sims et al., 2011; Vugia et al., 2013). In fact, the Centers for Disease Control and Prevention's (CDC) Foodborne Diseases Active Surveillance Network (FoodNet) reported an increase in incidence from 0.24 to 0.71 per 100,000 population, between 1998 and 2018 (CDC, 2021). Although more than half of all cases of vibriosis can be attributed to the ingestion of raw oysters contaminated with *Vibrio parahaemolyticus*, severe and fatal cases are more prevalent with *Vibrio vulnificus* infection and most likely due to water exposure (Baker-Austin & Oliver, 2018; Oliver, 2013; Shapiro et al., 1998).

Overall, the incidence of vibriosis is a complex issue that depends on many factors that behave at different scales; these include local scales such as consumer and recreational user behavior, access to quality shellfish, pre-harvest and post-production conditions, and more global effects like ocean warming. Particularly, recent works

indicate that pathogenic *Vibrio* spp. have increased in dominance in higher latitude sites like the North and Baltic Seas (Baker-Austin et al., 2013; Brehm et al., 2021; Fleischmann et al., 2022; Sterk et al., 2015; Vezzulli et al., 2012, 2013, 2015). Similarly, a study by DeLuca et al. (2020), applied environmental remote sensing techniques to show that *V. parahaemolyticus* has become more prevalent in the Chesapeake Bay, the largest estuary in North America, over the past decade. This study also suggested that the levels of *V. parahaemolyticus* in the water column are not only associated with sea surface temperature (SST) but also influenced by salinity, suspended solids, and chlorophyll-a concentrations. Moreover, a recent investigation in the Chesapeake Bay reported a long-term increase and extended seasonality of environmental and potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* strains (Brumfield et al., 2023). However, it is not entirely clear if pathogenic strains present in these waters have become more virulent over time, and how this translates to vibriosis risk. Specifically, there have been few studies devoted to the association between climate-induced changes to physico-chemical water parameters and the incidence and severity of vibriosis through water use in the Chesapeake Bay.

Although these knowledge gaps have begun to be addressed, there is still limited information concerning the pathogenicity of *Vibrio* species in the Chesapeake Bay in the context of ocean warming and climate-induced stressors, and how this translates to seasonal risk, especially with recreational and commercial water use. As a result, the overarching goal of my dissertation was to understand how the risk and severity of vibriosis associated with recreational and commercial water use in the

Chesapeake Bay has changed over time with climate induced-changes in the environment, and how to better protect the public from this potential threat. My specific goal was to utilize a mixed methods approach to address the following aims:

1. To evaluate changes in virulence factors among *V. parahaemolyticus* and *V. vulnificus* isolates recovered from the Chesapeake Bay between 2009-2022.
2. To evaluate changes to antibiotic susceptibility among *V. parahaemolyticus* and *V. vulnificus* isolates recovered from the Chesapeake Bay between 2009-2022.
3. To evaluate long-term trends in the incidence of severe vibriosis among water users (recreational and commercial) and shellfish consumers in Maryland between 2006-2019.

Each of the above-mentioned research aims is addressed in a separate manuscript included in this document, and the overall dissertation consists of six chapters that are described below.

Chapter 2 provides background information on vibriosis related gastrointestinal disease and wound infection, vibriosis surveillance systems, *Vibrio parahaemolyticus* and *Vibrio vulnificus* virulence factors and antibiotic drug resistance characteristics, and *Vibrio* spp. trends with climate change.

Chapter 3 is a manuscript, entitled “Long-term analysis of *Vibrio vulnificus* and *Vibrio parahaemolyticus* virulence factors and environmental associations in the Chesapeake Bay, Maryland, U.S.” that applies a retrospective longitudinal molecular

analysis using culture-based PCR methods to evaluate changes in *V. vulnificus* and *V. parahaemolyticus* virulence factors.

Chapter 4 is a manuscript, entitled "Long-term antibiotic resistance trends among *Vibrio vulnificus* and *Vibrio parahaemolyticus* isolated from the Chesapeake Bay, Maryland: A longitudinal study" that applies a retrospective longitudinal molecular analysis using culture-based PCR methods as well as antimicrobial susceptibility testing to evaluate changes in *V. vulnificus* and *V. parahaemolyticus* antibiotic resistance.

Chapter 5 is a manuscript, entitled "Vibriosis trends in Maryland, U.S., 2006-2019: Increased incidence of water-associated wound infections and hospitalization risk" that uses an epidemiological approach and logistic regression models to estimate the likelihood of hospitalization and vibriosis incidence rate trends.

Chapter 6 provides conclusions of my work, strengths and limitations of all three studies, a discussion of the public health significance of my findings, and future research directions.

Chapter 2: Background

Vibriosis

Vibrio Overview

Vibrio species (spp.) are widespread Gram-negative rod-shaped and facultative anaerobic bacteria that naturally occur in aquatic environments, including estuarine and marine ecosystems (Drake et al., 2007; Morris & Black, 1985; Thompson et al., 2004). There are currently more than 110 described *Vibrio* spp. but only a few are significant pathogens to humans (**Table 1**) and aquatic organisms (Baker-Austin et al., 2018; Grimes, 2020; Morris & Black, 1985). They are considered halophilic and with the exception of *Vibrio cholerae* and *Vibrio mimicus*, require sodium chloride supplementation for growth (Kaspar & Tamplin, 1993; Singleton et al., 1982). Vibrios are abundant in the sediment and water column and are known to attach to the surface of zooplankton crustaceans, i.e., copepods, which serve as a nutrient source, help facilitate DNA exchange, and act as vectors of disease transmission (Erken et al., 2015; Heidelberg et al., 2002; Huq et al., 1983; Pruzzo et al., 2008; Vezzulli et al., 2010). Important associations have also been found with algae (Rizzo et al., 2016), seagrass (Chase et al., 2015), sponges (Costa et al., 2021), corals (Bourne & Munn, 2005), mollusks (Destoumieux-Garzón et al., 2020; Mandel & Dunn, 2016), crustaceans (Caburlotto et al., 2016; Rodgers et al., 2014), fish (Halpern & Izhaki, 2017; Senderovich et al., 2010), and aquatic birds (Cardoso et al., 2018; Fernández-Delgado et al., 2016; Fu et al., 2019; Laviad-Shitrit et al., 2018, 2019). Indeed, these interactions may constitute an important survival strategy of

vibrios, allowing them to adapt to fluctuations in nutrient availability, resist antibiotics, evade predators, and tolerate other environmental stresses (Martinez-Urtaza et al., 2012; Thompson et al., 2004; Vezzulli et al., 2010).

Table 1. Pathogenic *Vibrio* spp. associated with human infections. Source of infection and clinical manifestations.

***Vibrio* species**	**Source of infection**			**Clinical manifestations[a]**			
	Seafood	**Seawater**	**Freshwater**	**Gastroenteritis**	**Wound infections**	**Primary septicemia**	**Eye and ear infections**
V. cholerae (O1-O139 strains)	Rare	Rare	Yes	+++	+	*	
V. cholerae (other strains)	Yes	Yes	No	+++	++	+	++
V. parahaemolyticus	Yes	Rare	No	+++	++	+	
V. vulnificus	Yes	Yes	No	++	+++	+++	
V. alginolyticus	No	Yes	No	+	+++	+	+++
V. fluvialis	No	Yes	No	+++	+	+	+
V. mimicus	Rare	Yes	Yes	+++	+	+	+
V. (*Grimontia*) *hollisae*	Yes	Yes	No	+++	++	+	
V. furnissii	Yes	Yes	No	+	*	*	
V. metschnikovii	No	Yes	No	+	+	+	
V. damsela (*P. damselae*)	Yes	Yes	No	*	+++	+	
V. harveyi (*V. carchariae*)	Yes	Yes	No		+		
V. cincinnatiensis	Rare	Rare	No	+	*	+	

[a] +++, infection most frequently reported; ++, possible association; +, rare association; *, the association remains to be firmly established. Adapted from Baker-Austin et al., 2018; Nishibuchi, 2006; West, 1989.

Other factors are known to influence the occurrence and distribution of *Vibrio* spp. in aquatic environments, including water temperature, salinity, nutrient

availability, total suspended solids, and tidal fluctuations (Johnson et al., 2012; Kaneko & Colwell, 1973; Namadi & Deng, 2023; Shaw, Jacobs, et al., 2014; Tantillo et al., 2004; Venkateswaran et al., 1989). Temperature has long been found to be strongly correlated with vibrio density, with the highest concentrations observed when water temperatures are between 20°C and 30°C (Kaneko & Colwell, 1973; Kaspar & Tamplin, 1993). Moreover, it has been shown that vibrios may enter a "viable but nonculturable" state (VBNC) when temperatures are lower than 15 °C (Oliver et al., 1995), a phenomenon first observed by Xu et al. (1982) with *V. cholerae* isolates. Particularly, the relationship between vibrio density and water temperature has a direct impact on their spatial and temporal variability across North America, Asia, and Europe (Baker-Austin et al., 2010; Ferchichi et al., 2021; Johnson et al., 2010, 2012; Julie et al., 2010; Kaspar & Tamplin, 1993; Ndraha & Hsiao, 2021; Tantillo et al., 2004). In temperate regions, vibrios are rarely cultured from the water column during colder months but are frequently detected in water and seafood samples during the summer season (Tantillo et al., 2004). Salinity and nutrient availability also influence bacterial growth; however, there is a wide salinity range among *Vibrio* spp. (5-30 ppt), and they may still grow despite salinities outside their optimum range or enter a VBNC state during periods of starvation (Colwell, 1996, 2000; Grimes, 2020; Tantillo et al., 2004).

Vibriosis Clinical and Epidemiologic Features

Human diseases caused by pathogenic *Vibrio* spp. can be categorized into two major groups: cholera and non-cholera infections (Baker-Austin et al., 2018; Tantillo et al., 2004). Cholera is a severe diarrheal disease caused by *V. cholerae* that is still

endemic in many developing nations due to poor sanitation and contaminated water and food sources, with an estimated 95,000 deaths annually, mostly in Sub-Saharan Africa (Ali et al., 2015; Carpenter, 1972; Colwell, 1996).

Non-cholera infections (vibriosis) are varied and depend on the pathogen species, source of infection and host susceptibility (Baker-Austin et al., 2018). They are caused by non-cholera *Vibrio* spp., primarily *V. parahaemolyticus* and *V. vulnificus*, and are either associated with the consumption of raw or undercooked seafood or wound exposure to contaminated water (Tantillo et al., 2004). Other *Vibrio* species such as *Vibrio alginolyticus* and *Vibrio fluvialis* have also increased in abundance over the last decade, and similarly to *V. vulnificus* infections, are most often associated with brackish water exposure (Baker-Austin et al., 2018). Clinical manifestations (**Table 1**) of vibriosis include mild to severe gastroenteritis or primary septicemia (following the ingestion of contaminated food), and wound infection or secondary septicemia (Baker-Austin et al., 2018; Drake et al., 2007).

Vibriosis cases have a marked seasonality and occur more frequently during the summer months when temperatures are warmer (Baker-Austin et al., 2018; Daniels et al., 2000; Shapiro et al., 1998). In the U.S., it is estimated that non-cholera *Vibrio* infections cause 80,000 illnesses and 100 fatalities each year (CDC, 2019), with noted increasing trends in annual incidence rates during the past two decades (A. Newton et al., 2012; Sims et al., 2011; Vugia et al., 2013). In 2014, of the 1,252 *Vibrio* infections reported to the CDC surveillance network, excluding toxigenic *V. cholerae* serogroups O1 and O139, 27% of patients were hospitalized and 4% died (CDC, 2014). The species most frequently reported were *V. parahaemolyticus* (48%),

V. alginolyticus (19%), and *V. vulnificus* (10%); of these cases the most likely to lead to hospitalization and/or death were related to *V. vulnificus* infections, with a hospitalization rate of 79% and a case fatality rate of 18%.

Vibriosis Risk Factors

Since the 1970s there has been a steady increase globally in the consumption of seafood, especially shellfish (Baker-Austin et al., 2010), increasing the number of individuals exposed to contaminated seafood. Ingestion of contaminated seafood (mainly raw oysters) is responsible for approximately 25% of all foodborne illness in the United States, and 95% of all seafood-related deaths are due to the ingestion of raw oysters contaminated with *V. vulnificus* (Newton et al., 2014; Shapiro et al., 1998). In particular, *Vibrio* spp. thrive in warm coastal waters and have the ability to concentrate in the tissue of filter feeding mollusks (e.g., oysters, clams, mussels) (Iwamoto et al., 2010). Larger bivalves such as oysters can accumulate more contaminants, they are also consumed raw or undercooked, which involves a higher risk of foodborne illness than seafood that is properly cooked (Graczyk & Schwab, 2000). Ingesting contaminated oysters can be especially problematic for individuals with underlying medical conditions, such as diabetes, liver disease, hemochromatosis, and immunodeficiencies, who will also have a higher risk of acquiring a severe vibriosis infection (Daniels & Shafaie, 2000; Iwamoto et al., 2010; Oliver, 2005; Weis et al., 2011).

The provenance of the harvested oysters is another important factor, as some coastal waters are much warmer year-round (e.g., Gulf of Mexico) and can lead to an increased risk of vibriosis. Oysters might also be contaminated by more than one

pathogen if they are harvested unlawfully from low quality waters (Rippey, 1994). Studies have also shown that the timing of oyster harvest is significant, and certain practices such as the intertidal harvest can allow *V. parahaemolyticus* to rapidly multiply when oysters are exposed to ambient air at low tide (Nordstrom et al., 2004).

Additional risk factors are related to inadequate practices during post-harvest handling of oysters, such as delays in refrigeration, and conditions at the marketplace (Graczyk & Schwab, 2000). Harvested oysters, especially those intended for raw consumption, should be kept at temperatures lower than 4 degrees Celsius to prevent *Vibrio* species and other bacteria from proliferating (Rippey, 1994). Moreover, cross-contamination can occur when infected seafood items are added to raw or undercooked oysters, leading to foodborne illness (Wallace et al. 1999).

Vibriosis Surveillance

FoodNet and COVIS Surveillance Systems Overview

Surveillance of *Vibrio* infections in the U.S. was initiated in 1989 for oysters harvested from four Gulf of Mexico states (Alabama, Florida, Louisiana, Texas) through the Cholera and Other Vibrio Illness Surveillance (COVIS) system managed by the CDC in conjunction with the Food and Drug Administration (FDA) (CDC, 2014). The program was initially focused on monitoring *V. cholerae* cases and in 2007 this surveillance expanded to include the monitoring of *V. parahaemolyticus* and *V. vulnificus* infections (CDC, 2019). Although this surveillance network is mostly passive, it allows for a better understanding of the risk of vibriosis and how it changes over time and has helped guide improved control strategies. In 1995 stricter

regulations were implemented by the FDA to try and curtail the number of *Vibrio* infections associated with oysters consumed from the Gulf of Mexico (Hlady, 1997). This new policy decreased the time allowed between oyster harvest and refrigeration during months when water temperatures exceeded 29 degrees Celsius, to a maximum of 6 hours. However, issues with this regulation were noted by Shapiro et al. (1998), whose findings indicated that few infections were the result of oysters harvested in waters these warm, and that only half of the illnesses occurred during months affected by this policy change.

It is also critical to understand the risk of exposure to *Vibrio* spp. from recreational and commercial water use. Importantly, a study by Shaw et al. (2015) evaluated the extent of dermal exposure to non-cholerae *Vibrio* spp. among recreational swimmers in the Chesapeake Bay. The methods used to assess the level of exposure to *Vibrio* spp. were successful in demonstrating that pathogens found in the estuarine waters may be detected dermally and add significantly to the risk of infection in swimmers. The authors also determined the level of ingested *Vibrio* spp. based on surface water collections. Nonetheless, the data regarding virulent strains was based on the presence and absence of the bacteria rather than a quantitative measurement. In addition, there was a lack of well-established data concerning the dose-response relationship and virulence markers needed to estimate the true overall risk associated with recreational exposures.

Vibriosis Monitoring and Reporting in Maryland

The Maryland Department of Health (MDH), together with the Maryland Department of the Environment (MDE) and the Maryland Department of Natural

Resources (DNR) participate in the National Shellfish Sanitation Program (NSSP), a cooperative federal/state program recognized by the U.S. Food and Drug Administration (FDA) that regulates the sanitation of shellfish produced and sold for human consumption (FDA, 2020). In addition, MDE and the DNR provide current information on the health status of Maryland's natural waters through water monitoring programs (Jones et al., 2013). Importantly, a study by Jones et al. (2013), is the most recent work to evaluate the *Vibrio* surveillance data in Maryland for quality and timeliness and describe case data by species as well as potential routes of exposure. Their results showed that vibriosis cases in Maryland related to both wound infection and seafood ingestion were predominantly associated with *V. parahaemolyticus*, followed by *V. vulnificus*. Their findings also suggested that recreational or professional shellfish and crab harvesters may be at greater risk of developing severe vibriosis. Nevertheless, the case data was only evaluated for a 6-year period (2002 to 2008) and case fatality rates and severe illness among recreational water users were not assessed.

Moreover, the work by Jones et al. (2013) and others (Shapiro et al., 1998; Weis et al., 2011), supports the need for targeted education and prevention strategies that help reduce the risk of vibriosis, especially when applied to high risk populations. For example, a CDC public health approach used in Gulf of Mexico states urged those at greater risk to avoid the consumption of raw oysters from the Gulf of Mexico as well as seawater exposure. This strategy was credited with the reduction of vibriosis incidence associated with raw oyster consumption, especially in Florida (Weis et al., 2011). In addition, a study by Vugia et al. (2013) also recommended that

prevention efforts should also include public health messages that focus on the risk of vibriosis from wound infections.

Vibrio spp. Virulence and Antibiotic Resistance

Virulence Factors

Thermostable direct hemolysin (*tdh*) is considered a major virulence factor of *V. parahaemolyticus,* capable of causing cytotoxicity and hemolytic activity and it is found in over 90% of all clinical strains (Hiyoshi et al., 2010; Nishibuchi et al., 1992). Thermostable direct-related hemolysin (*trh*) has also been identified as another significant virulence factor, which confers strains with the ability to produce urease (DePaola et al., 2000). Although the presence of these genes is typically used to identify pathogenic *V. parahaemolyticus* strains, studies have found that not all disease-causing isolates carry *tdh* and/or *trh*, and other factors are likely responsible (J. L. Jones et al., 2012; Mahoney et al., 2010; Raghunath, 2014). On the other hand, no single virulence gene that can successfully identify all pathogenic *V. vulnificus* strains has been found, and the mechanisms involved appear to be more complex (Baker-Austin & Oliver, 2018). Nevertheless, *V. vulnificus* isolates have been historically classified into biotype 1, responsible for most human infections and further categorized into genotypes *vcg*C (clinical origin) and *vcg*E (environmental origin); biotype 2, primarily an eel pathogen; and biotype 3, isolated from tilapia handlers in Israel (Baker-Austin & Oliver, 2018; Strom & Paranjpye, 2000). To date, the most significant virulence factors appear to be related to the bacteria's ability to form biofilms and exotoxins, including hemolysin cytolysin (*vvha*) found in most

strains, the RTX toxin (*rtx*A), and type IV pili (*pil*A) (Choi & Choi, 2022; Li & Wang, 2020).

Antibiotic Resistance

While the majority of vibriosis cases are mild and self-limiting and do not require clinical treatment, antibiotics are typically used to treat more severe infections and prompt administration, usually within 24-48 hrs., significantly improves case-fatality rates (Baker-Austin et al., 2018; Oliver, 2005). Tetracycline and third-generation cephalosporins (e.g., cefotaxime, ceftazidime) have been traditionally used in the treatment of both primary septicemia and wound infections caused by *V. vulnificus* and to a lesser degree *V. parahaemolyticus* (Oliver, 2005; Tang et al., 2002; Wong et al., 2015). According to Tang et al. (2002), cephalosporins and tetracyclines (including doxycycline) combined, rather than single drug regimens, may be more effective at treating severe *V. vulnificus* infections (18). In addition, newer fluoroquinolones (e.g., ciprofloxacin and levofloxacin), which display greater potency and a broader spectrum of antimicrobial activity and effectiveness, have been suggested as an alternative single agent treatment for severe vibriosis (Tang et al., 2002). Indeed, results from a long-term analysis of the U.S. Centers for Disease Control and Prevention's (CDC) *Vibrio* surveillance data, showed that mortality rates for *V. vulnificus* were significantly lower in patients taking either a fluoroquinolone only or a tetracycline combined with a third-generation cephalosporin (Wong et al., 2015). Conversely, for children in whom doxycycline and fluoroquinolones are contraindicated, trimethoprim-sulfamethoxazole plus an aminoglycoside (e.g.,

amikacin, gentamicin) are recommended instead (Centers for Disease Control and Prevention (CDC), 2023b).

Although *Vibrio* spp. are considered susceptible to common-use antibiotics (Oliver, 2005; Tang et al., 2002), resistance and intermediate-resistance has been increasingly recorded in both environmental (from seafood and seawater samples) and clinical isolates (Baker-Austin, McArthur, et al., 2009; Bier et al., 2015; Canellas et al., 2021; da Silva et al., 2021; Das Sarker et al., 2019; Elmahdi et al., 2018; Håkonsholm et al., 2020; Han et al., 2007; Jiang et al., 2014; Lopatek et al., 2018; Shaw, Rosenberg Goldstein, et al., 2014; Tan et al., 2020). Notably, a high percentage of resistance to penicillin and ampicillin has been observed in both *V. parahaemolyticus* and *V. vulnificus* (Han et al., 2007; Shaw, Rosenberg Goldstein, et al., 2014), and previous studies have suggested that these antibiotics may no longer be effective as a single antibiotic treatment for vibriosis (Ngasotter et al., 2022; Wong et al., 2015). Excessive use of antibiotics in humans, aquaculture, and agricultural settings (e.g., poultry farms) plays a major role in the selection of antibiotic resistance among many bacterial genera, including *Vibrio* spp. (Heng et al., 2017; Ngasotter et al., 2022). The persistence of antibiotics in aquatic environments, which function as critical reservoirs, promotes the evolution and transfer of antibiotic resistance genes among bacterial species and subsequently across the food chain (Kumarage et al., 2022).

Vibrio and Climate Change

Previous studies have shown that *Vibrio* species favor warm coastal waters, with bacterial levels rarely detected until seawater temperatures are greater than 15

degrees Celsius (Kelly 1982, Kaspar & Tamplin 1993, Cook 1994, McLaughlin et al. 2005). They also exhibit a strong seasonal pattern, with a higher risk of infection from oysters consumed during summer and fall months (Hlady 1997, Shapiro et al. 1998, Altekruse et al. 2000). Other studies have demonstrated that water temperature can serve as a predictor of infection, given the strong association between oysters harvested in warm water and vibriosis cases (Shapiro et al. 1998, Duan & Su 2005).

According to the 2014 Intergovernmental Panel on Climate Change (IPCC) report, the ocean and atmosphere have warmed significantly since 1950 as a direct result of climate change, and coastal ocean temperature is predicted to rise by a few degrees during this century (Harvell et al. 2002). There is also evidence of accelerated and unprecedented rates of warming in North Atlantic regional seas over the last 25 years (Reid et al. 2011). A long-term retrospective study by Vezzulli et al. (2012) showed that pathogenic *Vibrio* species have increased in dominance within the marine microbial community in response to the rapid warming in the North Sea. This finding coupled with other recent works (Baker-Austin et al. 2013, Vezzulli et al. 2013, Vezzulli et al. 2015) supports the view that ocean warming might be leading to the dispersion of *Vibrio* spp. and could help explain the worrisome global increase in vibriosis cases.

Chapter 3: Long-Term Analysis of *Vibrio vulnificus* and *Vibrio parahaemolyticus* Virulence Factors and Environmental Associations in the Chesapeake Bay, Maryland U.S.

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Abstract

A long-term increase and extended seasonality of environmental *V. parahaemolyticus* and *V. vulnificus* isolates from the Chesapeake Bay, Maryland, was recently noted. Hence, the present study aimed to analyze long-term trends in *Vibrio* spp. primary virulence factors associated with pathogenicity in Chesapeake Bay waters across two three-year sampling periods (2009-2012 and 2019-2022). *V. parahaemolyticus* (n=1,476) and *V. vulnificus* (n=806) isolates were tested for species-specific genes and virulence markers. The relative abundance of *V. parahaemolyticus* virulence factors, *tdh* and *trh*, was not significantly different between sampling periods but varied by season, being highest in the spring (n=9%) for both periods. The relative abundance of *V. vulnificus* virulence factors *pil*A and *rtx*A was significantly different between sampling periods, $p < 0.05$, with a higher percentage of *pil*A observed in isolates recovered between 2019-2022, and *rtx*A between 2009-2012. VcgC was only recorded for isolates recovered during 2019-2022, and was highest in the fall season (n=27%). Moreover, for both sampling periods (2009-2012 and 2019-2022) the relative abundance of *Vibrio* spp. virulence factors in collected isolates varied by section in the Bay. Our results indicate that potentially pathogenic *V.*

parahaemolyticus and *V. vulnificus* occur across the Bay throughout the year, including at frequencies that may still lead to a greater risk of infection in the fall and possibly early winter. Moreover, the mid and lower sections of the Bay, may harbor a greater risk of severe vibriosis from wound-associated water exposure, given the greater prevalence of *V. vulnificus* isolates displaying significant virulence factors.

Introduction

Vibrio spp. are ecologically important Gram-negative bacteria that are ubiquitous in marine and estuarine environments, and flourish in warm water with moderate salinity (Thompson et al. 2004, Singleton et al. 1982). *Vibrio* spp. incidence is strongly influenced by environmental parameters (Vezzulli et al. 2013, Colwell 1996, Brumfield et al. 2021), and they are known to be associated with aquatic invertebrates, especially zooplankton, crustaceans, and bivalves (Hlady 1997, Kaneko and Colwell 1973). Of the more than 110 described species, only a few are considered to be pathogenic to humans and animals (Daniels & Shafaie, 2000; Morris & Black, 1985). In particular, *Vibrio cholerae* is the well-known etiologic agent of cholera that continues to plague many developing nations (Colwell, 1996). However, in the United States and other developed countries, pathogenic non-cholera *Vibrio* species, including *Vibrio parahaemolyticus* and *Vibrio vulnificus*, are more likely to cause disease (Baker-Austin et al., 2017). Symptoms of non-cholera *Vibrio* illness (vibriosis) range from mild gastroenteritis to more severe cases that include wound infection and septicemia, leading to hospitalization and even death (Daniels & Shafaie, 2000).

According to the Centers for Disease Control and Prevention (CDC), *Vibrio* bacteria cause an estimated 80,000 illnesses each year in the U.S., with approximately 40% of cases being attributed to the consumption of *V. parahaemolyticus* contaminated shellfish (mainly raw oysters), and the remainder of cases to various *Vibrio* spp., including *V. vulnificus* (CDC 2023, Iwamoto et al. 2010, Ndraha et al 2020). The latter causes an estimated 150-200 cases each year but has a case-fatality rate among the highest of any waterborne pathogen, up to 50%, and infection is more commonly associated with exposure to contaminated brackish waters (Oliver, 2013). The pathogenicity of *Vibrio* spp. strains appears to be associated with their ability to produce multiple primary virulence factors that are often complex in nature and not yet entirely understood (Choi & Choi, 2022; Raghunath, 2014).

Thermostable direct hemolysin (*tdh*) is considered a major virulence factor of *V. parahaemolyticus*, capable of causing cytotoxicity and hemolytic activity and it is found in over 90% of all clinical strains (Hiyoshi et al., 2010; Nishibuchi et al., 1992). Thermostable direct-related hemolysin (*trh*) has also been identified as another significant virulence factor, which confers strains with the ability to produce urease (DePaola et al., 2000). Although the presence of these genes is typically used to identify pathogenic *V. parahaemolyticus* strains, studies have found that not all disease-causing isolates carry *tdh* and/or *trh*, and other factors are likely responsible (J. L. Jones et al., 2012; Mahoney et al., 2010; Raghunath, 2014). On the other hand, no single virulence gene that can identify all pathogenic *V. vulnificus* strains, and the mechanisms involved appear to be more complex (Baker-Austin & Oliver, 2018). Nevertheless, *V. vulnificus* isolates have been historically classified into biotype 1,

responsible for most human infections and further categorized into genotypes *vcg*C (clinical origin) and *vcg*E (environmental origin); biotype 2, primarily an eel pathogen; and biotype 3, isolated from tilapia handlers in Israel (Baker-Austin & Oliver, 2018; Strom & Paranjpye, 2000). To date, the most significant virulence factors appear to be related to the bacteria's ability to form biofilms and exotoxins, including hemolysin cytolysin (*vvha*) found in most strains, the RTX toxin (*rtx*A), and type IV pili (*pil*A) (Choi & Choi, 2022; Li & Wang, 2020).

In the past two decades, there have been noted increases in the annual incidence of vibriosis across the U.S. (Archer et al., 2023; A. Newton et al., 2012; Sims et al., 2011; Weis et al., 2011), and multiple studies have reported a significant geographic expansion of pathogenic *Vibrio* spp. and increased risk of infection, especially in more Northern ocean and brackish waters (Fleischmann et al. 2022, Sterk et al. 2015, Ford et al. 2020, Vezzulli et al. 2013, Baker-Austin et al. 2013). There are also concerns about the impact of climate related environmental changes (e.g., increased sea surface temperature) and the role of extreme weather events (e.g., hurricanes, heat waves) in the increase and proliferation of *Vibrio* spp. worldwide (Baker-Austin et al., 2017; Brehm et al., 2021; Deeb et al., 2018; Froelich & Daines, 2020). To this effect, a recent investigation in the Chesapeake Bay, the largest estuary in North America, reported a long-term increase and extended seasonality of environmental and potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* strains across two time periods (Brumfield et al., 2023). However, the extent of their pathogenicity was not fully evaluated for the later time period (2019-2022). Thus, the present study aimed to expand on this work and analyze long-term trends in *Vibrio*

spp. primary virulence factors associated with pathogenicity in this estuary, and the potential implications for human health.

Methods

Site description

Water and oyster samples were collected from the Chesapeake Bay, Maryland, U.S., during two separate three-year sampling events, namely 2009-2012 and 2019-2022. From June 2009 to August 2012 samples were collected at two locations, Chester River, and Tangier Sound, and between April 2019 and August 2022 samples were collected from 12 stations across the Chesapeake Bay (**Fig. 1**). The Chesapeake Bay is a commercial and recreationally important estuary characterized by a strong salinity gradient from the head of the Bay near fresh tidal rivers in the north to the opening of the Atlantic Ocean in the south where salinities average 25-30ppt (Roman et al., 2005). All chosen sampling stations were located in Maryland's portion of the Bay and were divided into the following sections based on latitude and average salinity: upper (Chester River, Sandy Point, South River, Cox Creek), mid (Miles River, Broad Creek, Choptank River, Upper Patuxent River, Lower Patuxent River, Wicomico River), and lower (St Mary's River, Tangier Sound). Between both sampling efforts, there were a total of 195 sampling events (111 from 2009-2012 and 85 from 2019-2022), with more than half of all samples collected during the warmer months of June through August.

Sample collection and processing

Brumfield et al. (2023), Chen et al. (2017), and Johnson et al. (2010, 2012) have previously described sample collection and processing methods that are summarized in this study. Briefly, physical, and chemical measurements were collected during each sampling event, including water temperature, pH, dissolved oxygen, salinity, and chlorophyll-*a*. Between 2009 and 2012, all sample collection occurred twice a month during the summer (June through August), and once a month for the rest of the year and between 2019-2022, samples were collected weekly during the summer and twice a month the rest of the year. For the latter, restrictions imposed during the COVID-19 pandemic impacted the collection of samples from March through July of 2020.

Water samples (12L) were collected just below the surface using a Van Dorn water sampler (WildCo, Buffalo, NY), and approximately thirty oysters were collected by dredging and stored in clean freezer bags. All samples were transported back to the laboratory on ice and kept refrigerated overnight until processing the following morning. Sample processing followed methods described in the *Bacteriological Analytical Manual* for food sampling/preparation of sample homogenate (Andrews & Hammack, 2022) and *Vibrio* (Kaysner et al. 2019). Water samples were shaken, and three volumes (10, 100, 1000 mL) were resuspended into alkaline peptone water (10X APW, pH 8.5). Oysters were rinsed and scrubbed under running water to remove debris from shells, shucked, and the tissue was homogenized in an equal amount of phosphate buffer solution (1X PBS, pH 7.4) in a sterile blender for 90 s. All samples were incubated at 33°C for 16-18 h, with shaking at 30 rpm.

Following incubation, a loopful from each overnight sample was removed and streaked individually onto selective media, including CHROMagar™ (CHROMagar, Springfield, NJ), thiosulfate citrate bile salts sucrose agar (TCBS, Oxoid, Ontario, Canada), and *V. vulnificus* agar (VVA). The plates were incubated at 37 °C for 16-18 h and based on growth media, presumptive colonies of *V. parahaemolyticus* and *V. vulnificus* were picked and streaked onto LB agar (BD Diagnostic Systems, Sparks, MD) to obtain pure cultures. Bacterial isolates were stored at −80°C in LB broth containing 50% (vol/vol) glycerol.

Vibrio species confirmation

DNA was extracted from presumptive isolates of *V. parahaemolyticus* and *V. vulnificus* following methods previously described in Chen et al. (2017) and confirmed using PCR targeting the *tox*R gene adapted from Bauer and Rørvik (2007) to differentiate between both vibrio species. In addition, samples testing positive for either bacterium were also tested for species-specific genes and virulence markers (*V. parahaemolyticus: tlh*, *tdh*, *trh*; and *V. vulnificus*: *vvha*, *vcg*E, *vcg*C, *rtx*A, *pil*A). *Vcg*C^{+} and *Vcg*E^{+} data were not available for isolates from the 2009-2012 sampling period.

PCR assays were performed using Promega GoTaq Green Master Mix 2x (Promega, Madison, WI, U.S.), and each reaction well contained a total of 25 µl, including 20 µl of mastermix solution (12.5 µl of GoTaq, 1 µl of each primer, and nuclease-free water to reaction volume) and 5 µl DNA template. The primer sequences, amplicon size, and conditions used for each PCR can be found in **Table 1**.

PCR products were kept at 4 °C until gel electrophoresis visualization. Positive controls included the following strains: *V. parahaemolyticus* ATCC 17803 (*tox*R$^+$), NIHCB0757 (*tlh*$^+$/*tdh*$^+$), AQ 4037 (*tlh*$^+$/*trh*$^+$), and *V. vulnificus* ATCC 27562 (*tox*R$^+$/*vcgE*$^+$/*vvhA*$^+$), ATCC 29307 (*vcgC*$^+$/*pilA*$^+$/*rtxA*$^+$). Nuclease-free water was used as a negative control in each reaction. PCR products were visualized using a 1.5% agarose gel at 110V for 60-90min and viewed under a UV transilluminator using a Gel Documentation System (GelDoc-IT^{TM}, UVP, LLC, CA, U.S.).

Data Analysis

The Wilcoxon rank sum test and the Kruskal-Wallis test were used to evaluate differences between environmental water parameters (water temperature, salinity, dissolved oxygen, pH, chlorophyll-*a*) measured in each sampling period (2009-2012 and 2019-2022) and season. A paired t-test and the repeated measures ANOVA (season and season*year interaction as the fixed effects; year as the repeated measure) were used to assess differences in *V. parahaemolyticus* and *V. vulnificus* virulence factors across seasons for each sampling period.

The relative abundance (%) of *Vibrio* spp. virulence factors per sampling event was calculated based on their frequency of detection in water and oyster samples from both longitudinal time periods. The Kruskal-Wallis test was used to evaluate how *Vibrio* spp. virulence factors differed between sections of the Bay (upper, mid, lower) for both sampling periods combined. The non-parametric two-sample t-test (Wilcoxon Rank Sum test) was used to assess any significant differences between the detection (absence/presence) of *Vibrio* spp. virulence factors and changes to environmental parameters. Correlation and regression analyses were also applied to

evaluate the association between the relative abundance of *Vibrio* spp. virulence factors and environmental parameters. All statistical analyses were performed using SAS 9.4 (Cary, NC USA).

Results

Water quality trends

The overall average water temperature, salinity, and dissolved oxygen concentrations were not significantly different ($p > 0.05$) between the two sampling periods (**Table 2**). By contrast, pH and chlorophyll-*a* concentrations were significantly different between sampling periods ($p < 0.001$), with higher average pH and chlorophyll-*a* levels observed during the 2019-2022 time period, although with a high degree of variability in chlorophyll-*a* measurements (**Table 2**).

Average water temperature and dissolved oxygen concentrations varied significantly by season ($p < 0.0001$) for both sampling periods and followed a similar pattern (**Table 2**). As expected, water temperatures were highest during the summer and lowest during the winter, ranging between 4.3-27.2 °C, while dissolved oxygen concentrations were lowest during the summer and highest during the winter, ranging from 6.4-11.9 mg/L. Average salinity, pH, and chlorophyll-*a* concentrations did not vary significantly between seasons for the 2009-2012 time period but were significantly different for 2019-2022 ($p < 0.05$). During this time, average salinity varied between 8.7 ppt during the spring and 12.6 ppt during the fall, while pH and chlorophyll-*a* concentrations were higher during the spring, and lower during the fall and winter, respectively (**Table 2**).

Average water temperature and pH did not vary significantly between the upper and lower sections for the 2009-2012 sampling period nor between upper, mid, and lower sections for the 2019-2022 time period ($p > 0.05$) (**Table 2**). On the other hand, average salinity and chlorophyll-*a* concentrations were significantly different between sections for both sampling periods ($p < 0.01$) and followed a similar trend. As expected, higher salinities close to 14 ppt were observed in the lower section, closer to the mouth of the Bay, and in the upper section (near fresh tidal waters) the lowest salinity was observed, an average of 8.5 ppt. By contrast, average chlorophyll-*a* concentrations declined moving from the upper section to the lower section, although it should be noted that there was a great degree of variability in the chlorophyll-*a* measurements per sampling event, especially during the 2019-2022 sampling period. Lastly, average dissolved oxygen concentrations did not vary significantly by section during the 2019-2022 sampling period ($p > 0.05$) but were significantly different between sections for the 2009-2012 time period ($p = 0.04$). During this time, dissolved oxygen concentrations were on average 1 mg/L higher in the lower section closest to the mouth of the Bay, compared to the upper section (**Table 2**).

Vibrio spp. abundance

There were a total of n=1,476 *V. parahaemolyticus* (n=715 from 2009-2012 and n=761 from 2019-2022) and n=806 *V. vulnificus* (n=565 from 2009-2012 and n=241 from 2019-2022) isolates recovered from water and oyster samples between both sampling periods, with most isolates collected during the summer and fall seasons. Moreover, more than 90% of all isolates that were positive for either vibrio

were collected from water samples. The average relative abundance of genetic markers per sampling event for *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvha*) was not significantly different between sampling periods or across seasons (**Table 3**).

Spatial-temporal trends in isolate virulence

The relative abundance of *V. parahaemolyticus* virulence factors *tdh* and *trh* was not found to be significantly different ($p > 0.05$) between sampling periods (**Table 3**). On the other hand, the relative abundance of *V. vulnificus* virulence factors *pil*A and *rtx*A was significantly different between sampling periods, $p = 0.02$ and $p < 0.0001$, respectively, with a higher overall percentage of *pil*A observed in isolates recovered between 2019-2022, and *rtx*A during the 2009-2012 time period (**Table 3**).

The relative abundance of *V. parahaemolyticus* virulence factors, *tdh* and *trh*, varied by season, but only significantly for the 2009-2012 sampling period ($p = 0.02$) (**Table 3**). During the 2009-2012 sampling period, the relative abundance of *tdh* and *trh* positive isolates was highest in the spring (n=9%) and lowest in the fall. For the 2019-2022 time period, *tdh* was infrequently observed in isolates across each season, and *trh* abundance was highest in the spring (n=9%) and lowest in the winter (**Table 3**).

The relative abundance of *V. vulnificus* virulence factors, *vcg*C, *pil*A, and *rtx*A, varied by season but not significantly for either sampling period ($p > 0.05$) (**Table 3**). *Vcg*C was only recorded in isolates collected during the 2019-2022 time period and was highest in the fall (n=27%) and not detected during the spring. The abundance of *pil*A ranged between 39% in the spring to non-detectable in the winter for the 2009-2012 sampling period, while it was highest during the summer (n=44%)

and lowest in the spring (n=11%) for isolates collected during 2019-2022. Lastly, r*tx*A abundance for both longitudinal sampling periods was lowest in isolates collected during the winter (not detected) and highest during the fall (n=48% in 2009-2012 and n=5% in 2019-2022) (**Table 3**).

For both sampling periods combined (2009-2012 and 2019-2022) the relative abundance of *Vibrio* spp. virulence factors in collected isolates varied by section in the Chesapeake Bay, although only significantly for *tdh* ($p = 0.04$), *pil*A ($p = 0.0002$) and *rtx*A ($p < 0.0001$) (**Fig. 2**). Overall, the relative abundance of *tdh* (n=5%), *trh* (n=5%), *and rtxA* (n=33%) was highest in isolates collected from the upper section, which included Chester River, Sandy Point, South River, and Cox Creek stations. On the other hand, *Vcg*C and *pil*A were more frequently detected in isolates, n=18% and n=48%, respectively, collected from the mid-section, which included Miles River, Broad Creek, Choptank River, Upper Patuxent River, Lower Patuxent River, and Wicomico River stations (**Fig 2**).

Environmental associations with virulence

Between both sampling periods, only 1% of *V. parahaemolyticus* isolates (n=15), all cultured from water samples, tested positive for *tdh* (n=9) and/or *trh* (n=15) virulence factors. The detection of *tdh* and *trh* (absence/presence) was not found to be significantly different ($p > 0.05$) with changes to average water temperature, dissolved oxygen, pH, and chlorophyll-*a*. Additionally, the relative abundance of both virulence factors did not show a significant linear relationship or a strong correlation with average water temperature, pH, dissolved oxygen, and chlorophyll-*a* concentrations. However, there was a significant but weak negative

correlation between the detection of *trh* and average salinity (ρ = -0.21, p = 0.01), while there was no such correlation observed for *tdh*.

Between both sampling periods, 45% of *V. vulnificus* isolates (n=363), 95% of them cultured from water samples, tested positive for vcg*C* (n=37), pil*A* (n=155), and/or rtx*A* (n=217) virulence factors. Interestingly, n=16 isolates also tested positive for both *vcg*C and *vcg*E virulence factors. The detection of *vcg*C, *pil*A and *rtx*A was not found to be significantly associated ($p > 0.05$) with changes to environmental water parameters. Moreover, the relative abundance of *vcg*C and *pil*A did not show a significant linear relationship or a strong correlation with any of the measured environmental water parameters. Conversely, the relative abundance of *rtx*A was significantly negatively correlated, although weakly, with both average chlorophyll-*a* concentration (ρ = -0.23, p = 0.02) and pH levels (ρ = -0.28, p = 0.007). Notwithstanding, there was no observable significant linear relationship between *rtx*A and chlorophyll-*a* and pH levels, nor was there any significant effect with changes to average water temperature, salinity, and dissolved oxygen concentration.

Although there wasn't a significant or strong linear relationship between average water temperature and salinity, and the relative abundance of *Vibrio* spp. virulence factors, an interesting pattern denoting potential optimum environmental conditions per species can be observed in a bubble plot (**Fig. 3**). For *V. parahaemolyticus* isolates with virulence factors, *tdh* and/or *trh* (**Fig. 3A**), the highest observed average relative abundance was included within water temperatures between 17-25 °C and salinities of 1-7 ppt. By contrast, *V. vulnificus* isolates with

virulence factors, *vcg*C (**Fig. 3B**), *pil*A (**Fig. 3C**), and *rtx*A (**Fig. 3D**), occurred more frequently in water temperatures ranging between 23-30 °C and salinities of 7-14 ppt.

Discussion

Similarly to previous works by Parveen et al. (2008), Banakar et al. (2011), Johnson et al. (2012), Jacobs et al. (2014), Chen et al. (2017), Davis et al. (2017), Parveen et al. (2020), and Brumfield et al. (2023), this study evaluated reported associations between pathogenic *V. parahaemolyticus* and *V. vulnificus* and environmental determinants in the Chesapeake Bay. The findings presented here confirm known associations but given the long-term nature of the sampling efforts, also expand on changes and possible trends in the distribution and seasonality of potentially pathogenic *Vibrio* spp. in this important estuarine system.

Understanding how physico-chemical parameters change over time across coastal ecosystems is a critical aspect in the prevention of severe *Vibrio* spp. infections worldwide, as their abundance in the environment has been shown to be strongly associated with certain environmental conditions (Brumfield et al. 2021, Davis et al. 2017, Williams et al. 2017, Tran et al. 2020). These conditions differ between vibrios and may also change over time as the bacteria adapt to environmental stressors and increasingly adverse environments, including their ability to enter a viable but non-culturable state (VBNC) (Colwell, 2000; Davis et al., 2017; Nowakowska & Oliver, 2013). Not only have studies noted some variability in optimal growing conditions between *Vibrio* spp. but they also suggest that their abundance could shift depending on the environmental host (e.g., water, sediment, or oyster populations) (Chen et al. 2017, Brumfield et al. 2023, Ndraha and Hsiao 2021,

DePaola et al. 2003, Johnson et al. 2010, 2012). In the present study, environmental and potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* were mostly found in water samples, compared to oysters, especially in the later time period (2019-2022). This could reflect an increasing trend of *Vibrio* spp. concentrations in the water compared to oyster populations but may also be a limitation of using culture-based methods and the ability of vibrios to enter a VBNC state (Chen et al., 2017).

In this study, the average relative abundance of *V. parahaemolyticus* and *V. vulnificus*, denoted by *tlh* and *vvha* concentrations, respectively, was not found to be significantly different between sampling periods or across seasons. By contrast, a study by Brumfield et al. (2023) that quantified the log CFU/g of *Vibrio* spp. based on similar culture methods at the same locations and collection periods, found that while *vvha* concentrations were not significantly different between both sampling periods, *tlh* concentrations were significantly higher during 2019-2022 compared to 2009-2012. Moreover, *tlh* concentrations had the greatest observable increase during the fall, whereas *vvha* did not show a significant change across seasons. Based on these findings it was suggested that *V. parahaemolyticus* isolates in the Chesapeake Bay may have an extended seasonality.

According to a study by Najjar et al. (2010) that evaluated the potential impact of climate change in the Chesapeake Bay, the greatest changes by the end of the 21st century were predicted to be increased sea-level variability and water temperature, between 2-6 °C, increased precipitation, especially in the winter and spring, and greater winter and spring streamflow. In the current study, overall average water temperatures were not significantly higher in the later sampling period of 2019-2022,

and were in fact slightly lower, however, it should be noted that the average water temperature was greater in the fall and winter compared to the earlier sampling period of 2009-2012. It is also important to recognize that there can be inter-decadal and intra-annual variability in temperature and precipitation patterns as well as the potential impact of extreme weather events, such as heat waves and hurricanes, that can lead to deviations from established trends (Banakar et al. 2011, Jacobs et al. 2014, Shaw et al. 2014).

An interesting change between sampling periods was the observed decrease in both average water temperature and salinity during the spring, which might correlate to increased precipitation and streamflow. Other potential environmental changes forecasted by Najjar et al. (2010) included increased hypoxia, which was not observed between sampling periods, and altered phytoplankton dynamics, which would in turn impact the timing of zooplankton blooms, a known reservoir of *Vibrio* spp. (Kaneko & Colwell, 1973). To this point, a noticeable increase and a greater variability in average chlorophyll-*a* levels was observed in the later sampling period across all seasons compared to the earlier time, and especially during the spring and summer. An additional concern includes the increase in acidification (lower pH) across the Chesapeake Bay (Najjar et al., 2010). A study by Waldbusser et al. (2011) found that pH has remained mostly unchanged in mesohaline waters across the Chesapeake Bay compared to more polyhaline areas further south. Moreover, the pH in tributaries that once supported large oyster populations showed an increase over time, which may help explain the increased average pH levels observed during the later sampling period (2019-2022) compared to the earlier one (2009-2012).

Importantly, recent studies have found that *V. vulnificus* thrives in salinities between 5-25 ppt and at temperatures at or above 25 °C (Baker-Austin & Oliver, 2018; Williams et al., 2017). Our results appear to support these observations in terms of temperature preferences for *V. vulnificus* isolates that harbor virulence factors *vcg*C, *pil*A, and/or *rtx*A. However, the salinity range appeared to be narrower, between 7-14 ppt, which would have implications in terms of the pathogen's preferred location within the Chesapeake Bay and optimal seasonal conditions. Notwithstanding, sampling efforts were not performed in more polyhaline areas of the Bay further south, which limited our ability to fully evaluate *V. vulnificus* virulence factor trends across the estuary. Interestingly, the significant, albeit weak, negative association observed between isolates *rtx*A positive and average pH and chlorophyll-*a* levels, may be indicative of optimal growing conditions in areas of lower pH and chlorophyll-*a* concentrations, i.e. more polyhaline areas closer to the mouth of the Bay, as well as the fall season when these conditions may be more likely. In fact, stations located in the mid-section and the lower section of the Bay appeared to harbor a greater percentage *V. vulnificus* strains with virulence factors, compared to the upper section. These findings differ somewhat from a hindcast prediction for a 15-year period, 1991-2005, by Banakar et al. (2011), which found that the greatest hotspots of *V. vulnificus* were mostly located in the upper Bay, parts of western estuaries and the mid Bay, with peaks in July and a likely decrease in presence until November. This particular study and findings by Jacobs et al. (2014), also noted a significant trend in which the mean probability of *V. vulnificus* occurrence in years with excessive precipitation (wet years) shifted southwards in the Bay towards more

polyhaline areas, and the reverse happened during drier years, where the shift occurred northward.

Earlier works have noted that optimal growing conditions for *V. parahaemolyticus* can vary depending on the medium (water, oyster, sediment) as well as the geographic location (Johnson et al., 2012; Parveen et al., 2008). For instance, Johnson et al. (2012) found that with respect to *tlh* concentrations, Gulf of Mexico strains had a greater affinity towards higher salinities and water temperatures. However, similarly to our study they did not find that sea surface temperature was a strong predictor of the density for potentially pathogenic strains carrying *tdh* and/or *trh*. In fact, as suggested by others, it may be that total *V. parahaemolyticus* populations are not a suitable indicator of the presence of isolates containing these virulence genes, as they have shown to be variable and maybe even inversely related to temperature (Davis et al., 2021; DePaola et al., 2003; Williams et al., 2017). Although limited by the low percentage of *tdh* and/or *trh* positive isolates cultured between sampling periods, our study showed a greater prevalence of potentially pathogenic subpopulations in temperatures at or around 20 °C and at lower salinities, between 1-7 ppt, and an inverse association was observed with average salinity for *trh* positive isolates. The reduced isolation of strains containing virulence genes is in line with multiple studies that have found anywhere between 1 and 1.9% of pathogenic *V. parahaemolyticus* concentrations in environmental samples, however, unlike previous works pathogenic subpopulations were not detected in oyster samples (Davis et al., 2017; Parveen et al., 2020; Williams et al., 2017). In terms of location and seasonality, potentially pathogenic *V. parahaemolyticus* was more frequently

observed in the upper Bay stations, and appeared to have two peaks, one in the spring and another in the fall, mainly for the later sampling period (2019-2022).

It is important to note that our study used direct colony DNA hybridization methods without the use of enrichment or real-time PCR, and as reported by previous works, the VBNC vibrio will not be isolated using this method and the subpopulations of pathogenic *V. parahaemolyticus* with *tdh* and *trh* genes may be too sparse to be properly enumerated, which may underrepresent their abundance (Chen et al., 2017; Johnson et al., 2012; Parveen et al., 2008). On the other hand, solely relying on real-time PCR methods may overestimate the abundance of potentially pathogenic strains in the environment, as it will not be able to differentiate between living and non-living populations. As such, a combination of culture-dependent and culture-independent methods may yield the best results, as it would provide a more complete picture of pathogenic *Vibrio* spp. concentrations, including the culturable and the VBNC populations, which may dominate during the colder months and possibly in other mediums (e.g., sediment and oysters). Other limitations of our study include the lack of continuous sampling between longitudinal studies, which led to a 10-year gap in data. This highlights the importance of continued monitoring, especially given the intra-and inter-annual variability and rapidly changing physico-chemical conditions in the Chesapeake Bay.

Acknowledgements

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Chapter 3 Tables

Table 1. List of primer sequences, amplicon size, and PCR conditions used for the detection of *V. parahaemolyticus* and *V. vulnificus* species-specific and virulence genes.

Primers	Primer sequence (5′-3′)	Amplicon (bp)	PCR conditions	Source
utox-F	GASTTTGTTTGGCGYGARCAAGGTT			
vptox-R	GGTTCAACGATTGCGTCAGAAG	297	95°C for 4 m; 34x: 95°C for 30 s, 55°C for 30 s, 72°C for 60 s; 72°C 5 m	(Bauer & Rørvik, 2007)
vvtox-R	AACGGAACTTAGACTCCGAC	435	95°C for 4 m; 30x: 95°C for 30 s, 60°C for 30 s, 72°C for 60 s; 72°C 7 m	
tlh-F	AAAGCGGATTATGCAGAAGCACTG	173	94°C for 3 m; 30x: 94°C for 60 s, 58°C for 60 s, 72°C for 60 s; 72°C 5 m	(Bej et al., 1999; Rizvi & Bej, 2010)
tlh-R	TGTGCCTTGATGAACTCGTTC			
tdh-F	GTAAAGGTCTCTGACTTTTGGAC	270		
tdh-R	TGGAATATGAACCTTCATCTTCACC			
trh-F	TTGGCTTCGATATTTTCAGTATCT	500		
trh-R	CATAACAAACATATGCCCATTTCCG			
vvh-F	AGCGGTGATTTCAACG	411	94°C for 3 m; 34x: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C 5 m	(Warner and Oliver 2008, Panicker and Bej 2005, Panicker et al. 2004, Rosche et al. 2005, Brasher et al. 1998
vvh-R	GGCCGTCTTTGTTCACT			
vcgC-F	AGCTGCCGATAGCGATCT	97	94°C for 3 m; 30x: 94°C for 40 s, 57°C for 40 s, 72°C for 40 s; 72°C 5 m	
vcgC-R	TGAGCTAACGCGAGTAGTGAG			
vcgE-F	CTCAGAAAGGCTCAATTGAC	199		
vcgE-R	GATTAACGCTGTAAGGCCG			
pilA-F	TGGCTGCTGTTGCTATTC	217	94°C for 3 m; 30x: 94°C for 60 s, 60°C for 60 s, 72°C for 60 s; 72°C 5 m	(Natividad-Bonifacio et al., 2013; Paranjpye & Strom, 2005)
pilA-R	GGTCCACCACTAGTACCAAC			
rtxA-F	CGGGATCCTATGGCGTGAACGGCGAAG	1440	94°C for 3 m; 30x: 94°C for 30 s, 68°C for 30 s, 72°C for 60 s; 72°C 5 m	(Natividad-Bonifacio et al. 2013, Lee et al. 2008, Kim et al. 2008)
rtxA-R	CGGGATCCAGCAGCCACAAGCGATTC			

Table 2. Characteristics by sampling period (2009-2012 and 2019-2022), season and section, including number of sampling events with positive *Vibrio* spp. cultures (#), average temperature (T), salinity (S), pH, dissolved oxygen (DO), and chlorophyll-*a* concentrations (chla) per sampling event with ± standard deviation.

Study / Season, Section	#	T (°C)	S (ppt)	pH	DO (mg/l)	Chla (µg/L)
2009-2012						
Season[a]						
Spring	12	16.3 ± 5.0	11.0 ± 4.4	7.3 ± 0.8	10.0 ± 4.1	14.0 ± 7.8
Summer	45	27.2 ± 1.6	11.0 ± 3.3	7.5 ± 0.6	6.6 ± 1.2	14.1 ± 5.4
Fall	15	17.6 ± 5.4	11.3 ± 4.4	7.7 ± 0.5	8.4 ± 1.6	12.0 ± 6.2
Winter	10	4.3 ± 3.2	11.0 ± 3.6	7.4 ± 0.2	11.8 ± 1.3	12.8 ± 6.7
Section[b]						
Upper	40	21.7 ± 7.7	8.2 ± 2.4	7.4 ± 0.6	7.6 ± 2.2	15.5 ± 6.9
Mid	---	---	---	---	---	---
Lower	42	20.4 ± 9.2	13.6 ± 2.6	7.6 ± 0.6	8.6 ± 3.0	11.8 ± 4.6
Overall (2009-2012)	**82**	**21.0 ± 8.5**	**11.1 ± 3.7**	**7.5 ± 0.6**	**8.1 ± 2.7**	**13.6 ± 6.1**
2019-2022						
Season[a]						
Spring	11	13.3 ± 4.7	8.7 ± 2.6	8.3 ± 0.4	10.5 ± 1.6	39.6 ± 35.3
Summer	34	27.2 ± 1.8	10.3 ± 3.0	7.9 ± 0.4	6.4 ± 1.0	21.1 ± 12.2
Fall	22	20.1 ± 5.5	12.6 ± 2.1	7.8 ± 0.4	7.8 ± 1.4	15.6 ± 7.6
Winter	13	5.0 ± 2.1	12.4 ± 2.9	8.0 ± 0.6	11.9 ± 1.5	14.1 ± 9.4
Section[b]						
Upper	8	21.6 ± 8.8	9.4 ± 3.8	8.0 ± 0.5	8.4 ± 2.1	30.0 ± 25.0
Mid	56	19.1 ± 8.8	10.4 ± 2.2	7.9 ± 0.4	8.2 ± 2.7	21.8 ± 17.9
Lower	16	20.4 ± 9.6	13.7 ± 3.1	8.1 ± 0.4	8.4 ± 1.9	13.6 ± 8.2
Overall (2019-2022)	**80**	**19.6 ± 8.9**	**11.0 ± 3**	**7.9 ± 0.4**	**8.3 ± 2.5**	**21.0 ± 17.7**

[a]Seasons defined as: spring (March, April, May); summer (June, July, August); fall (September, October, November); winter (December, January, February).

[b]Stations included in each section: Upper (Chester River, Sandy Point, South River, Cox Creek), Mid (Miles River, Broad Creek, Choptank River, Upper Patuxent River, Lower Patuxent River, Wicomico River), and Lower (St Mary's River, Tangier Sound).

Table 3. Number of *V. parahaemolyticus* and *V. vulnificus* isolates and relative abundance (%) of species-specific and virulence factors (*V. parahaemolyticus*: *tlh*$^+$, *tdh*$^+$, *trh*$^+$; *V. vulnificus*: *vvha*$^+$, *vcg*E$^+$, *vcg*C$^+$, *pil*A$^+$, *rtx*A$^+$) by longitudinal sampling period (2009-2012 and 2019-2022) and season.

Study/season	*V. parahaemolyticus*				*V. vulnificus*					
	N	***tlh*$^+$ (%)**	***tdh*$^+$ (%)**	***trh*$^+$ (%)**	**N**	***vvha*$^+$ (%)**	***vcgE*$^+$ (%)**	***vcgC*$^+$ (%)**	***pilA*$^+$ (%)**	***rtxA*$^+$ (%)**
2009-2012										
Spring	79	100	9	9	71	85	---*	---*	39	45
Summer	391	100	2	2	311	97	---*	---*	17	40
Fall	153	100	0	1	182	99	---*	---*	17	48
Winter	92	100	7	7	1	100	---*	---*	0	0
Overall	**715**	**100**	**3**	**3**	**565**	**96**	---*	---*	**20**	**41**
2019-2022										
Spring	37	88	0	9	9	87	87	0	11	0
Summer	362	97	0.1	0.3	133	96	92	12	44	0.6
Fall	274	100	0.2	0.2	95	97	85	27	27	5
Winter	88	100	0	0	4	67	67	17	33	0
Overall	**761**	**97**	**0.1**	**2**	**241**	**94**	**88**	**17**	**36**	**2**

* Data not available.

Chapter 3 Figures

Figure 1. Map of the Chesapeake Bay, Maryland, showing locations for both longitudinal sampling periods (2009-2012 and 2019-2022).

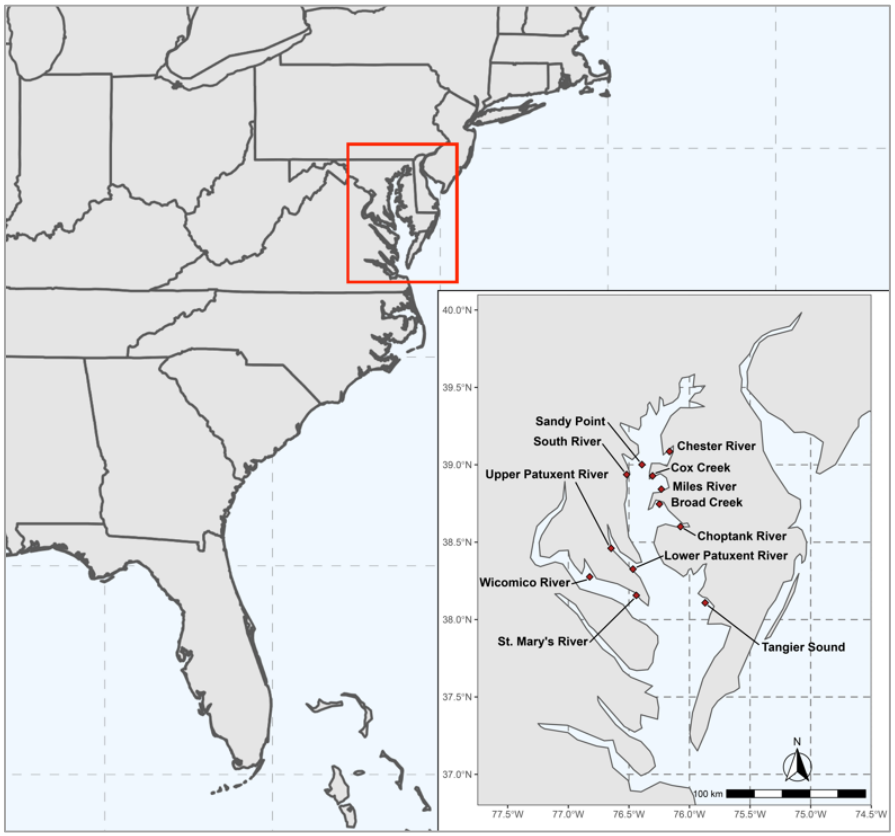


Figure 2. Average relative abundance of *Vibrio* spp. virulence factors (*V. parahaemolyticus*: *tdh*, *trh*; *V. vulnificus*: *vcg*C, *pil*A, *rtx*A) by section location from both sampling periods combined. Stations included in each section: Upper (Chester River, Sandy Point, South River, Cox Creek), Mid (Miles River, Broad Creek, Choptank River, Upper Patuxent River, Lower Patuxent River, Wicomico River), and Lower (St Mary's River, Tangier Sound).

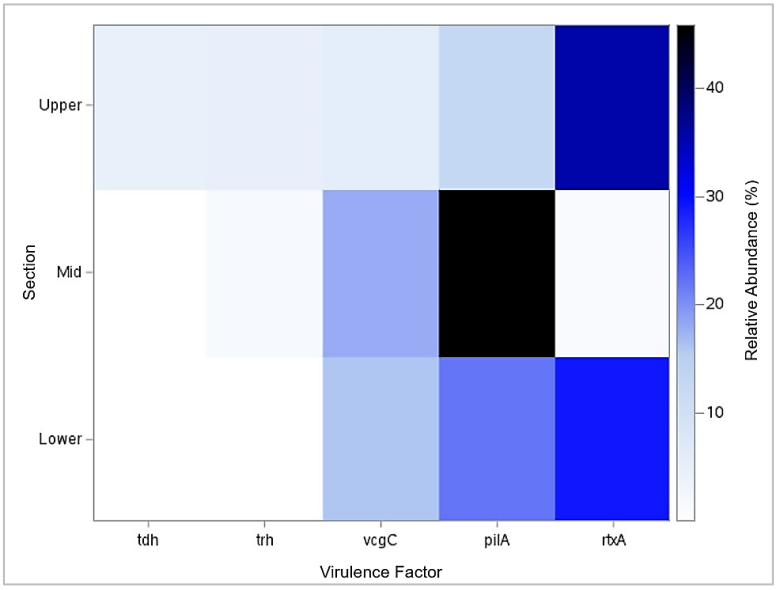
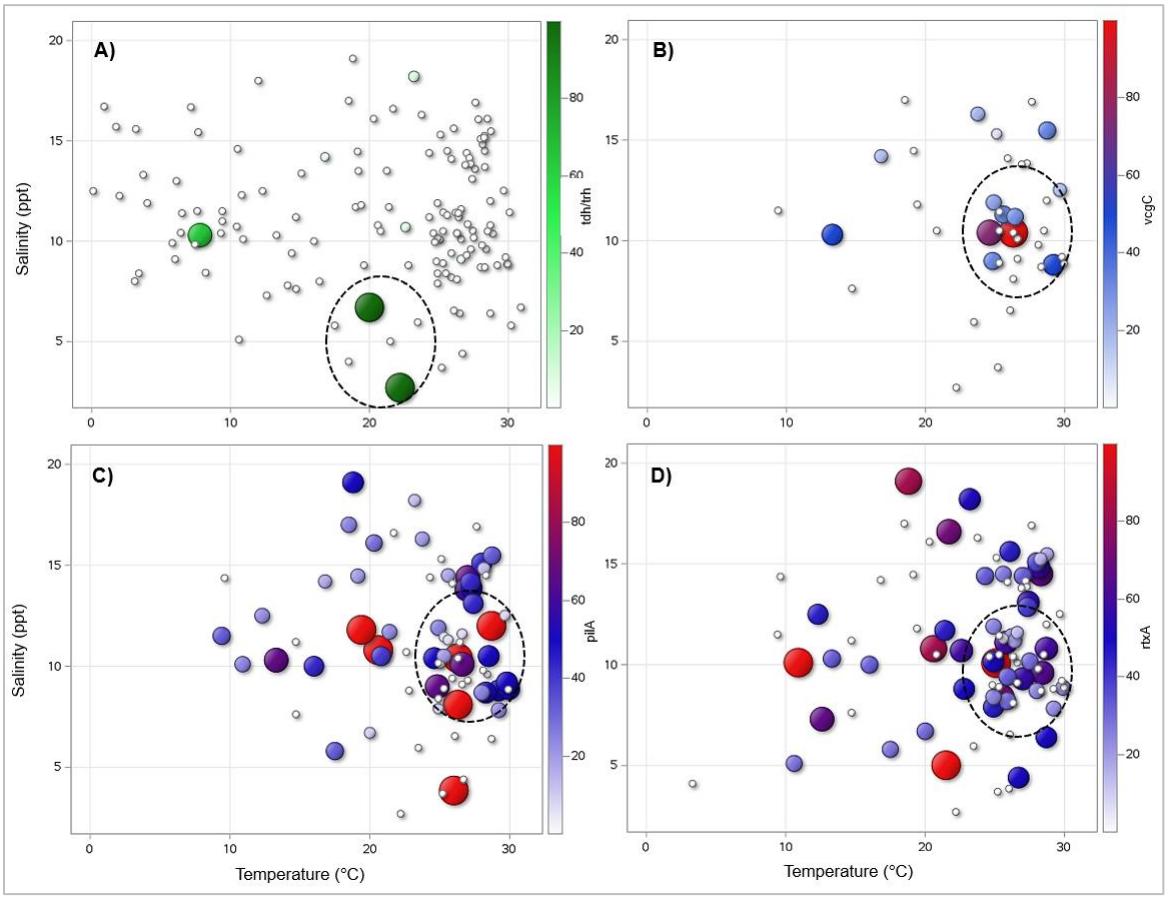


Figure 3. Association between average temperature/salinity and the occurrence of *V. parahaemolyticus* and *V. vulnificus* virulence factors for both sampling periods combined. *Vcg*C data only from 2019-2022. Disks in each bubble plot represent a single sampling event; disk size and color are indicative of detection and average relative abundance in percentage of *tdh* and/or *trh* (A), *vcg*C (B), *pil*A (C), and *rtx*A (D). The smallest white disks represent sampling events that did not detect the virulence factors but reported isolates positive for either *tlh* (*V. parahaemolyticus*) or *vvha* (*V. vulnificus*). The dotted black circles denote an area of greater virulence factor aggregation.



Chapter 4: Long-Term Antibiotic Resistance Trends Among *Vibrio vulnificus* and *Vibrio parahaemolyticus* Isolated from the Chesapeake Bay, Maryland: A Longitudinal Study

(Michele E. Morgado, Kyle D. Brumfield, Suhana Chattopadhyay, Leena Malayil, Taiwo Alawode, Ibiyinka Amokeodo, Xin He, Anwar Huq, Rita R. Colwell, Amy R. Sapkota)

Abstract

Antibiotics are often used to treat severe *Vibrio* infections, with third generation cephalosporins and tetracyclines combined or fluoroquinolones alone being recommended by the US Centers for Disease Control and Prevention. Increases in antibiotic resistance of both environmental and clinical vibrios are of concern; however, limited longitudinal data have been generated among environmental isolates to inform how resistance patterns may be changing over time. Hence, we evaluated long-term trends in antibiotic resistance of vibrios isolated from Chesapeake Bay waters (Maryland) across two three-year sampling periods (2009-2012 and 2019-2022). *V. parahaemolyticus* (n=134) and *V. vulnificus* (n=94) t*ox*R-confirmed isolates were randomly selected from both sampling periods and tested for antimicrobial susceptibility against eight antibiotics using the Kirby-Bauer disk diffusion method. A high percentage (94-96%) of *V. parahaemolyticus* isolates from both sampling periods were resistant to ampicillin and only 2-6% of these isolates expressed intermediate resistance or resistance to third generation cephalosporins, amikacin, tetracycline, and trimethoprim-sulfamethoxazole. Even lower percentages of resistant

V. vulnificus isolates were observed and those were mostly recovered from 2009-2012. The frequency of multi-drug resistance was relatively low (6-8%) but included resistance against antibiotics used to treat severe vibriosis in adults (e.g., third generation cephalosporins and tetracycline) and children (e.g., trimethoprim-sulfamethoxazole and amikacin). All isolates were susceptible to ciprofloxacin, a fluoroquinolone, indicating its sustained efficacy as a first-line agent in the treatment of severe vibriosis. Overall, our data indicate that antibiotic resistance patterns among *V. parahaemolyticus* and *V. vulnificus* recovered from the lower Chesapeake Bay have remained relatively stable since 2009.

Importance

Vibrio spp. have historically been susceptible to most clinically-relevant antibiotics; however, resistance and intermediate-resistance has been increasingly recorded in both environmental and clinical isolates. Our data showed that while the percentage of multi-drug resistance and resistance to antibiotics was relatively low, *Vibrio* isolates displayed resistance and intermediate resistance to antibiotics typically used to treat severe vibriosis (e.g., third generation cephalosporins, tetracyclines, sulfamethoxazole-trimethoprim, and aminoglycosides). Also, given the high case fatality rates observed with *V. vulnificus* infections, the presence of multiple virulence factors in the tested isolates is concerning. Nevertheless, the continued susceptibility for all tested isolates against ciprofloxacin, a fluoroquinolone, is indicative of its use as an effective first-line treatment of severe infections stemming from exposure to Chesapeake Bay waters. Overall, our data indicate that antibiotic resistance patterns

among *V. parahaemolyticus* and *V. vulnificus* recovered from the lower Chesapeake Bay have remained relatively stable since 2009.

Introduction

Non-cholera *Vibrio* spp., primarily pathogenic *Vibrio vulnificus* and *Vibrio parahaemolyticus*, are responsible for an estimated 80,000 illnesses and 100 fatalities each year in the United States (Centers for Disease Control and Prevention (CDC), 2019). These Gram-negative bacteria are causative agents of gastroenteritis, wound infections, and primary septicemia associated with seafood consumption and brackish or ocean water exposure (Daniels & Shafaie, 2000; Horseman & Surani, 2011; Iwamoto et al., 2010; Morris & Black, 1985). Although more than half of all cases of *Vibrio* illness (vibriosis) in the U.S. can be attributed to the ingestion of *V. parahaemolyticus* contaminated shellfish (Iwamoto et al., 2010; Ndraha et al., 2020; Shapiro et al., 1998), severe and fatal cases are more frequent following *V. vulnificus* infection from water exposure (Baker-Austin et al., 2010; Baker-Austin & Oliver, 2018; Oliver, 2005, 2013). Individuals with underlying medical conditions, such as diabetes, liver disease and immunocompromised systems, are at a greater risk of acquiring severe vibriosis (Daniels & Shafaie, 2000; Iwamoto et al., 2010; Oliver, 2005). Most infections occur during the summer months, when water temperatures are highest (Altekruse et al., 2000; Baker-Austin et al., 2018; Daniels et al., 2000; Shapiro et al., 1998), with noted increasing annual incidence rates during the last few decades (A. Newton et al., 2012; Vugia et al., 2013; Wong et al., 2015).

While the majority of vibriosis cases are mild and self-limiting and do not require clinical treatment, antibiotics are typically used to treat more severe infections

and prompt administration, usually within 24-48 hrs., significantly improves case-fatality rates (Baker-Austin et al., 2018; Oliver, 2005). Tetracycline and third-generation cephalosporins (e.g., cefotaxime, ceftazidime) have been traditionally used in the treatment of both primary septicemia and wound infections caused by *V. vulnificus* and to a lesser degree *V. parahaemolyticus* (Oliver, 2005; Tang et al., 2002; Wong et al., 2015). According to Tang et al. (2002), cephalosporins and tetracyclines (including doxycycline) combined, rather than single drug regimens, may be more effective at treating severe *V. vulnificus* infections (18). In addition, newer fluoroquinolones (e.g., ciprofloxacin and levofloxacin), which display greater potency and a broader spectrum of antimicrobial activity and effectiveness, have been suggested as an alternative single agent treatment for severe vibriosis (Tang et al., 2002). Indeed, results from a long-term analysis of the U.S. Centers for Disease Control and Prevention's (CDC) *Vibrio* surveillance data, showed that mortality rates for *V. vulnificus* were significantly lower in patients taking either a fluoroquinolone only or a tetracycline combined with a third-generation cephalosporin (Wong et al., 2015). Conversely, for children in whom doxycycline and fluoroquinolones are contraindicated, trimethoprim-sulfamethoxazole plus an aminoglycoside (e.g., amikacin, gentamicin) are recommended instead (Centers for Disease Control and Prevention (CDC), 2023b).

Although *Vibrio* spp. are considered susceptible to common-use antibiotics (Oliver, 2005; Tang et al., 2002), resistance and intermediate-resistance has been increasingly recorded in both environmental (from seafood and seawater samples) and clinical isolates (Baker-Austin, McArthur, et al., 2009; Bier et al., 2015; Canellas

et al., 2021; da Silva et al., 2021; Das Sarker et al., 2019; Elmahdi et al., 2018; Håkonsholm et al., 2020; Han et al., 2007; Jiang et al., 2014; Lopatek et al., 2018; Shaw, Rosenberg Goldstein, et al., 2014; Tan et al., 2020). Notably, a high percentage of resistance to penicillin and ampicillin has been observed in both *V. parahaemolyticus* and *V. vulnificus* (Han et al., 2007; Shaw, Rosenberg Goldstein, et al., 2014), and previous studies have suggested that these antibiotics may no longer be effective as a single antibiotic treatment for vibriosis (Ngasotter et al., 2022; Wong et al., 2015). Excessive use of antibiotics in humans, aquaculture, and agricultural settings (e.g., poultry farms) plays a major role in the selection of antibiotic resistance among many bacterial genera, including *Vibrio* spp. (Heng et al., 2017; Ngasotter et al., 2022). The persistence of antibiotics in aquatic environments, which function as critical reservoirs, promotes the evolution and transfer of antibiotic resistance genes among bacterial species and subsequently across the food chain (Kumarage et al., 2022).

Shaw et al. (2014) completed the most recent antimicrobial susceptibility study of *V. parahaemolyticus* and *V. vulnificus* environmental isolates recovered from Chesapeake Bay waters in Maryland, and included strains collected during the summer of 2009 (21). Results indicated that while antibiotics used to treat adult vibriosis were fully effective at suppressing the growth of recovered isolates, pediatric-use antibiotics (e.g., aminoglycosides such as amikacin, apramycin, and streptomycin) were less so. Moreover, low-level intermediate resistance to newer generation cephalosporins was also observed. However, it’s unclear whether

antibiotic resistance patterns in environmental *Vibrio* isolates recovered from the Chesapeake Bay have changed over time.

To address this data gap, we conducted a longitudinal study to analyze trends in antibiotic resistance of *V. parahaemolyticus* and *V. vulnificus* isolates collected from Chesapeake Bay waters during two three-year sampling periods that took place a decade apart (2009-2012 and 2019-2022). Of particular interest were the potential antimicrobial resistance patterns associated with changes in environmental parameters or the presence of virulence factors.

Methods

Site description and source selection

Water samples were collected from Tangier Sound in the Chesapeake Bay, Maryland, USA, during two separate three-year sampling events, namely 2009-2012 and 2019-2022. For spatial-temporal comparison purposes, only isolates from water samples collected during the summer months (June, July, August) were selected for this study. Tangier Sound (38°10.97'N, 75°57.90'W) is a mesohaline region of the lower Chesapeake Bay, just west of Maryland's southernmost bay county of the Eastern Shore (Somerset County) and close to the Virginia border (**Fig. 1**). The land area adjacent to Tangier Sound is characterized by heavy agricultural use and ranks first in the State of Maryland for broiler poultry production (University of Maryland Extension, 2023). Tangier Sound is also a popular location for recreational and commercial fishing, including crabbing and oyster harvesting (Paolisso, 2002).

Sample collection and processing

Methods used for sample collection and processing have been previously described by Brumfield et al. (Brumfield et al., 2023), Chen et al. (Chen et al., 2017) and Johnson et al. (Johnson et al., 2010, 2012); a summary of methods related to this study are provided here. Physical and chemical measurements were collected during each sampling event, including water temperature, pH, dissolved oxygen, salinity, and chlorophyll-*a*. Water samples (12 L) were collected just below the surface, transported back to the laboratory on ice, and kept refrigerated overnight until processing the following morning. Collected water was shaken, and three volumes (1000 mL, 100 mL, 10 mL) were inoculated into 10X alkaline peptone water (APW, pH 8.5) and incubated at 33°C for 16-18 hr, with shaking at 30 rpm. Following incubation, a loopful was removed from each sample and streaked individually into selective media, including CHROMagar™ (CHROMagar, Springfield, NJ), thiosulfate citrate bile salts sucrose agar (TCBS, Oxoid, Ontario, Canada), and *V. vulnificus* agar (VVA). The plates were incubated at 37 °C for 16-18 hr and based on growth media, presumptive colonies of *V. parahaemolyticus* and *V. vulnificus* were picked and streaked onto LB agar (BD Diagnostic Systems, Sparks, MD) to obtain pure cultures. Bacterial isolates were stored at −80°C in LB broth containing 50% (vol/vol) glycerol.

Vibrio species confirmation

DNA was extracted from presumptive isolates of *V. parahaemolyticus* and *V. vulnificus* following methods described in Chen et al. (Chen et al., 2017) and confirmed using PCR targeting the *tox*R gene adapted from Bauer and Rørvik (Bauer

& Rørvik, 2007) to differentiate between the two vibrios. Additionally, samples testing positive for either species were further tested for species-specific genes and virulence markers (*V. parahaemolyticus*: thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), thermostable direct-related hemolysin (*trh*); *V. vulnificus*: hemolysin cytolysin (*vvha*), virulence correlated gene environmental variant (*vcg*E), virulence correlated gene clinical variant (*vcg*C), RTX toxin (*rtx*A), type IV pili (*pil*A)). *Vcg*C^{+} data were not available for the *V. vulnificus* isolates selected from the 2009-2012 longitudinal study.

PCR assays were performed using Promega GoTaq Green Master Mix 2x (Promega, Madison, WI, U.S.); each reaction well contained a total of 25 µl, including 20 µl of master mix solution (12.5 µl of GoTaq, 1 µl of each primer, and nuclease-free water to reaction volume) and 5 µl DNA template. The primer sequences, amplicon sizes, and conditions used for each PCR assay can be found in **Table 1**.

PCR products were kept at 4°C until gel electrophoresis visualization. Positive controls included *V. parahaemolyticus* ATCC 17803 (*tox*R^{+}), NIHCB0757 (*tlh*$^{+}$/*tdh*$^{+}$), and AQ 4037 (*tlh*$^{+}$/*trh*$^{+}$); and *V. vulnificus* ATCC 27562 (*tox*R^{+}/*vcg*E^{+}/*vvh*A^{+}), and ATCC 29307 (*vcg*C^{+}/*pil*A^{+}/*rtx*A^{+}). Nuclease-free water was used as a negative control in each reaction. PCR products were visualized using a 1.5% agarose gel at 110V for 60-90min and viewed under a UV transilluminator using a Gel Documentation System (GelDoc-IT^{TM}, UVP, LLC, CA, U.S.).

Antimicrobial Susceptibility Testing

A total of n=134 *V. parahaemolyticus tox*R-confirmed isolates (n=84 from 2009-2012; n=50 from 2019-2022) and n=94 *V. vulnificus tox*R-confirmed isolates (n=51 from 2009-2012; n=43 from 2019-2022) were subjected to antimicrobial susceptibility testing. These isolates were randomly selected from samples recovered from Tangier Sound during the summer season (June, July, and August) during both sampling periods. All tested isolates were also positive for their respective species identifying markers, namely the *tlh* marker (*V. parahaemolyticus*) and the *vvhA* marker (*V. vulnificus*). Isolates kept at -80°C were streaked onto Luria-Bertani (LB) agar (Miller, USA) plates and incubated at 37°C between 16-18hr overnight. Antibiotic susceptibility testing was carried out using the Kirby-Bauer disk diffusion method on Muller-Hinton (MH) agar (BD, USA), according to Clinical and Laboratory Standards Institute guidelines for *Vibrio* spp. (Clinical and Laboratory Standards Institute (CLSI), 2015) and *Enterobacteriaceae* (Clinical and Laboratory Standards Institute (CLSI), 2020). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains; pure water was used as a negative control.

All isolates were tested for susceptibility to 8 antibiotics from 7 different antibiotic classes frequently used to treat severe *Vibrio* spp. infections (Wong et al., 2015). This included ampicillin (AMP, 10 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), imipenem (IPM, 10 μg), amikacin (AK, 30 μg), tetracycline (TE, 30 μg), ciprofloxacin (CIP, 5 μg), and trimethoprim-sulfamethoxazole (SXT, 23.75 and 1.25 μg, respectively). The multiple antibiotic resistance (MAR) index was calculated

by dividing the number of antibiotics to which the isolate expressed resistance (x) by the total number of antibiotics to which the isolate was tested (y) (Krumperman, 1983). A MAR index greater than 0.2 suggests an area with increased sources of antibiotic contamination in the environment, and a greater likelihood for the spread of antibiotic resistance genes among bacterial pathogens (Canellas et al., 2021; Krumperman, 1983). Isolates were further classified as multi-drug resistant (MDR) when they expressed resistance to two or more antibiotic classes. Isolates that could not be revived during antimicrobial susceptibility testing were omitted from the data analysis (n=16 *V. vulnificus* from the 2019-2022 study period).

Data Analysis

The Wilcoxon rank sum test and the Kruskal-Wallis test were used to assess whether the environmental water parameters measured in each longitudinal sampling period (2009-2012 vs. 2019-2022) and month differed significantly from each other. The Wilcoxon signed rank test as well as the Friedman test (non-parametric repeated measures ANOVA) were used to examine the effects of the longitudinal sampling period (2009-2012 vs. 2019-2022), month and year on the MAR index of *V. parahaemolyticus* and *V. vulnificus* isolates. Linear mixed models (LMM) were also developed to characterize the effect of sampling month, year, and changes to environmental water parameters (i.e., water temperature, salinity, pH, dissolved oxygen, and chlorophyll-*a*) over time on the MAR index in both studies combined. Data were aggregated by month for *V. parahaemolyticus*, and models were assessed adding each environmental water parameter at a time and evaluating the lowest AIC, simplest model, estimates of coefficients, confidence limits and *p*-values. Random

intercept was used as a random effect, and year was the repeated measure. LMM were not developed for *V. vulnificus* due to insufficient data available. Correlation and regression analyses were performed to examine the relationship between *V. vulnificus* virulence markers and the MAR index. Statistical analyses were performed using SAS 9.4 (Cary, NC USA).

Results

Water quality parameters

The average water temperature, salinity, pH, and dissolved oxygen concentrations were relatively uniform between longitudinal sampling periods (2009-2012 and 2019-2022) during the summer season (June, July, and August) (**Table 2**). On the other hand, chlorophyll-*a* concentrations were significantly different between sampling periods ($p < 0.0001$), with higher overall concentrations observed during the 2019-2022 time period (**Table 2**).

During the 2009-2012 sampling period, dissolved oxygen and chlorophyll-*a* concentrations were not significantly different between months. Whereas water temperature, salinity and pH were significantly different across each month ($p < 0.001$). The highest average water temperature, salinity and pH were recorded during the month of August, while the lowest were recorded during the month of June (**Table 2**). During the 2019-2022 sampling period, pH concentrations were not significantly different between months. Conversely, water temperature, salinity, dissolved oxygen, and chlorophyll-*a* were significantly different across each month ($p < 0.001$) (**Table 2**). The highest average water temperature and salinity were observed during the months of July and August, respectively, while the lowest were recorded

during the month of June. Average dissolved oxygen and chlorophyll-*a* concentrations were highest during the month of June, and lowest during the month of August and July, respectively (**Table 2**).

Vibrio spp. Virulence Factors

All tested *V. parahaemolyticus* isolates were negative for the presence of *V. parahaemolyticus* associated virulence factors, *tdh* and *trh* (**Table 3**). The prevalence of *V. vulnificus* associated virulence factors varied between sampling periods, with the exception of *pilA* which was present in roughly the same number of isolates during each sampling period (n=14% and n=15%, 2009-2012 and 2019-2022, respectively) (**Table 3**). The prevalence of *pilA* was highest in isolates collected in June during the earlier sampling period (n=40%) and August during the later sampling period (n=22%). *VcgC* was present in 15% of isolates recovered from 2019-2022, particularly during the month of August (n=22%), but data were not available for the isolates collected in 2009-2012 (**Table 3**). The prevalence of *rtxA* was much greater in isolates from the 2009-2012 sampling period (n=33%), especially in the month of June (n=50%), but it was only present in 4% of isolates collected during the later sampling period (2019-2022), and only during August (**Table 3**).

Antimicrobial Resistance in V. parahaemolyticus

During the 2009-2012 sampling period, all *V. parahaemolyticus* isolates tested were fully susceptible to 2 antibiotics (ciprofloxacin and trimethoprim-sulfamethoxazole) out of the 8 antibiotics tested (**Fig. 2**). During the 2019-2022 sampling period, all *V. parahaemolyticus* were fully susceptible to 3 antibiotics

(imipenem, tetracycline, and ciprofloxacin). Intermediate resistance was infrequently observed among the *V. parahaemolyticus* isolates tested, with the greatest intermediate resistance observed against ampicillin (2009-2012: 1%, 2019-2022: 4%) and amikacin (both sampling periods: 4%) (**Fig. 2**).

In terms of complete resistance, a high percentage of resistance was observed during both sampling periods for ampicillin (2009-2012: 94%, 2019-2022: 96%). Lower percentages of resistance were also seen against tetracycline (2009-2012: 6%), cefotaxime (2019-2022: 2%), ceftazidime (both sampling periods: 2%), amikacin (2019-2022: 2%), and ciprofloxacin (2019-2022: 2%) (**Fig. 2**).

Antimicrobial Resistance in V. vulnificus

During the 2009-2012 sampling period, all tested *V. vulnificus* isolates were susceptible to 1 antibiotic (ciprofloxacin) out of the 8 antibiotics tested (**Fig 3**). During the 2019-2022 sampling period, all *V. vulnificus* were susceptible to 7 antibiotics (ampicillin, cefotaxime, imipenem, amikacin, tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole) (**Fig. 3**).

Low levels of intermediate resistance were observed among the tested *V. vulnificus* isolates with regard to ceftazidime (2019-2022: 4%), imipenem (2009-2012: 2%), amikacin (2009-2012: 2%), and tetracycline (2009-2012: 4%) (**Fig. 3**). In terms of complete resistance, the 2019-2022 isolates did not express resistance against any of the antibiotics tested, while the 2009-2012 isolates expressed low levels of resistance to ampicillin (16%), cefotaxime (6%), trimethoprim-sulfamethoxazole (4%), ceftazidime (2%), and amikacin (2%) (**Fig. 3**).

Multiple Antibiotic Resistance Profile for Vibrio spp.

The overall percentage of multi-drug resistant *V. parahaemolyticus* isolates was similar between both sampling periods: 8% during 2009-2012 and 6% during 2019-2022 (**Fig. 4**). However, trends differed by month; August had a greater percentage of MDR *V. parahaemolyticus* isolates in the earlier sampling period (n=14%), while in the later sampling period the month of June had the highest percentage of MDR isolates (n=17%) (**Fig. 4**).

During the 2009-2012 sampling period, 8% of *V. vulnificus* isolates were multi-drug resistant, with the greatest percentage of MDR isolates (20%) collected during the month of June, whereas the *V. vulnificus* isolates selected from 2019-2022 did not display multi-drug resistance (**Fig. 4**).

The MAR index for isolated *V. parahaemolyticus* from both sampling periods (2009-2012 and 2019-2022, n=134) ranged between 0 and 0.38, with an average of 0.13 and with 10 isolates (7%) exhibiting a MAR value greater than 0.2 (**Table 4**). Resistance to ampicillin was common for all 10 isolates and most frequently combined with resistance against tetracycline (n=5). Only 1 isolate was resistant to more than 2 antibiotics (ampicillin, cefotaxime, and ceftazidime) with a MAR value of 0.38 (**Table 4**).

The MAR index for isolated *V. vulnificus* from both sampling periods (2009-2012 and 2019-2022, n=78) also ranged between 0 and 0.38, with an average of 0.02 and with 4 isolates (5%) exhibiting a MAR value greater than 0.2 (**Table 4**). Resistance against ampicillin and cefotaxime was observed in 3 out of 4 of these

isolates. A MAR value of 0.38 was observed in 2 isolates with resistance against ampicillin, cefotaxime, and trimethoprim-sulfamethoxazole (**Table 4**).

MAR Index Analysis

The MAR index was not found to be statistically significantly different between the two sampling periods (2009-2012 vs. 2019-2022) for tested *V. parahaemolyticus* isolates but was statistically significantly different between sampling periods for *V. vulnificus* (p=0.004); with the earlier time period characterized by a higher overall MAR index average. The sampling month, after adjusting for repeated measures over time (year), did not significantly affect the MAR index among *V. parahaemolyticus* (p=0.756) or *V. vulnificus* (p=0.737). The sampling year, after adjusting for repeated measures over time (month), did not significantly impact the MAR index among *V. parahaemolyticus* (p=0.359) or *V. vulnificus* (p=0.065).

Linear mixed models developed for tested *V. parahaemolyticus* isolates, while controlling for repeated measures of year and month, did not yield statistically significant effects for any environmental water parameter (e.g., water temperature, salinity, dissolved oxygen, salinity, pH, chlorophyll-*a*), or combination of miscellaneous parameters on the MAR index ($p > 0.05$).

V. vulnificus virulence factors (*vcg*C, *pilA*, *toxA*) and the MAR index were not significantly correlated, nor did they display a significant linear relationship ($p > 0.05$).

Discussion

This study represents the first long-term survey of antibiotic resistance among environmental *Vibrio* spp. isolates recovered from the Chesapeake Bay. Consistent with earlier studies (Han et al., 2007; Shaw, Rosenberg Goldstein, et al., 2014), a high percentage of ampicillin resistance was found in *V. parahaemolyticus* isolates during both sampling periods, 2009-2012 and 2019-2022, especially in the latter where all tested isolates expressed resistance (96%) or intermediate resistance (4%). As mentioned by Han et al. (Han et al., 2007) and others (Han et al., 2007; Shaw, Rosenberg Goldstein, et al., 2014; Wong et al., 2015), given the high levels of resistance found in *V. parahaemolyticus* against ampicillin, penicillins are likely not effective as a single use antibiotic to treat severe vibriosis and are no longer recommended by the CDC for this purpose (Centers for Disease Control and Prevention (CDC), 2023b). Ampicillin resistance was also observed for *V. vulnificus* isolates in the current study, but only for samples collected during 2009-2012 (16%). This percentage was higher than previously reported for Chesapeake Bay waters in 2014, where only 1% of *V. vulnificus* isolates were resistant against ampicillin (Shaw, Rosenberg Goldstein, et al., 2014), and is closer to findings by Elmahdi et al. (Elmahdi et al., 2018) where 26% of *V. vulnificus* recovered from oysters collected in Maryland in 2018 were ampicillin-resistant.

Of the CDC recommended antibiotics used to treat severe vibriosis (tetracyclines combined with third generation cephalosporins, fluoroquinolones alone, and aminoglycosides combined with trimethoprim-sulfamethoxazole), only fluoroquinolones demonstrated full efficacy against all *V. parahaemolyticus* and *V.*

vulnificus isolates tested (Centers for Disease Control and Prevention (CDC), 2023b). These findings are similar to previous studies on the East Coast of the U.S. that found that most or all environmental *Vibrio* spp. tested were susceptible to fluoroquinolones (Baker-Austin, Mcarthur, et al., 2009; da Silva et al., 2021; Elmahdi et al., 2016; Shaw, Rosenberg Goldstein, et al., 2014) and it suggests, as recommended by Wong et al. (Wong et al., 2015), that fluoroquinolones should be considered by health care professionals as a first-line agent to treat severe and life-threatening vibriosis. Notwithstanding, increased levels of intermediate resistance and resistance to ciprofloxacin (fluoroquinolone), ranging from 7-67%, have been documented for *V. parahaemolyticus, V. alginolyticus,* and *V. harveyi* isolated from seafood in Malaysia (Tan et al., 2020) and India (Sony et al., 2021) as well as from seawater samples in Brazil (Canellas et al., 2021), indicating the ability for fluoroquinolone resistance to be acquired by *Vibrio* spp.

In terms of potential pathogenicity, all *V. parahaemolyticus* isolates tested negative for *tdh* and *trh* virulence genes. Although present in a large percentage of clinical strains, these genes are often found in less than 1% of all environmental strains (DePaola et al., 2003; Elmahdi et al., 2018), and may not always reflect the bacteria's true ability to cause illness (Mahoney et al., 2010). Namely, Mahoney et al. (2010) found that many environmental isolates lacking *tdh* and *trh* were still highly cytotoxic to human gastrointestinal cells and had the ability to horizontally acquire and regulate new virulence factors (Mahoney et al., 2010). On the other hand, *V. vulnificus* isolates from both sampling periods tested positive for virulence factors believed to be important in causing disease, namely *vcg*C, *rtx*A, and *pil*A (Choi &

Choi, 2022; Li & Wang, 2020; Liu & Crosa, 2012; Rosche et al., 2010). The *vcgC* gene found in most pathogenic strains was present in 15% of the 2019-2022 isolates, which is similar to findings by Elmahdi et al. (Elmahdi et al., 2018) and Warner and Oliver (Warner & Oliver, 2008), who reported prevalence rates for the *vcg*C gene of 20.9% and 15.6%, respectively, among *V. vulnificus* recovered from oyster samples. *Pil*A was also found in approximately 15% of all environmental strains and at a similar rate during both sampling periods, while *rtx*A was more prevalent in the 2009-2012 isolates tested, 33% compared to only 4% for the 2019-2022 isolates. While a direct association between virulence factors and antimicrobial resistance has not been established, previous studies suggest that vibrios, including non-pathogenic strains, have the ability to simultaneously acquire virulence factors and antimicrobial resistance genes from other bacteria and their surrounding environment (Gao et al., 2022; Gennari et al., 2012; Pérez-Duque et al., 2021). In this study, we did not observe a significant correlation between the presence of virulence factors and the presence of multiple antibiotic resistance. However, our findings suggest that *V. vulnificus* isolates with the potential to cause disease may be prevalent in this important recreational and commercial watershed.

Comparing 2009-2012 to 2019-2022, the percentage of multi-drug resistant *V. parahaemolyticus* isolates found was not significantly different between sampling periods, 8% and 6%, respectively. Interestingly, the number of MDR strains observed increased throughout the summer season during the earlier sampling period but decreased in the latter, which may warrant further study into possible seasonal trends and temperature regulated antibiotic resistance. Multi-drug resistance in *V. vulnificus*

was also found in less than 10% of isolates but was only observed for samples from 2009-2012 and not for the later sampling period. Nonetheless, the absence of MDR *V. vulnificus* isolates between 2019-2022 may not be indicative of a decreasing trend in the lower Chesapeake Bay, but rather a result of the limited number of isolates tested (n=16 could not be revived). Overall, these results were similar to those observed in Maryland Coastal Bays by Shaw et al. (Shaw, Rosenberg Goldstein, et al., 2014) and Elmahdi et al. (Elmahdi et al., 2018) but lower than findings from Da Silva et al. (da Silva et al., 2021) and Baker-Austin et al (Baker-Austin, McArthur, et al., 2009), where approximately 40% of *V. parahaemolyticus* and *V. vulnificus* isolates were characterized by multi-drug resistance.

Importantly, the percentage of isolates displaying a MAR index greater than 0.2 was relatively low for both *V. parahaemolyticus* and *V. vulnificus* from both sampling periods combined, namely 7% and 5%, respectively. Furthermore, only 1 *V. parahaemolyticus* isolate and 2 *V. vulnificus* isolates were resistant against more than two antibiotics, which included ampicillin, 3rd generation cephalosporins, and sulfamethoxazole-trimethoprim. As mentioned previously, a MAR index greater than 0.2 is indicative of an area with increased sources of antibiotic contamination in the environment, and a greater likelihood for the spread of antibiotic resistance genes among bacterial pathogens (Canellas et al., 2021; Krumperman, 1983). Although Tangier Sound is adjacent to a land-area with a history of heavy agricultural use and ranks first in the State of Maryland for broiler poultry production (University of Maryland Extension, 2023), our findings did not demonstrate the same high prevalence of antimicrobial resistance as in other comparable sites (Baker-Austin,

McArthur, et al., 2009; da Silva et al., 2021; Shaw, Rosenberg Goldstein, et al., 2014). However, it should be noted that Tangier Sound, which is located in the lower Chesapeake Bay, may benefit from increased tidal flow compared to other sites in the upper and mid-Bay or in more inland waterways (Valle-Levinson et al., 2003) and may not be representative of conditions across the Bay or throughout the seasons.

Limitations of our study included the selection of *Vibrio* spp. isolates that were collected only during the peak vibrio abundance season, which restricted our ability to analyze antimicrobial resistance trends throughout the year. Moreover, our analysis included water samples that were processed using culture-dependent methods with selective media, which has been shown to result in more false negatives during PCR testing of virulence factors (Parveen et al., 2020). Of the *V. vulnificus* isolates selected from the 2019-2022 sampling period, 16 could not be revived and our results may underrepresent changes to antimicrobial resistance during this time period.

Acknowledgements

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Chapter 4 Tables

Table 1. List of primer sequences, amplicon sizes, and PCR conditions used for the detection of *V. parahaemolyticus* and *V. vulnificus* species-specific genes and virulence genes.

Primers	Primer sequence (5′-3′)	Amplicon (bp)	PCR conditions	Source
utox-F	GASTTTGTTTGGCGYGARCAAGGTT			
vptox-R	GGTTCAACGATTGCGTCAGAAG	297	95°C for 4 m; 34x: 95°C for 30 s, 55°C for 30 s, 72°C for 60 s; 72°C 5 m	(Bauer & Rørvik, 2007)
vvtox-R	AACGGAACTTAGACTCCGAC	435	95°C for 4 m; 30x: 95°C for 30 s, 60°C for 30 s, 72°C for 60 s; 72°C 7 m	
tlh-F	AAAGCGGATTATGCAGAAGCACTG	173	94°C for 3 m; 30x: 94°C for 60 s, 58°C for 60 s, 72°C for 60 s; 72°C 5 m	(Bej et al., 1999; Rizvi & Bej, 2010)
tlh-R	TGTGCCTTGATGAACTCGTTC			
tdh-F	GTAAAGGTCTCTGACTTTTGGAC	270		
tdh-R	TGGAATATGAACCTTCATCTTCACC			
trh-F	TTGGCTTCGATATTTTCAGTATCT	500		
trh-R	CATAACAAACATATGCCCATTTCCG			
vvh-F	AGCGGTGATTTCAACG	411	94°C for 3 m; 34x: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C 5 m	(Brasher et al., 1998; Panicker et al., 2004; Panicker & Bej, 2005; Rosche et al., 2005; Warner & Oliver, 2008)
vvh-R	GGCCGTCTTTGTTCACT			
vcgC-F	AGCTGCCGATAGCGATCT	97	94°C for 3 m; 30x: 94°C for 40 s, 57°C for 40 s, 72°C for 40 s; 72°C 5 m	
vcgC-R	TGAGCTAACGCGAGTAGTGAG			
vcgE-F	CTCAGAAAGGCTCAATTGAC	199		
vcgE-R	GATTAACGCTGTAAGGCCG			
pilA-F	TGGCTGCTGTTGCTATTC	217	94°C for 3 m; 30x: 94°C for 60 s, 60°C for 60 s, 72°C for 60 s; 72°C 5 m	(Natividad-Bonifacio et al., 2013; Paranjpye & Strom, 2005)
pilA-R	GGTCCACCACTAGTACCAAC			
rtxA-F	CGGGATCCTATGGCGTGAACGGCGAAG	1440	94°C for 3 m; 30x: 94°C for 30 s, 68°C for 30 s, 72°C for 60 s; 72°C 5 m	(Y. R. Kim et al., 2008; B. C. Lee et al., 2008; Natividad-Bonifacio et al., 2013)
rtxA-R	CGGGATCCAGCAGCCACAAGCGATTC			

Table 2. Water quality characteristics by sampling period (2009-2012 and 2019-2022) and month, including average temperature (T), salinity (S), pH, dissolved oxygen (DO), and chlorophyll-*a* concentration (chla) with ± standard deviation.

Study/month	T (°C)	S	pH	DO (mg/l)	Chla (µg/L)
2009-2012					
June	25.5 ± 1.5	12.4 ± 2.1	7.4 ± 0.6	6.6 ± 1.0	12.8 ± 4.2
July	27.4 ± 1.0	13.8 ± 2.4	7.8 ± 0.4	7.0 ± 0.7	13.7 ± 4.5
August	28.0 ± 1.3	14.3 ± 1.9	8.0 ± 0.4	6.8 ± 0.4	12.3 ± 3.3
Overall	**27.1 ± 1.6**	**13.6 ± 2.0**	**7.8 ± 0.5**	**6.8 ± 0.7**	**13.1 ± 3.6**
2019-2022					
June	25.3 ± 0.0	11.4 ± 0.0	8.0 ± 0.0	7.6 ± 0.0	19.8 ± 0.0
July	29.1 ± 1.6	13.8 ± 3.1	7.9 ± 0.1	7.0 ± 0.3	13.7 ± 4.1
August	26.6 ± 2.5	15.4 ± 0.1	8.0 ± 0.1	6.6 ± 0.4	17.7 ± 2.5
Overall	**27.1 ± 2.0**	**13.9 ± 2.3**	**8.0 ± 0.1**	**7.0 ± 0.5**	**16.8 ± 5.0**

Table 3. Number of selected *V. parahaemolyticus* and *V. vulnificus* isolates and associated virulence factors (*V. parahaemolyticus*: *tdh*$^+$, *trh*$^+$; *V. vulnificus*: *vcg*C$^+$, *pil*A$^+$, *rtx*A$^+$) by sampling period (2009-2012 and 2019-2022) and month.

Study/month	*V. parahaemolyticus*			*V. vulnificus*			
	N	*tdh*$^+$ (%)	*trh*$^+$ (%)	N	*vcgC*$^+$ (%)	*pilA*$^+$ (%)	*rtxA*$^+$ (%)
2009-2012							
June	24	0	0	10	---*	40	50
July	32	0	0	18	---*	17	28
August	28	0	0	23	---*	0	30
Overall	**84**	**0**	**0**	**51**	**---***	**14**	**33**
2019-2022							
June	12	0	0	5	0	20	0
July	17	0	0	13	15	8	0
August	21	0	0	9	22	22	11
Overall	**50**	**0**	**0**	**27**	**15**	**15**	**4**

*Data not available.

Table 4. Multiple antibiotic resistance (MAR) index frequency among *V. parahaemolyticus* and *V. vulnificus* isolates and resistance profiles for both sampling periods (2009-2012 and 2019-2022) combined.

Vibrio	Resistance profile[a]	MAR index	Frequency # (%)
V. parahaemolyticus			
N=134	None	0.00	7 (5%)
	AMP10	0.13	117 (87%)
	AMP10, TE30	0.25	5 (4%)
	AMP10, CAZ30	0.25	2 (1%)
	AMP10, AK30	0.25	1 (1%)
	AMP10, SXT25	0.25	1 (1%)
	AMP10, CTX30, CAZ30	0.38	1 (1%)
V. vulnificus N=78			
	None	0.00	69 (88%)
	AMP10	0.13	5 (6%)
	AMP10, AK30	0.25	1 (1%)
	CTX30, CAZ30	0.25	1 (1%)
	AMP10, CTX30, SXT25	0.38	2 (3%)

[a]Abbreviations used: AK30, amikacin; AMP10, ampicillin; CAZ30, ceftazidime; CTX30, cefotaxime; SXT25, trimethoprim-sulfamethoxazole; TE30, tetracycline.

Chapter 4 Figures

Figure 1. Map of the Chesapeake Bay showing sampling site in Tangier Sound.

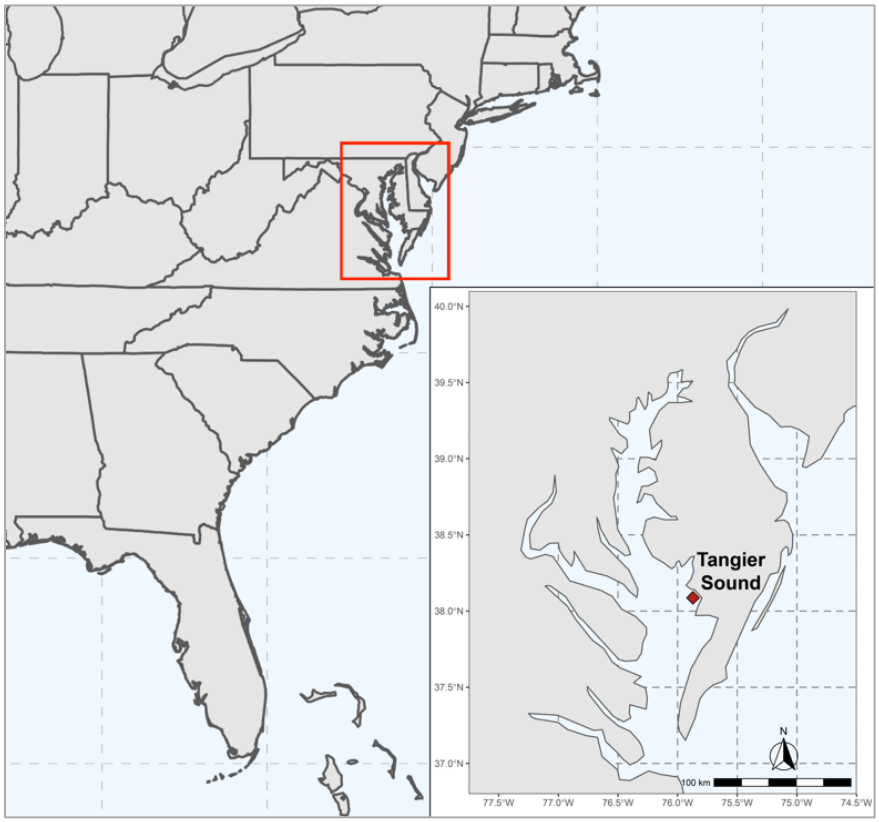


Figure 2. Antibiotic resistance patterns among *V. parahaemolyticus* isolates (n=134). List of abbreviations: AK30, amikacin; AMP10, ampicillin; CAZ30, ceftazidime; CIP5, ciprofloxacin; CTX30, cefotaxime; IPM10, imipenem; SXT25, trimethoprim-sulfamethoxazole; TE30, tetracycline.

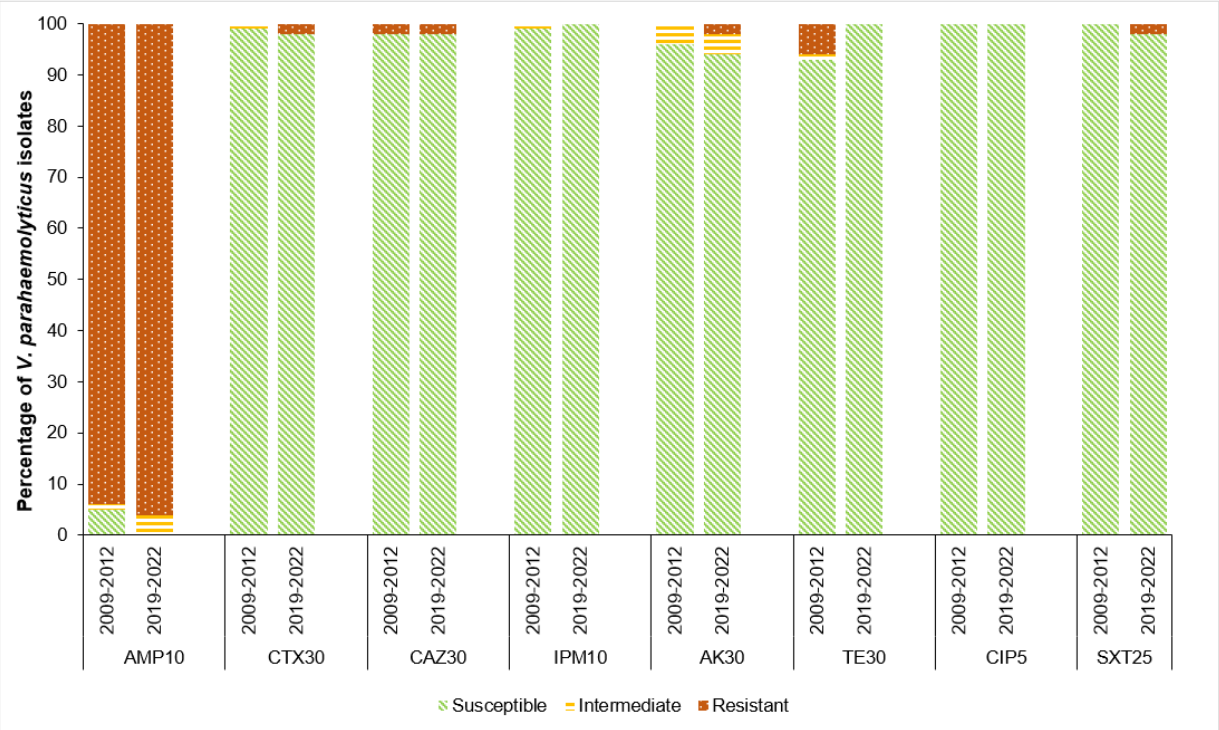


Figure 3. Antibiotic resistance patterns among *V. vulnificus* isolates (n=78). List of abbreviations: AK30, amikacin; AMP10, ampicillin; CAZ30, ceftazidime; CIP5, ciprofloxacin; CTX30, cefotaxime; IPM10, imipenem; SXT25, trimethoprim-sulfamethoxazole; TE30, tetracycline.

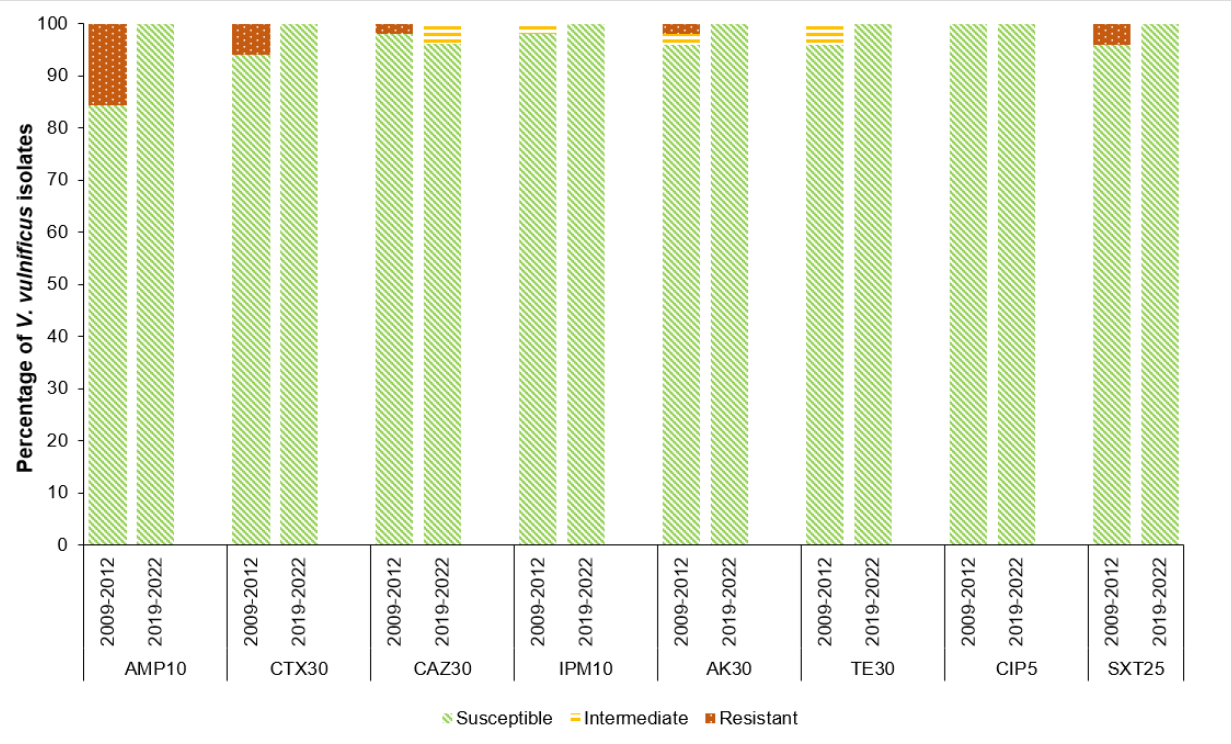
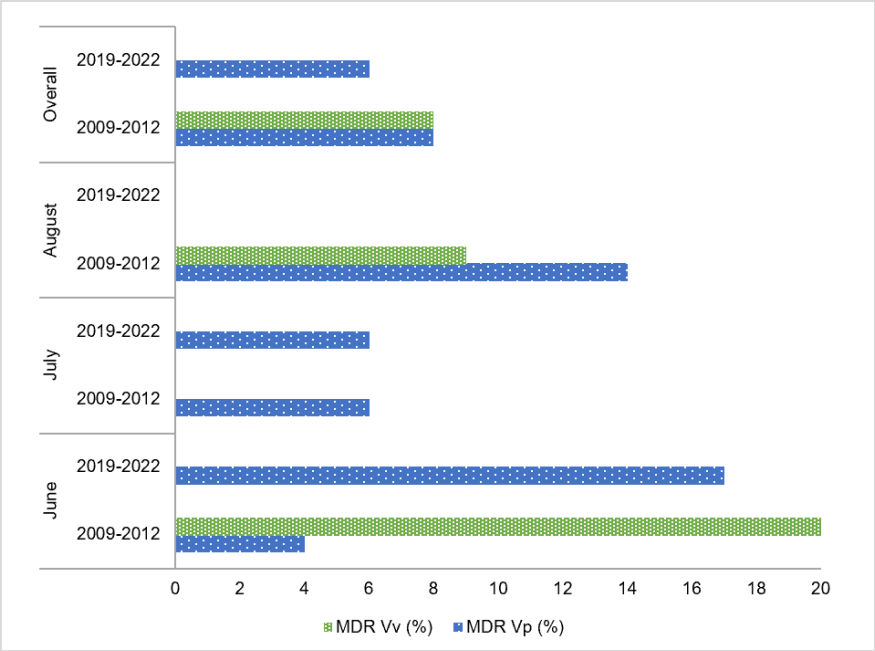


Figure 4. Percentage of multi-drug resistant (MDR) isolates among *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) selected from each sampling period (2009-2012 and 2019-2022) and month.



Chapter 5: Vibriosis Trends in Maryland, U.S., 2006-2019: Increased Incidence of Water-Associated Wound Infections and Hospitalization Risk

(Michele E. Morgado, Kyle D. Brumfield, Clifford Mitchell, Michelle M. Boyle, Rita R. Colwell, Amy R. Sapkota)

Abstract

Background. *Vibrio* spp. naturally occur in warm water with moderate salinity. Infections with non-cholera *Vibrio* (vibriosis) cause an estimated 80,000 illnesses and 100 fatalities each year in the United States. Climate associated changes to environmental parameters in aquatic ecosystems are largely promoting *Vibrio* growth, and increased incidence of vibriosis is being reported globally. However, vibriosis trends in the northeastern U.S. (e.g., Maryland) have not been evaluated since 2008.

Methods. Vibriosis case data for Maryland (2006-2019; n=611) were obtained from the COVIS database. Incidence rates were calculated using U.S. Census Bureau population estimates for Maryland. A logistic regression model, including region, age group, race, gender, occupation, and exposure type, was used to estimate the likelihood of hospitalization.

Results. Comparing the 2006-2012 and 2013-2019 periods, there was a 39% (p=0.01) increase in the average annual incidence rate (per 100,000 population) of vibriosis, with *V. vulnificus* infections seeing the greatest percentage increase (53%, p=0.01), followed by *V. parahaemolyticus* (47%, p=0.05). The number of hospitalizations increased by 58% (p=0.01). Since 2010, the average hospital duration for vibriosis was frequently greater than 5 days. Patients from the upper eastern shore region and

those over the age of 65 were more likely (OR=6.8 and 12.2) to be hospitalized compared to other patients.

Conclusions. Long-term increases in *Vibrio* infections, notably *V. vulnificus* wound infections, are occurring in Maryland. This trend, along with increased rates in hospitalizations and average hospital durations, underscore the need to improve public awareness, water monitoring, post-harvest seafood interventions, and environmental forecasting ability.

Introduction

Vibrio spp. are Gram-negative rod-shaped bacteria that include more than 110 described species, many of which are known to be pathogenic to humans and animals (Baker-Austin et al., 2017, 2018; Daniels et al., 2000; Morris & Black, 1985). These bacteria occur naturally in marine and estuarine environments, flourishing in warm water with moderate salinity, especially along the coast (Kaspar & Tamplin, 1993; Singleton et al., 1982; Vezzulli et al., 2013, 2016). *Vibrio* spp. incidence is strongly influenced by environmental parameters (Hlady, 1997; Iwamoto et al., 2010); they are also associated with aquatic invertebrates, such as crustaceans, zooplankton, and bivalves, and play an important role in biogeochemical processes (Colwell, 1996; Huq et al., 1983; Krantz et al., 1969; Lovelace et al., 1968). *Vibrio cholerae, primarily serogroups O1 and O139,* is well-documented as the etiological agent of cholera which continues to plague developing nations (Colwell, 1996). On the other hand, pathogenic non-cholera *Vibrio* species are more commonly the cause of disease in developed countries (Baker-Austin et al., 2017). In the United States, the Centers for Disease Control and Prevention (CDC) estimates that non-cholera *Vibrio* illness

(vibriosis) causes 80,000 illnesses and 100 fatalities each year (CDC, 2019), with noted increasing annual incidence rates during the past two decades (A. Newton et al., 2012; Sims et al., 2011; Vugia et al., 2013).

Vibrio spp. concentrate in filter-feeding shellfish, especially oysters, and more than half of all cases of vibriosis in developed countries, including the U.S., are attributed to ingestion of raw oysters or shellfish contaminated with vibrios, mainly *Vibrio parahaemolyticus* (Iwamoto et al., 2010; Ndraha et al., 2020; Shapiro et al., 1998). However, severe, and fatal cases are more prevalent with *Vibrio vulnificus* infection, which is usually associated with brackish or ocean water exposure containing the bacterium (Baker-Austin et al., 2010, 2018; Oliver, 2013; Shapiro et al., 1998). *V. vulnificus* has a case fatality rate among the highest of waterborne pathogens, ranging from 30-48% (Horseman & Surani, 2011; Strom & Paranjpye, 2000). Other vibrios such as *Vibrio alginolyticus* and *Vibrio fluvialis*, have increased in abundance over the last decade, and similarly to *V. vulnificus* infections, are typically associated with brackish water exposure, leading to ear and wound infections or gastroenteritis (Baker-Austin et al., 2018). Vibriosis cases have a strong seasonal component, with most infections occurring during the warmer months (Baker-Austin et al., 2018; Daniels et al., 2000; Shapiro et al., 1998). Symptoms of vibriosis can range from mild gastroenteritis to more severe cases including wound infection and septicemia, which can lead to hospitalization, amputation, or death (Daniels & Shafaie, 2000; Horseman & Surani, 2011; Morris & Black, 1985; Shapiro et al., 1998). Populations at greater risk for severe vibriosis include those with preexisting liver disease, alcohol use disorder, diabetes, hemochromatosis, or

immunodeficiencies (Baker-Austin et al., 2018; Daniels & Shafaie, 2000; Oliver, 2005; Weis et al., 2011).

Surveillance of *Vibrio* infections in the U.S. was initiated in 1989 through the Cholera and Other Vibrio Illness Surveillance (COVIS) system managed by the CDC (CDC, 2014). This program initially focused on monitoring *V. cholerae* cases from four Gulf Coast states (Alabama, Florida, Louisiana, Texas), but in 2007 all vibriosis cases became nationally notifiable (CDC, 2014; Sims et al., 2011). In 1996, the CDC also initiated the Foodborne Diseases Active Surveillance Network (FoodNet), which conducts active surveillance of *Vibrio* infections and other important foodborne pathogens in 10 U.S. sites, including the Maryland Department of Health (MDH) (CDC, 2021; Jones et al., 2007). MDH, together with the Maryland Department of the Environment (MDE) and the Maryland Department of Natural Resources (DNR) also participate in the National Shellfish Sanitation Program (NSSP), a cooperative federal/state program recognized by the U.S. Food and Drug Administration (FDA) that regulates the sanitation of shellfish produced and sold for human consumption (FDA, 2020). In addition, MDE and the DNR provide current information on the health status of Maryland's natural waters through water monitoring programs (E. H. Jones et al., 2013). Forecasting models have also been used successfully for global risk prediction of cholera (Usmani et al., 2023), and other models, such as the National Centers for Coastal Ocean Science (NCCOS) Probability Model (Jacobs et al., 2014), project *V. parahaemolyticus* and *V. vulnificus* levels in the Chesapeake Bay, MD.

Recently there is increased concern regarding the role of climate change-associated shifts in the geographical range of microbial species and the emergence and re-emergence of disease. Notably, several studies have documented a significant geographic expansion of pathogenic *Vibrio* spp., with increased numbers of reported vibriosis cases (Archer et al., 2023; Baker-Austin et al., 2017, 2013; Brehm et al., 2021; Fleischmann et al., 2022; Sterk et al., 2015; Brumfield et al., 2023, 2021; Colwell, 1996; Ford et al., 2020; Vezzulli et al., 2015, 2013, 2012). Moreover, a recent investigation in the Chesapeake Bay reported a long-term increase and extended seasonality of pathogenic *Vibrio* spp. between 2009 and 2022 (Brumfield et al., 2023). These works also highlight the potential role of extreme weather events (e.g., heatwaves and hurricanes) in the increase of more severe gastrointestinal illness and wound infection stemming from vibriosis outbreaks. It is therefore of significance to understand how *Vibrio* infection trends have changed in more northern U.S. states under warming conditions, and the implications for local healthcare costs. Of note, a study by Jones et al. (2013) covering the 2002-2008 time period is the most recent work to evaluate *Vibrio* spp. infection trends in Maryland. The current study aimed to analyze current long-term vibriosis case data, from 2006-2019, including changes to hospitalization risk and case fatality rates, and the relative importance of exposure type to patient infections. The latter is especially relevant given that a study from our group (Shaw et al., 2015) demonstrated that *Vibrio* spp. detected dermally from Chesapeake Bay waters added significantly to the risk of infection in recreational swimmers and might reflect a growing trend in the acquisition of severe vibriosis.

Methods

Data Sources

To examine clinical and epidemiological information on *Vibrio* spp. infections, case data from 2006-2019 were obtained from the CDC's COVIS database, which receives case reports through the Maryland FoodNet program, housed at MDH. This program represents 1 of 10 FoodNet sites funded by the CDC that conduct active, population-based surveillance since 1996 on laboratory-diagnosed infections, identified by culture or culture-independent diagnostic tests (CIDT), caused by *Vibrio* as well as 7 other pathogens. Cases are defined as an individual whose biological specimen (stool, blood, wound, or other) was culture-confirmed for the presence of *Vibrio*, regardless of symptoms or date of onset. To determine whether a case was associated with seafood consumption or water exposure (leading to blood or wound infection), we reviewed variables including specimen source, evidence of a preexisting wound, exposure to brackish or ocean water, consumption of or exposure to raw seafood as well as drippings (uncooked seafood item residues that may contaminate other cooked items), occupational exposure, and date of illness onset relative to the exposure. These variables were used to make a subjective determination about the possible association (seafood or water associated infection) and followed similar methods used in a previous study (E. H. Jones et al., 2013). For instance, cases determined from a stool specimen, with consumption or exposure to raw seafood and drippings were considered to be associated with seafood consumption; while cases determined from a blood specimen and with evidence of a preexisting wound or exposure to brackish/ocean water were

categorized as wound-water associated exposures. Cases without enough information to make this determination (n=15) were excluded from analysis, as well as those related to known foreign or domestic travel (n=34). On the basis of the 2010 census and 2020 census by the US Bureau of the Census, the population estimates for the State of Maryland of 5,773,552 and 6,177,224, respectively, were used to calculate incidence which was expressed per 100,000 population.

Data Analysis

A two-sample t-test was used to evaluate the differences between percentage change in 2013-2019 compared to 2006-2012, for the incidence rate, number of hospitalizations, average hospital duration, and the number of seafood and wound/water associated cases. A comparison of the average vibriosis cases in the first seven years versus the last seven was chosen in order to balance interannual climatic changes over a similar period of time. For data that were not approximately normal, or for which the sample size was smaller than 20, the Mann-Whitney test was used. Additionally, analysis of variance (ANOVA) was performed to determine the significant differences between multiple variables, and the non-parametric Kruskal-Wallis test was used for non-normal data. Correlation and regression analyses were also applied to evaluate the strength of the association between the number of vibriosis cases and year of exposure, and other continuous variables. In addition, the mean annual change in cases at the county level between 2006 and 2019 was calculated for all culture-confirmed *Vibrio* spp. infections reported by the State of MD (n=4 cases with unknown county). Vibriosis case data by county represent the county of residence.

To estimate the probability of hospitalization, we used a logistic regression model that included region (western, capital, south, central, upper eastern shore, lower eastern shore), patient age group (0-4, 5-17, 18-65, and ≥65 years of age), race (White, Black, Asian, other, unknown), gender (female, male), occupation (child/student, retired, maritime related, non-maritime related, unknown), and the attributed exposure type (wound/water, seafood contamination). The PROC LOGISTIC command (SAS) was used, controlling for mentioned exposure variables, and the outcome group was whether or not a patient was hospitalized. Regions are defined as those including the following counties: *Western*: Allegany and Washington; *Capital*: Frederick, Montgomery and Prince George's; *Central*: Carroll, Baltimore, Harford, Howard and Anne Arundel; *South*: Charles, Calvert and St. Mary's; *Upper Eastern Shore*: Cecil, Kent, Queen Anne's, Caroline and Talbot; and *Lower Eastern Shore*: Dorchester, Wicomico, Worcester and Somerset. Of the 611 reported cases, this analysis excluded n=69 cases of vibriosis due to incomplete information for one or more variables included in the model. Statistical analyses were performed using SAS 9.4 (Cary, NC USA).

Results

Epidemiologic Observations and Trends

From 2006 to 2019, there were 611 culture-confirmed cases of vibriosis reported in Maryland (**Table 1**), with an annual average of 44 cases per year and an average annual incidence rate of 0.72 cases per 100,000 population. The most commonly reported *Vibrio* spp. were *V. parahaemolyticus* (39%, 3.90 IR), *V. vulnificus* (23%, 2.31 IR), and *Vibrio alginolyticus* (12%, 1.17 IR), but other species

of *Vibrio*, including *Vibrio fluvialis* and *V. cholerae* non-O1/non-O139, were also reported with some frequency, ranging from 11 to 7%, respectively.

Demographic characteristics of patients reported were similar among species, with a median age of 55 years and males accounting for 67% of all illnesses. The youngest median age reported was 38 years for *V. alginolyticus* infections, and the oldest was 67 years for both *V. vulnificus* and *V. fluvialis* infections (**Table 1**).

Although *V. parahaemolyticus* infections were most frequently reported, they led to hospitalization in only 30% of cases and were rarely fatal, with a case fatality rate (CFR) of 1% (**Table 1**). *V. vulnificus*, by contrast, led to hospitalization in 81% of cases and also had the highest CFR of 11%. Infections with *V. fluvialis* and other species of *Vibrio* (*V.* other) also led to higher hospitalization rates among patients, with 60% and 59%, respectively. The overall CFR for vibriosis cases was 4% between 2006 and 2019.

The frequency of isolated *Vibrio* spp. varied by year. However, the number of vibriosis cases was positively correlated with year ($r = 0.75$, $p = 0.002$), and increased significantly between 2006 and 2019 (**Fig. 1**). The year with the most *Vibrio* infections was 2016 (n= 69) followed by 2013 (n=57), with *V. parahaemolyticus* and *V. vulnificus* contributing to over 65% of all cases. The year with the least reported cases was 2007 (n= 26), with over 50% of infections being attributed to *V. parahaemolyticus* and *V. alginolyticus*. With the exception of 2011, where *V. vulnificus* and *V. alginolyticus* contributed to most cases, *V. parahaemolyticus* was the most frequently reported species each year (**Fig.1**).

From 2006-2019, there were 266 (48.3%) cases associated with wound/water infections and 285 (51.7%) associated with seafood consumption or contamination (**Fig. 2**). The *Vibrio* species most frequently associated with seafood related infections was *V. parahaemolyticus* (58.1%), followed by *V. fluvialis* (11.4%) and *V. vulnificus* (10.4%). *V. vulnificus* was the most frequent cause of infection associated with wound/water exposure (41.2%), followed by *V. parahaemolyticus* (21.7%) and *V. alginolyticus* (18.7%) (**Fig. 2**).

The season with the most reported infections, both from wound/water and seafood consumption, was the summer (n=349), including June, July, and August; followed by the fall (n=122) season, including September, October, and November. During the summer and fall seasons, wound-water related infections (n=242) were slightly higher than those associated with seafood consumption (n=229). By contrast, in the winter (December, January, February) and spring (March, April, May) seasons, seafood related infections (n=56) dominated the reported cases compared with wound/water infections (n=24). Seasonality did not vary significantly by species (**Fig. 2**), data not shown.

Vibriosis Percentage and Average Annual Changes

Comparing the percentage change in *Vibrio* spp. infections reported in 2013-2019 with those in 2006-2012 (**Table 2**), there was an overall 39% (p=0.01) increase in the average annual incidence rate (per 100,000 population), with *V. vulnificus* infections seeing the greatest percentage increase (53%, p=0.01), followed by *V. parahaemolyticus* (47%, p=0.05).

The number of hospitalizations as well as the average hospital duration (in days) for all vibriosis cases also increased in the 2013-2019 period compared to 2006-2012, by 58% (p=0.01) and 35% (*p*=0.08), respectively (**Table 2**). This increase was observed for most *Vibrio* species, although statistically significant only for *V. vulnificus* (63%, *p*=0.03 for hospitalizations and 92%, *p*=0.02% for average hospital duration) and infections with multiple *Vibrio* species (100%, *p*=0.01 for hospitalizations). Seafood associated cases and water-associated infections doubled between both time periods, with a 56% (*p*=0.02) increase in the former and a 50% (*p*=0.03) increase in the latter. *V. parahaemolyticus* infections as well as those with multiple species represented the greatest increase in seafood associated cases between 2013-2019 compared to 2006-2012, with 63% (*p*=0.04) and 144% (*p*=0.004), respectively; while *V. vulnificus* infections were responsible for the greatest increase (82%, *p*=0.01) in water-associated wound cases (**Table 2**). The overall ratio of wound/water to seafood associated cases also increased slightly between both time periods, although not significantly, by 4.1% (*p*=0.43), data not shown.

The map in **Fig. 3** shows the average annual change in vibriosis cases in Maryland at the county level between 2006 and 2019. Anne Arundel and Baltimore (central region), and Wicomico (lower eastern shore) counties, showed the greatest increase in average *Vibrio* infections per year, with approximately 0.3 new cases reported each year. By comparison, slight decreases in average vibriosis cases per year were reported in Harford County (central region; 0.2 case decrease), as well as counties in the south region including Calvert (0.07 case decrease) and St. Mary's

(0.15 case decrease). For all counties collectively, the average annual number of vibriosis cases increased by approximately two cases per year, data not shown.

Hospital Duration and Hospitalization Risk Trends

The average hospital duration in Maryland for vibriosis cases between 2006-2019, regardless of exposure type, was approximately 5 days. While we did not observe a statistically significant increase in average hospital duration over time (p= 0.60), after 2010 there were more reported cases with a hospital duration stay of at least 10 days (**Fig. 4**). This was more frequently observed for infections with *V. fluvialis*, *V. alginolyticus*, and *V. vulnificus*. In particular, *V. fluvialis* had the highest annual average hospital duration of approximately 9 days and led to average hospital durations above 10 days in 2010, 2014 and 2016 (**Fig. 4**).

In Maryland from 2006-2019, 259 (48%) vibriosis case patients out of 542 were hospitalized for whom enough data were available. The proportion of patients hospitalized varied by region, from 1% in the western counties (Allegany, Washington) to 50% in the central counties (Carroll, Baltimore, Harford, Howard, Anne Arundel) (**Table 3**). The highest rates of hospitalization were observed for those over 65 years of age (46%), followed by those aged 45-64 (39%), and among white (69%), males (71%), and those listed as retired (37%). Fifty-two percent of patients with water-associated wound infections were hospitalized, compared to 48% for seafood-associated contamination (**Table 3**).

From the logistic regression model, patients in the upper eastern shore (Cecil, Kent, Queen Anne's, Caroline, Talbot counties) were more likely (OR = 6.8) to be hospitalized than those in the western region (**Table 3**). Patients over the age of 65

years and those aged 45-64 were more likely (OR = 12.2 and 10.7, respectively), to be hospitalized than those ages 0-4; Black patients were more likely to be hospitalized (OR = 2.3) than White patients and those retired were more likely (OR = 2.2) to be hospitalized than those whose occupation was non-maritime related. Moreover, patients with wound/water associated infections were more likely (OR = 1.8) to be hospitalized than those with seafood associated vibriosis (**Table 3**).

Discussion

Our analysis of surveillance data from the Maryland FoodNet program that monitors *Vibrio* illness indicates that the incidence of culture-confirmed vibriosis increased between 2006 and 2019. Not only did the average annual incidence rate increase 39% in 2013-2019 compared with 2006-2012, but the overall average annual incidence of 0.72 cases/100,000 population was 35% higher than the average rate of 0.47 reported for 2002-2008, in the previous Maryland study of *Vibrio* infection trends (E. H. Jones et al., 2013). The annual incidence of vibriosis was similar to that reported by two other FoodNet sites during the same time period (California and Connecticut), albeit higher than the national average of 0.42/100,000 population for all 10 FoodNet sites (CDC, 2021). Previous studies have noted higher rates of *Vibrio* infection in coastal U.S. states compared to inland states, as well as a greater contribution of *V. parahaemolyticus* towards infections (CDC, 2014; Jones et al., 2013; Newton et al., 2012; Sims et al., 2011; Weis et al., 2011), with the exception of Florida where *V. vulnificus* is the leading cause of *Vibrio* illness (Weis et al., 2011). Despite national trends, Maryland has seen a change in the most common *Vibrio* species, type of exposure, hospitalization risk, and average hospital duration.

Compared to Maryland vibriosis data from 2002-2008 (E. H. Jones et al., 2013), the frequency of *V. parahaemolyticus* infections decreased by 4%, while *V. alginolyticus* related cases increased by 2%, and *V. fluvialis* surpassed *V. cholerae* non-O1/non-139 infections to become the fourth most common species to cause illness in the State. Although the relative contribution of *V. vulnificus* infections remained unchanged, the incidence, number of cases that led to hospitalization, as well as the average hospital duration increased significantly, by 53%, 63% and 92%, respectively, in 2013-2019 compared with 2006-2012. Interestingly, the case fatality rate (CFR) observed from 2006-2019 (CFR=11%) for *V. vulnificus* was approximately 20% lower than that reported in previous studies (A. Newton et al., 2012; Oliver, 2005, 2013; Sims et al., 2011; Weis et al., 2011), although the median age of infected patients was still among mostly male, older, and those more likely to have comorbidities. This likely represents greater awareness among health care providers in treating wound and blood infection cases considered to be associated with *V. vulnificus*. In fact, several studies have noted the importance of early recognition of nonfoodborne *Vibrio* infections and timely and aggressive treatment, especially within 24 hours of hospitalization, to reduce mortality rates (Bross et al., 2007; Chao et al., 2013; Dechet et al., 2008; J. S. Kim et al., 2022; Yun & Kim, 2018). On the other hand, the significant increase in *V. vulnificus* incidence and higher number of cases associated with wound infection in summer and fall, highlights the need for improved public awareness of infection risk.

Currently, the “Maryland Healthy Beaches” program run by the MDE (MDE, 2023), provides *Vibrio* infection information on their website and flyers, including

avoiding water contact if there are any open wounds, wearing water shoes to avoid cuts, wearing gloves when crabbing or fishing, and showering after swimming in natural waters. However, it is unclear how readily available this information is in local creeks and waterways surrounding the Chesapeake Bay or if more remote water access points also have "*Vibrio* facts" flyers posted.

Similarly, increased hospitalization rates between 2006 and 2019 and average hospital durations were observed with the less common *V. fluvialis.* However, most infections were associated with seafood contamination and lower CFR (2%) than *V. vulnificus* related cases. Notably, incidence of vibriosis caused by *V. fluvialis* remains relatively less frequent across other FoodNet sites (averaging 0.03 per 100,000 population) (CDC, 2021), and is considerably higher in Maryland, with the potential to become a greater public health concern. In the United States, severe cases of illness from *V. fluvialis* infection are still rare (Allton et al., 2006; Daniels & Shafaie, 2000; Klontz & Desenclos, 1990) but have been more frequently reported across other countries, e.g., Taiwan, South Korea, India, and South Africa (Huang et al., 2005; Igbinosa & Okoh, 2010; Lai et al., 2006; J. Y. Lee et al., 2008; Ramamurthy et al., 2014; Shravan et al., 2021). A few of the more severe and concerning cases included symptoms of cholera-like diarrhea and acidosis, acute infectious peritonitis, and fatal bacteremia. There have also been earlier reports of *V. fluvialis* associated gastroenteritis cases among infants (Ramamurthy et al., 2014). Owing to its ability to cause significant and severe diarrhea similar to *V. cholerae* (Igbinosa & Okoh, 2010), there is a need to promptly recognize infections with this pathogen, especially in the

very young and those with underlying medical conditions, and to administer aggressive antibiotic therapy.

As the incidence of vibriosis increases in the State of Maryland, there may be a greater recognition of symptoms of illness, as well as more individuals seeking medical attention, and better access to medical care over time. Notwithstanding, some areas across the State may incur higher healthcare costs related to longer hospital stays, especially among Maryland's Eastern Shore counties, where the likelihood of being hospitalized was higher than in other locations. Although there were more observed *Vibrio* infection cases in central and less rural counties (e.g., Anne Arundel, Baltimore), it is concerning that more remote locations with a higher poverty level and less access to healthcare resources (Sangaramoorthy & Guevara, 2017) may have to treat more severe cases of vibriosis in years to come. According to recent estimations for waterborne-related illness in the United States, the cost per hospital stay for a *Vibrio* infection was approximately $16,000 (Collier et al., 2021). Moreover, Sheahan et al. (2022) estimated the average national cost of vibriosis under a climate warming scenario, to be around $3.9 billion per year by 2050. The burden and direct healthcare costs will likely vary by state and region but may disproportionately impact those under Medicare (over 65 years of age), who are more likely to experience complications following infection.

In Europe and across other parts of the world, including the eastern seaboard of the U.S., there are shared concerns regarding the increased burden of disease from vibriosis cases linked to rising ocean temperatures (Archer et al., 2023; Baker-Austin et al., 2010, 2018; Banerjee et al., 2018; Ferchichi et al., 2021; Sims et al., 2011;

Vezzulli et al., 2016; Yun & Kim, 2018). For instance, previous studies have noted the effect of severe heatwaves and warmer water temperatures on the increased number of *Vibrio* infections, especially from *V. vulnificus*, in German North and Baltic Sea coasts (Brehm et al., 2021; Fleischmann et al., 2022; Le Roux et al., 2015), as well as a significant increase in the risk of *Vibrio*-related illness in northern European waters with projected temperature increases under a warming climate (Sterk et al., 2015). Moreover, a recent study reported on the occurrence of *V. parahaemolyticus* and *V. vulnificus* in Tangier Sound (lower eastern bay) whereby a long-term increase in and extended seasonality of these bacteria were observed (Brumfield et al., 2023). This study is of particular interest since Wicomico County (lower eastern shore) was among the locations reporting the greatest increase in average *Vibrio* infections per year (**Fig. 3**). These cases are likely not only associated with increased levels of seafood contamination but also increased *Vibrio* levels in natural waters, where commercial and recreational activities, such as swimming and fishing, take place. This underscores the need to improve forecasting capabilities for environmental parameters that influence occurrence and abundance of pathogenic *Vibrio* spp. (Brumfield et al., 2023; Colwell, 1996; Jutla et al., 2013; Lobitz et al., 2000; Usmani et al., 2023), as well as to raise public awareness of risks associated with open wound infection or eating shellfish during warmer months.

As mentioned in past studies (E. H. Jones et al., 2013; A. Newton et al., 2012; Shapiro et al., 1998; Weis et al., 2011), there should also be more targeted education strategies aimed at populations with higher risk of developing severe *Vibrio* infections (males, over the age of 45, with underlying medical conditions), or with a

greater likelihood of being exposed (e.g., professional, and recreational crabbers). For example, a CDC public health approach used in Gulf of Mexico states urged those at greater risk to avoid consumption of raw oysters from the Gulf of Mexico as well as seawater exposure. This strategy was associated with a reduction in vibriosis incidence linked to raw oyster consumption, notably in Florida, where state law requires that food establishments that sell raw oysters include visible information on the risks associated with their consumption (Weis et al., 2011). In a subsequent study by Vugia et al. (2013), a similar strategy was also successful in reducing the number of cases or deaths caused by *V. vulnificus* infections from raw oyster consumption in California. However, previous studies have also recommended that prevention efforts should include public health messages that focus on the risk of vibriosis from wound infections(A. Newton et al., 2012; Weis et al., 2011). Moreover, it was noted that awareness among clinicians, including prompt diagnosis, was imperative to improve patient outcomes. The data show that public health education alone may be insufficient to control vibriosis (A. Newton et al., 2012; Vugia et al., 2013) and other measures such as improved monitoring of natural waters, awareness of pre-harvest conditions, and post-harvest decontamination of shellfish may also be needed to mitigate *Vibrio* illness. Research into newer and safer post-harvest interventions is encouraging, and methods such as high hydrostatic pressure show effectiveness in reducing the abundance of *V. parahaemolyticus and V. vulnificus* in oysters (Distefano et al., 2011; Spaur et al., 2020; Vu et al., 2018; Ye et al., 2012).

A limitation of this study is that while the MD FoodNet system can be considered representative of more severe cases where a *Vibrio* infection is culture-

confirmed, mild or self-limiting cases are less likely to be represented. This has also been recognized as a limitation of the national vibriosis surveillance system (COVIS). In addition, *Vibrio* spp. are known to enter a protective state, namely viable but nonculturable (VBNC), whereby the cells become metabolically dormant and cannot be cultured using routine enteric media (Colwell, 2000). Hence, the numbers reported here are likely an underestimation of the actual total number of *Vibrio* spp. infections for the State. It is also important to note that there isn't a standardized method of classifying vibriosis by exposure type; therefore, the determination of whether a case resulted from wound or seafood contamination was subjective. Moreover, vibriosis cases are recorded based on a patient's city and county of residence and may not necessarily reflect the county in which they sought medical attention or where they were exposed to the pathogen. Lastly, certain key variables were sometimes missing, such as age, gender, race, or occupation, and results may not fully reflect the population at highest risk across the State of Maryland. Future studies would benefit from inclusion of important metrics such as underlying medical conditions and antibiotics used during treatment.

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Chapter 5 Tables

Table 1. Vibriosis cases, incidence rate, selected patient demographic characteristics, outcomes (hospitalizations, deaths, and case fatality rate), by species, Maryland, 2006 to 2019.

Vibrio species	Cases			Age (years)		Gender		Hospitalizations		Deaths	
	N	%	IR[§]	Median	Range	Male (n/N)	%	n/N	%	n	CFR (%)
V. parahaemolyticus	241	39	3.90	49	1-98	160/241	66	72/238	30	2	1
V. vulnificus	143	23	2.31	67	1-90	115/143	80	113/140	81	16	11
V. alginolyticus	72	12	1.17	38	2-84	50/72	69	7/71	10	1	1
V. fluvialis	47	8	0.76	67	15-93	28/47	60	28/47	60	1	2
V. cholerae Non O1/139	42	7	0.68	50	5-87	19/42	45	20/41	49	2	5
V. other*	66	11	1.07	57	4-87	38/66	58	39/66	59	3	5
TOTAL	**611**	**100**	**9.89**	**55**	**1-98**	**410/611**	**67**	**253/603**	**42**	**25**	**4**

Abbreviation: IR, incidence rate; CFR, case fatality rate.
[*] Includes *Photobacterium damselae* subsp. *damselae* (formerly known as *V. damsela*), *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. metschnikovii*, multiple species, and species not identified.
[§] Per 100,000 population.

Table 2. The percentage change in 2013-2019 vibriosis cases compared with 2006-2012 for average annual incidence rate, number of hospitalizations, average hospital duration, and number of seafood and wound-water associated cases by species, Maryland.

Vibrio species	Percentage change 2013-2019 compared with 2006-2012, % (*p*-value)[¶]				
	IR[§]	No. of hospitalizations	Average hospital duration (days)	Seafood associated cases	Wound/water associated cases
V. parahaemolyticus	+47 (0.05)	+32 (0.11)	+4 (0.44)	+63 (0.04)	+23 (0.29)
V. vulnificus	+53 (0.01)	+63 (0.03)	+92 (0.02)	0 (0.50)	+82 (0.01)
V. alginolyticus	+20 (0.18)	-25 (0.35)	+47 (0.34)	0 (0.50)	+38 (0.15)
V. fluvialis	+27 (0.33)	+80 (0.19)	+84 (0.16)	+54 (0.21)	+67 (0.30)
V. cholerae Non O1/139	+41 (0.16)	+86 (0.09)	+110 (0.08)	+38 (0.34)	+13 (0.42)
V. other*	+15 (0.21)	+100 (0.01)	-37 (0.18)	+144 (0.004)	+40 (0.10)
TOTAL	**+39 (0.01)**	**+58 (0.01)**	**+35 (0.08)**	**+56 (0.02)**	**+50 (0.03)**

Abbreviation: IR, incidence rate.
[*] Includes *Photobacterium damselae* subsp. *damselae* (formerly known as *V. damsela*), *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. metschnikovii*, multiple species, and species not identified.
[§] Per 100,000 population.
[¶] Average annual percentage change, for each 7-year block, reported as increase or decrease.

Table 3. Hospitalization rate and associated odds ratio for patients with culture-confirmed *Vibrio* spp. infections by region and demographic/epidemiologic characteristics, Maryland, 2006 to 2019[§].

Region* or characteristic	Hospitalized n (%)	Logistic regression model results[¶]		
		Odds ratio	95% CI	*p*-value
Region				
Western	2 (1)	Reference	---	---
Capital	32 (12)	2.80	0.49-15.74	0.24
Central	128 (50)	3.28	0.62-17.30	0.16
South	32 (12)	4.93	0.85-28.65	0.08
Upper Eastern Shore	37 (14)	6.82	1.13-41.07	0.04
Lower Eastern Shore	28 (11)	3.71	0.64-21.43	0.14
Age group, years				
0-4	1 (1)	Reference	---	---
5-17	6 (2)	0.93	0.08-11.36	0.95
18-44	32 (12)	3.58	0.36-35.87	0.28
45-64	100 (39)	10.66	1.06-107.04	0.04
≥65	120 (46)	12.15	1.19-124.48	0.04
Race				
White	179 (69)	Reference	---	---
Black	58 (2)	2.33	1.36-3.95	0.002
Asian	10 (4)	1.78	0.63-4.99	0.28
Other	2 (1)	4.19	0.15-113.74	0.39
Unknown	10 (4)	0.52	0.22-1.26	0.15
Gender				
Male	184 (71)	Reference	---	---
Female	75 (29)	0.94	0.60-1.46	0.77
Occupation				
Child or student	6 (2)	0.81	0.21-3.12	0.76
Retired	97 (37)	2.20	1.21-4.00	0.01
Non-maritime related	69 (27)	Reference	---	---
Maritime related	17 (7)	2.28	0.77-6.77	0.14
Unknown	70 (27)	1.35	0.82-2.25	0.24
Exposure type				
Seafood	125 (48)	Reference	---	---
Wound-water	134 (52)	1.78	1.15-2.75	0.01

Abbreviation: CI, confidence interval.

* Counties included in each region- *Western*: Allegany, Washington; *Capital*: Frederick, Montgomery, Prince George's; *Central*: Carroll, Baltimore, Harford, Howard, Anne Arundel; *South*: Charles, Calvert, St. Mary's; *Upper Eastern Shore*: Cecil, Kent, Queen Anne's, Caroline, Talbot; *Lower Eastern Shore*: Dorchester, Wicomico, Worcester, Somerset.

§ Excludes cases with incomplete variable information (n=69).

¶ Estimates controlling for the variables shown.

Chapter 5 Figures

Figure 1. Laboratory-confirmed cases of *Vibrio* illness, by species and association, Maryland, 2006 to 2019. *Includes *Photobacterium damselae* subsp. *damselae* (formerly known as *V. damsela*), *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. metschnikovii*, multiple species, and species not identified.

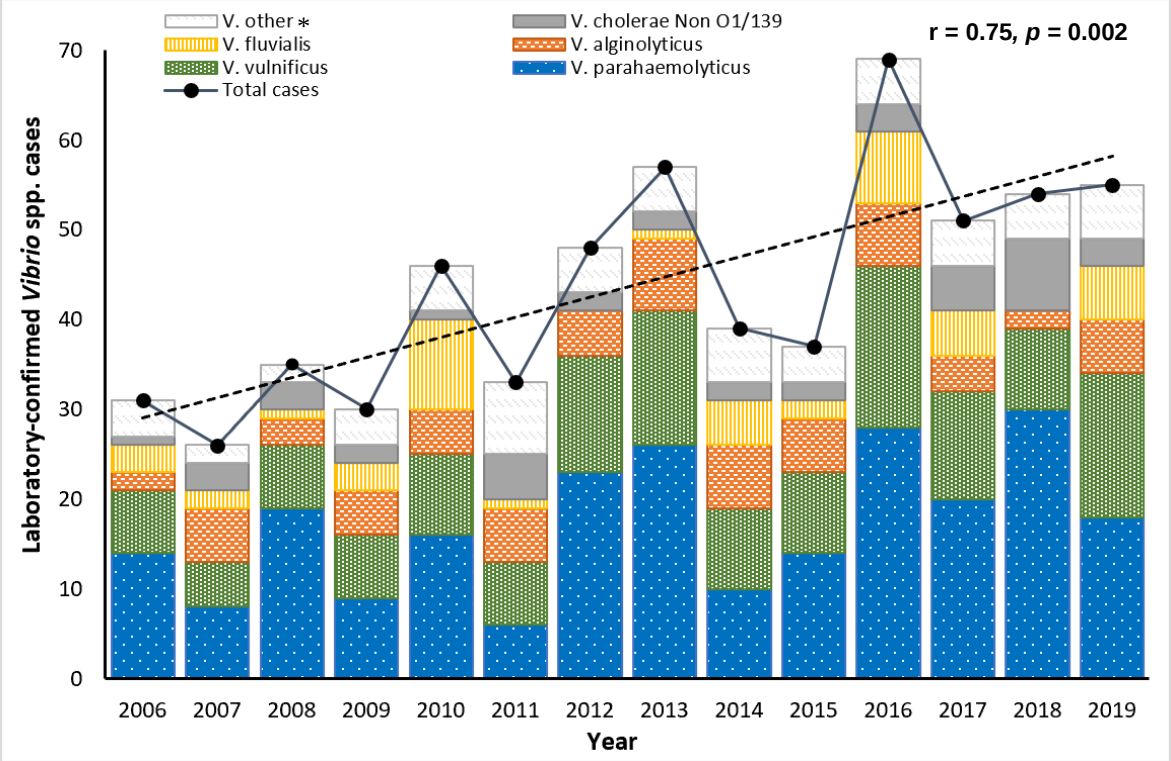


Figure 2. Laboratory-confirmed cases of *Vibrio* illness, by species, season and association, Maryland, 2006 to 2019. PAR: *V. parahaemolyticus*, VUL: *V. vulnificus*, ALG: *V. alginolyticus*, FLU: *V. fluvialis*, CHN: *V. cholerae* Non O1/139, OTH: *V.* other. Not including n=60 cases of undetermined association, known foreign/domestic travel or missing season information. *Includes *Photobacterium damselae* subsp. *damselae* (formerly known as *V. damsela*), *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. metschnikovii*, multiple species, and species not identified.

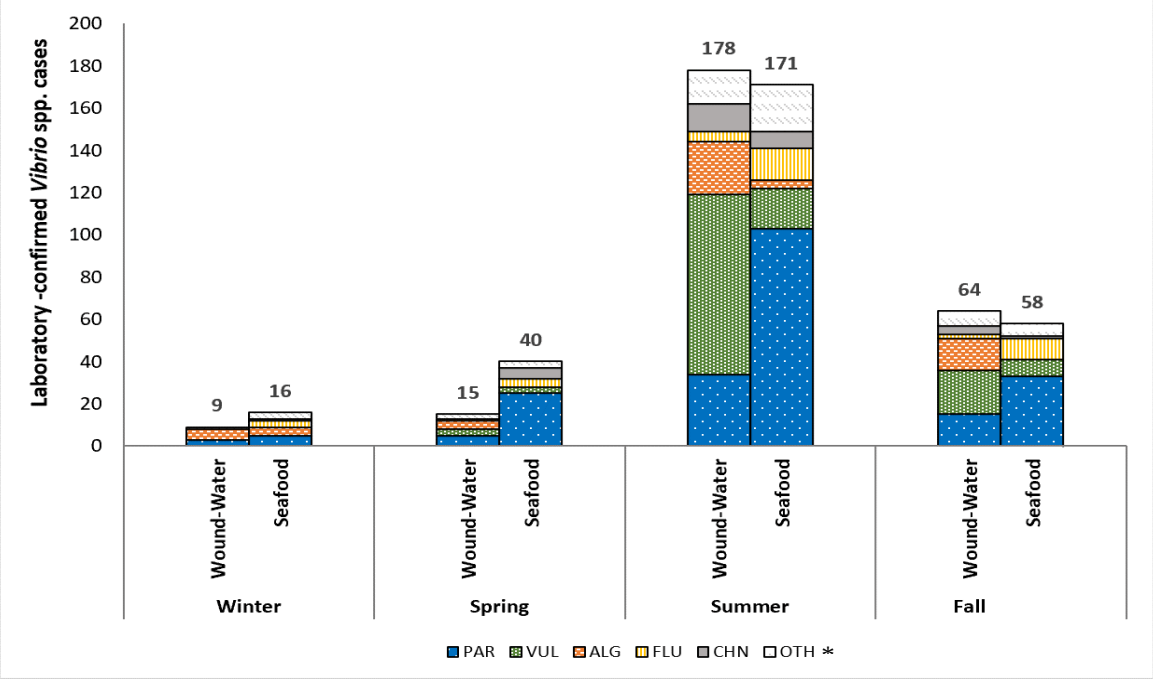


Figure 3. Average annual change in vibriosis cases by county for the State of Maryland, 2006-2019. Counties showing the highest increase in average annual *Vibrio* infections are indicated. Baltimore City and Baltimore County are listed together as "Baltimore". Scale bar corresponds to distance according to World Map Data from Natural Earth (Massicotte & South, 2023).

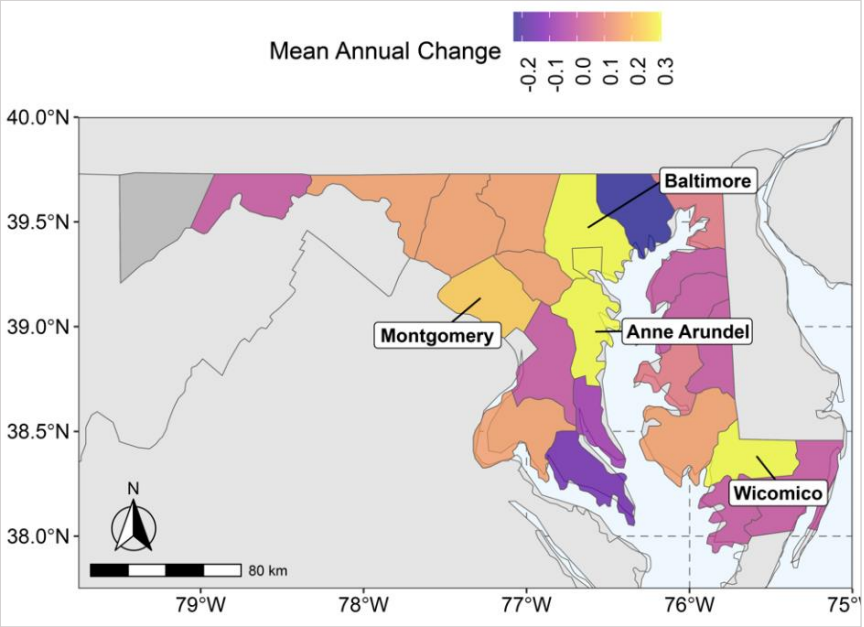
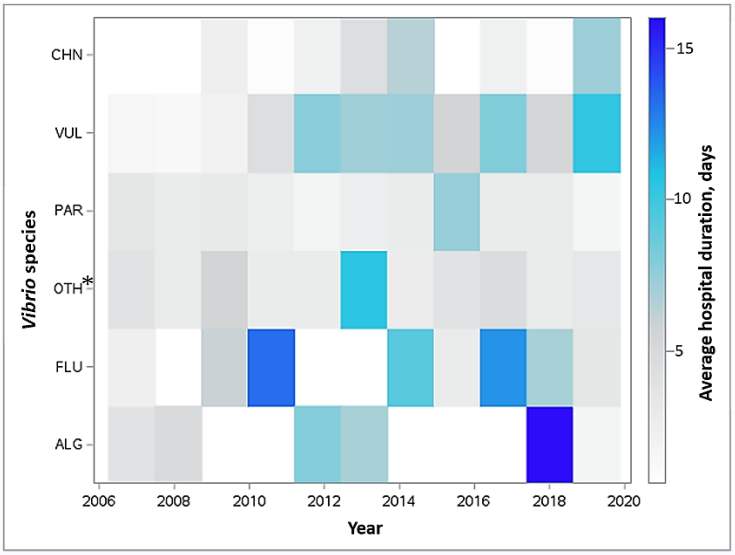


Figure 4. Average hospital duration in days, by *Vibrio* species and year, Maryland, 2006-2019. PAR: *V. parahaemolyticus*, VUL: *V. vulnificus*, ALG: *V. alginolyticus*, FLU: *V. fluvialis*, CHN: *V. cholerae* Non O1/139, OTH: *V.* other. *Includes *Photobacterium damselae* subsp. *damselae* (formerly known as *V. damsela*), *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. metschnikovii*, multiple species, and species not identified.



Chapter 6: Conclusions, Strengths and Limitations, Public Health Significance and Directions of Future Research

Conclusions

My epidemiological analysis (Chapter 5) demonstrated that there has been a long-term increase in *Vibrio* spp. infections in the State of Maryland, with the most significant impacts observed in coastal counties. Furthermore, the significant increase in the incidence of *V. vulnificus* between 2006-2019, increased risk of hospitalization, and average hospital duration, are noteworthy. Although infections with *V. fluvialis* are relatively rare, increasing trends in average hospital duration are concerning and may indicate more severe clinical strains associated with locally available seafood. These findings underscore the need to develop early warning systems, improve public awareness campaigns for individuals most-at-risk as well as clinicians, increase water monitoring and "*Vibrio* facts" flyers in local creeks and waterways, and invest in newer and more effective post-harvest seafood interventions.

Regarding my laboratory-based molecular analyses, the overarching context is that the presence of virulence genes and the prevalence of antimicrobial resistance in environmental *V. vulnificus* and *V. parahaemolyticus* isolates have a direct impact on the prevention and management of vibriosis. Although the percentage of multi-drug resistance observed in my study was relatively low, isolates tested showed varying levels of resistance and intermediate resistance to antibiotics typically used to treat severe vibriosis, including third generation cephalosporins, tetracyclines, sulfamethoxazole-trimethoprim, and aminoglycosides. Exceptionally, all isolates were susceptible against ciprofloxacin, a fluoroquinolone. *V. vulnificus* isolates also

displayed the presence of multiple virulence factors which have been found in disease-causing pathogenic strains. Thus, prompt diagnosis and treatment by health care professionals with an effective antibiotic, e.g., fluoroquinolones, is imperative when treating severe vibrio infections stemming from exposure to lower Chesapeake Bay waters and possibly across the Bay. Overall, my data indicate that antibiotic resistance patterns among *V. parahaemolyticus* and *V. vulnificus* recovered from the lower Chesapeake Bay have remained relatively stable since 2009.

Ultimately, my results indicate that potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* occur across the Chesapeake Bay throughout the year, including at frequencies that may still lead to a greater risk of infection in the fall and possibly early winter. Moreover, the mid and lower sections of the Bay, which include many commercial and recreationally important areas, may harbor a greater risk of severe vibriosis from wound-associated water exposure, given the greater prevalence of *V. vulnificus* isolates displaying significant virulence factors.

Strengths and Limitations

A limitation of my epidemiological research is that while the MD FoodNet system can be considered representative of more severe cases where a *Vibrio* infection is culture-confirmed, mild or self-limiting cases are less likely to be represented. This has also been recognized as a limitation of the national vibriosis surveillance system (COVIS). In addition, *Vibrio* spp. are known to enter a protective state, namely viable but nonculturable (VBNC), whereby the cells become metabolically dormant and cannot be cultured using routine enteric media (Colwell, 2000). Hence, the numbers reported here are likely an underestimation of the actual

total number of *Vibrio* spp. infections for the State. It is also important to note that there isn't a standardized method of classifying vibriosis by exposure type; therefore, the determination of whether a case resulted from wound or seafood contamination was subjective. Moreover, vibriosis cases are recorded based on a patient's city and county of residence and may not necessarily reflect the county in which they sought medical attention or where they were exposed to the pathogen. Lastly, certain key variables were sometimes missing, such as age, gender, race, or occupation, and results may not fully reflect the population at highest risk across the State of Maryland. Future studies would benefit from inclusion of important metrics such as underlying medical conditions and antibiotics used during treatment.

With regard to my laboratory-based, molecular analyses, there were also several limitations. Although using direct colony DNA hybridization methods may underestimate the presence of *V. parahaemolyticus* virulence genes, *trh* and *tdh*, due to their limited presence in environmental strains, culture-dependent methods are important because they estimate the percentage of live and viable *Vibrio* spp. populations. Ideally, these methods should be supplemented by qPCR-most probable number (MPN) or whole genome sequencing (WGS), unfortunately the added costs can often be prohibitive in long-term monitoring projects.

Other limitations include lack of continuity of samples: I used data from two three-year longitudinal sampling periods that were 10 years apart but without sampling throughout the entire time, which led to a substantial data gap. This underscores the importance of continued monitoring, especially given the intra-and

inter-annual variability and rapidly changing physico-chemical conditions in the Chesapeake Bay.

Also, shallower coastal systems that are heavily impacted by conditions on land, will change more dramatically. As a result, forecasting models that help predict the abundance of total and pathogenic *V. parahaemolyticus* and *V. vulnificus* might need to have environmental and *Vibrio* spp. data updated every few years. This can be accomplished with targeted water monitoring of pre-determined stations that represent the upper, mid, and lower sections of the Chesapeake Bay, as well as seasonal sampling. A combination of culture-dependent and culture-independent methods would yield the best results, as it would provide a picture of the culturable and viable, as well as the VBNC populations, which may dominate during the colder months and possibly in other mediums (e.g. sediment and oysters)

Public Health Significance

Ultimately, my results indicate that potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* occur across the Chesapeake Bay throughout the seasons, including at frequencies that may still lead to a greater risk of infection in the fall and possibly early winter. Moreover, the mid and lower sections of the Bay, which include many commercial and recreationally important areas, may harbor a greater risk of severe vibriosis from wound-associated water exposure, given the greater prevalence of *V. vulnificus* isolates displaying significant virulence factors. Although the "Maryland Healthy Beaches" program run by the MDE provides *Vibrio* infection information on their website and flyers, including avoiding water contact if there are any open wounds, wearing water shoes to avoid cuts, wearing gloves when

crabbing or fishing, and showering after swimming in natural waters; it is unclear how readily available this information is in local creeks and waterways surrounding the Chesapeake Bay or if more remote water access points also have "*Vibrio* facts" flyers posted. It is therefore of significance to increase the awareness of risk of infection among recreational and commercial users across the Bay, especially in areas that may harbor more pathogenic *Vibrio* spp., beyond the summer season. There should also be more targeted education strategies aimed at populations with a higher risk of developing severe *Vibrio* infections (males, over the age of 45, with underlying medical conditions), or with a greater likelihood of being exposed (e.g., professional, and recreational crabbers).

Furthermore, as the incidence of vibriosis increases in the State of Maryland, there may be more individuals seeking medical attention, and as a result a greater need for awareness among clinicians of severe vibriosis symptoms and prompt treatment with effective first-line antibiotics, e.g., fluoroquinolones.

Future Research

It is imperative to continue to understand how vibrios continue to evolve in the face of environmental stressors and disruptions. As their abundance and distribution shift, other important associations may occur, including those with other physico-chemical parameters and pollutants that are less understood, e.g., dissolved oxygen, pH, nutrients, heavy metals. This work also highlights the importance of continued monitoring, as gaps in data availability will limit our ability to properly forecast and predict how this important bacterial pathogen will adapt over time in critical aquatic ecosystems around the world. Additionally, as the cost of new

technologies such as whole genome sequencing (WGS) become less prohibitive, their application in future studies will allow us to have a more complete picture of how virulence genes may evolve.

Bibliography

Ali, M., Nelson, A. R., Lopez, A. L., & Sack, D. A. (2015). Updated global burden of cholera in endemic countries. *PLoS Neglected Tropical Diseases*, *9*(6). https://doi.org/10.1371/journal.pntd.0003832

Allton, D. R., Forgione, M. A., & Gros, S. P. (2006). Cholera-like Presentation in Vibrio fluvialis Enteritis. *Southern Medical Journal*, *99*(7), 765–766.

Altekruse, S. F., Bishop, R. D., Baldy, L. M., Thompson, S. G., Wilson, S. A., Ray, B. J., & Griffin, P. M. (2000). Vibrio gastroenteritis in the US Gulf of Mexico region: The role of raw oysters. *Epidemiology and Infection*, *124*(3), 489–495. https://doi.org/10.1017/S0950268899003714

Andrews, W. H., & Hammack, T. S. (2022). *BAM Chapter 1: Food Sampling/Preparation of Sample Homogenate*. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-1-food-samplingpreparation-sample-homogenate

Archer, E. J., Baker-Austin, C., Osborn, T. J., Jones, N. R., Martínez-Urtaza, J., Trinanes, J., Oliver, J. D., González, F. J. C., & Lake, I. R. (2023). Climate warming and increasing Vibrio vulnificus infections in North America. *Scientific Reports*, *13*(1), 3893. https://doi.org/10.1038/s41598-023-28247-2

Baker-Austin, C., McArthur, J. V., Lindell, A. H., Wright, M. S., Tuckfield, R. C., Gooch, J., Warner, L., Oliver, J., & Stepanauskas, R. (2009). Multi-site analysis reveals widespread antibiotic resistance in the marine pathogen Vibrio

vulnificus. *Microbial Ecology*, *57*(1), 151–159. https://doi.org/10.1007/s00248-008-9413-8

Baker-Austin, C., Mcarthur, J. V, Lindell, A. H., Wright, M. S., Tuckfield, R. C., Gooch, J., Warner, L., Oliver, J., & Stepanauskas, R. (2009). Multi-site Analysis Reveals Widespread Antibiotic Resistance in the Marine Pathogen Vibrio vulnificus. *Microbial Ecology*, *57*, 151–159. https://doi.org/10.1007/s00248-008-9413-8

Baker-Austin, C., & Oliver, J. D. (2018). Vibrio vulnificus: new insights into a deadly opportunistic pathogen. *Environmental Microbiology*, *20*(2), 423–430. https://doi.org/10.1111/1462-2920.13955

Baker-Austin, C., Oliver, J. D., Alam, M., Ali, A., Waldor, M. K., Qadri, F., & Martinez-Urtaza, J. (2018). Vibrio spp. infections. *Nature Reviews Disease Primers*, *4*(1), 1–19. https://doi.org/10.1038/s41572-018-0005-8

Baker-Austin, C., Stockley, L., Rangdale, R., & Martinez-Urtaza, J. (2010). Environmental occurrence and clinical impact of Vibrio vulnificus and Vibrio parahaemolyticus: A European perspective. *Environmental Microbiology Reports*, *2*(1), 7–18. https://doi.org/10.1111/j.1758-2229.2009.00096.x

Baker-Austin, C., Trinanes, J. A., Taylor, N. G. H., Hartnell, R., Siitonen, A., & Martinez-Urtaza, J. (2013). Emerging Vibrio risk at high latitudes in response to ocean warming. *Nature Climate Change*, *3*(1), 73–77. https://doi.org/10.1038/nclimate1628

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., & Martinez-Urtaza, J. (2017). Non-Cholera Vibrios: The Microbial Barometer of Climate Change. *Trends in Microbiology*, *25*(1), 76–84. https://doi.org/10.1016/j.tim.2016.09.008

Banakar, V., Constantin de Magny, G., Jacobs, J., Murtugudde, R., Huq, A., Wood, R. J., & Colwell, R. R. (2011). Temporal and spatial variability in the distribution of Vibrio vulnificus in the Chesapeake Bay: a hindcast study. *EcoHealth*, *8*(4), 456–467. https://doi.org/10.1007/s10393-011-0736-4

Banerjee, S. K., Rutley, R., & Bussey, J. (2018). Diversity and dynamics of the Canadian coastal Vibrio community: An emerging trend detected in the temperate regions. *Journal of Bacteriology*, *200*(15). https://doi.org/10.1128/JB.00787-17

Bauer, A., & Rørvik, L. M. (2007). A novel multiplex PCR for the identification of Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus. *Letters in Applied Microbiology*, *45*(4), 371–375. https://doi.org/10.1111/j.1472-765X.2007.02195.x

Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C. L., Jones, D. D., & Kaysner, C. A. (1999). Detection of total and hemolysin-producing Vibrio parahaemolyticus in shellfish using multiplex PCR amplification of tl, tdh and trh. *Journal of Microbiological Methods*, *36*(3), 215–225. https://doi.org/10.1016/S0167-7012(99)00037-8

Bier, N., Schwartz, K., Guerra, B., & Strauch, E. (2015). Survey on antimicrobial resistance patterns in Vibrio vulnificus and Vibrio cholerae non-O1/non-O139 in Germany reveals carbapenemase-producing Vibrio cholerae in coastal waters. *Frontiers in Microbiology*, *6*(OCT), 1–11. https://doi.org/10.3389/fmicb.2015.01179

Bourne, D. G., & Munn, C. B. (2005). Diversity of bacteria associated with the coral Pocillopora damicornis from the Great Barrier Reef. *Environmental Microbiology*, *7*(8), 1162–1174. https://doi.org/10.1111/j.1462-2920.2005.00793.x

Brasher, C. W., DePaola, A., Jones, D. D., & Bej, A. K. (1998). Detection of Microbial Pathogens in Shellfish with Multiplex PCR. *Current Microbiology*, *37*, 101–107. https://doi.org/10.1007/s002849900346

Brehm, T. T., Berneking, L., Martins, M. S., Dupke, S., Jacob, D., Drechsel, O., Bohnert, J., Becker, K., Kramer, A., Christner, M., Aepfelbacher, M., Schmiedel, S., Rohde, H., Balau, V., Baufeld, E., Brechmann, S., Briedigkeit, L., Diedrich, S., Ebert, U., … Zaki, W. (2021). Heatwave-associated Vibrio infections in Germany, 2018 and 2019. *Eurosurveillance*, *26*(41), 1–11. https://doi.org/10.2807/1560-7917.ES.2021.26.41.2002041

Bross, M. H., Soch, K., Morales, R., & Mitchell, R. B. (2007). Vibrio vulnificus Infection: Diagnosis and Treatment. *American Family Physician*, *76*(4), 539–544. www.aafp.org/afp.

Brumfield, K. D., Chen, A. J., Gangwar, M., Usmani, M., Hasan, N. A., Jutla, A. S., Huq, A., & Rita R Colwell. (2023). Environmental Factors Influencing Occurrence of Vibrio parahaemolyticus and Vibrio vulnificus. *Environmental Microbiology*.

Brumfield, K. D., Usmani, M., Chen, K. M., Gangwar, M., Jutla, A. S., Huq, A., & Colwell, R. R. (2021a). Environmental parameters associated with incidence and transmission of pathogenic Vibrio spp. *Environmental Microbiology*, *23*(12), 7314–7340. https://doi.org/10.1111/1462-2920.15716

Brumfield, K. D., Usmani, M., Chen, K. M., Gangwar, M., Jutla, A. S., Huq, A., & Colwell, R. R. (2021b). Environmental parameters associated with incidence and transmission of pathogenic Vibrio spp. *Environmental Microbiology*, *00*. https://doi.org/10.1111/1462-2920.15716

Caburlotto, G., Suffredini, E., Toson, M., Fasolato, L., Antonetti, P., Zambon, M., & Manfrin, A. (2016). Occurrence and molecular characterisation of Vibrio parahaemolyticus in crustaceans commercialised in Venice area, Italy. *International Journal of Food Microbiology*, *220*, 39–49. https://doi.org/10.1016/j.ijfoodmicro.2015.12.007

Canellas, A. L. B., Lopes, I. R., Mello, M. P., Paranhos, R., de Oliveira, B. F. R., & Laport, M. S. (2021). Vibrio species in an urban tropical estuary: Antimicrobial susceptibility, interaction with environmental parameters, and possible public health outcomes. *Microorganisms*, *9*(5). https://doi.org/10.3390/microorganisms9051007

Cardoso, M. D., Lemos, L. S., Roges, E. M., de Moura, J. F., Tavares, D. C., Matias, C. A. R., Rodrigues, D. P., & Siciliano, S. (2018). A comprehensive survey of Aeromonas sp. and Vibrio sp. in seabirds from southeastern Brazil: outcomes for public health. *Journal of Applied Microbiology*, *124*(5), 1283–1293. https://doi.org/10.1111/jam.13705

Carpenter, C. C. I. (1972). Cholera and Other Enterotoxin-related Diarrheal Diseases. *THE JOURNAL OF INFECTIOUS DISEASES* , *126*(5), 551–563. https://academic.oup.com/jid/article/126/5/551/924754

Centers for Disease Control and Prevention (CDC). (2011). Vital Signs: Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 1996–2010. *MMWR Morb Mortal Wkly Rep*, *60*(22), 749–755.

Centers for Disease Control and Prevention (CDC). (2014). *National Enteric Disease Surveillance: COVIS Annual Summary, 2014*. http://www.cdc.gov/

Centers for Disease Control and Prevention (CDC). (2019, March 5). *Vibrio Species Causing Vibriosis*. CDC, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases. https://www.cdc.gov/vibrio/index.html

Centers for Disease Control and Prevention (CDC). (2021). *Foodborne Diseases Active Surveillance Network (FoodNet)*. CDC's FoodNet Fast. https://wwwn.cdc.gov/foodnetfast/

Centers for Disease Control and Prevention (CDC). (2023a). *Severe Vibrio vulnificus Infections in the United States Associated with Warming Coastal Waters*. https://emergency.cdc.gov/han/2023/han00497.asp#print

Centers for Disease Control and Prevention (CDC). (2023b). *Vibrio Species Causing Vibriosis: Information for Health professionals and Laboratorians*. CDC 24/7: Saving Lives, Protecting People. https://www.cdc.gov/vibrio/healthcare.html#wounds

Chao, W. N., Tsai, C. F., Chang, H. R., Chan, K. S., Su, C. H., Lee, Y. T., Ueng, K. C., Chen, C. C., Chen, S. C., & Lee, M. C. (2013). Impact of timing of surgery on outcome of Vibrio vulnificus-related necrotizing fasciitis. *American Journal of Surgery*, *206*(1), 32–39. https://doi.org/10.1016/j.amjsurg.2012.08.008

Chase, E., Young, S., & Harwood, V. J. (2015). Sediment and vegetation as reservoirs of Vibrio vulnificus in the Tampa Bay Estuary and Gulf of Mexico. *Applied and Environmental Microbiology*, *81*(7), 2489–2494. https://doi.org/10.1128/AEM.03243-14

Chen, A. J., Hasan, N. A., Haley, B. J., Taviani, E., Tarnowski, M., Brohawn, K., Johnson, C. N., Colwell, R. R., & Huq, A. (2017). Characterization of pathogenic Vibrio parahaemolyticus from the Chesapeake Bay, Maryland. *Frontiers in Microbiology*, *8*(DEC), 1–10. https://doi.org/10.3389/fmicb.2017.02460

Choi, G., & Choi, S. H. (2022). Complex regulatory networks of virulence factors in Vibrio vulnificus. *Trends in Microbiology*, *30*(12), 1205–1216. https://doi.org/10.1016/j.tim.2022.05.009

Clinical and Laboratory Standards Institute (CLSI). (2015). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, 3rd ed., with 19 June 2017 correction. CLSI guideline M45 (ISBN 1-56238-918-1).* Clinical and Laboratory Standards Institute. www.clsi.org.

Clinical and Laboratory Standards Institute (CLSI). (2020). *Performance standards for antimicrobial susceptibility testing, 30th ed. CLSI supplement M100 (ISBN 978-1-68440-067-6).* Clinical and Laboratory Standards Institute.

Collier, S. A., Deng, L., Adam, E. A., Benedict, K. M., Beshearse, E. M., Blackstock, A. J., Bruce, B. B., Derado, G., Edens, C., Fullerton, K. E., Gargano, J. W., Geissler, A. L., Hall, A. J., Havelaar, A. H., Hill, V. R., Hoekstra, R. M., Reddy, S. C., Scallan, E., Stokes, E. K., … Beach, M. J. (2021). Estimate of burden and direct healthcare cost of infectious waterborne disease in the United States. *Emerging Infectious Diseases*, *27*(1), 140–149. https://doi.org/10.3201/eid2701.190676

Colwell, R. R. (1996). Global Climate and Infectious Disease: The Cholera Paradigm. *Science*, *274*(5295), 2025–2031. https://www.science.org

Colwell, R. R. (2000). Viable but nonculturable bacteria: a survival strategy. *J Infect Chemother*, *6*, 121–125.

Costa, W. F., Giambiagi-Demarval, M., & Laport, M. S. (2021). Antibiotic and heavy metal susceptibility of non-cholera vibrio isolated from marine sponges and sea urchins: Could they pose a potential risk to public health? *Antibiotics*, *10*(12). https://doi.org/10.3390/antibiotics10121561

Daniels, N. A., Mackinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., Thompson, S., Wilson, S., Bean, N. H., Griffin, P. M., & Slutsker, L. (2000). Vibrio parahaemolyticus Infections in the United States, 1973-1998. *THE JOURNAL OF INFECTIOUS DISEASES*, *181*, 1661–1666. https://academic.oup.com/jid/article-abstract/181/5/1661/2191420

Daniels, N. A., & Shafaie, A. (2000). A Review of Pathogenic Vibrio Infections for Clinicians. *Infectious Medicine*, *17*(10), 665–685.

da Silva, L. V., Ossai, S., Chigbu, P., & Parveen, S. (2021). Antimicrobial and Genetic Profiles of Vibrio vulnificus and Vibrio parahaemolyticus Isolated From the Maryland Coastal Bays, United States. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.676249

Das Sarker, M. K., Ahammed, T., Sahabuddin, Md., Akter, P., Haque, A., Hossain, Md. R., Mosaib, Md. G., Islam, Md. R., Mondol, G. C., & Alam, Md. F. (2019). Antibiotic Resistance Analysis of Vibrio spp Isolated from Different Types of Water Sources of Bangladesh and Their Characterization. *European Journal of Medical and Health Sciences*, *1*(4), 19–29. https://doi.org/10.34104/ejmhs.01929

Davis, B. J. K., Corrigan, A. E., Sun, Z., Atherly, E., DePaola, A., & Curriero, F. C. (2021). A case-control analysis of traceback investigations for Vibrio parahaemolyticus infections (vibriosis) and pre-harvest environmental conditions in Washington State, 2013–2018. *Science of the Total Environment*, *752*, 141650. https://doi.org/10.1016/j.scitotenv.2020.141650

Davis, B. J. K., Jacobs, J. M., Davis, M. F., Schwab, K. J., DePaola, A., & Curriero, F. C. (2017). Environmental determinants of Vibrio parahaemolyticus in the Chesapeake Bay. *Applied and Environmental Microbiology*, *83*(21). https://doi.org/10.1128/AEM.01147-17

Dechet, A. M., Yu, P. A., Koram, N., & Painter, J. (2008). Nonfoodborne Vibrio infections: An important cause of morbidity and mortality in the United States, 1997-2006. *Clinical Infectious Diseases*, *46*(7), 970–976. https://doi.org/10.1086/529148

Deeb, R., Tufford, D., Scott, G. I., Moore, J. G., & Dow, K. (2018). Impact of Climate Change on Vibrio vulnificus Abundance and Exposure Risk. *Estuaries and Coasts*, *41*(8), 2289–2303. https://doi.org/10.1007/s12237-018-0424-5

DeLuca, N. M., Zaitchik, B. F., Guikema, S. D., Jacobs, J. M., Davis, B. J. K., & Curriero, F. C. (2020). Evaluation of remotely sensed prediction and forecast models for Vibrio parahaemolyticus in the Chesapeake Bay. *Remote Sensing of Environment*, *250*(May 2019), 112016. https://doi.org/10.1016/j.rse.2020.112016

DePaola, A., Kaysner, C. A., Bowers, J., & Cook, D. W. (2000). Environmental investigations of Vibrio parahaemolyticus in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). *Applied and Environmental Microbiology*, *66*(11), 4649–4654. https://doi.org/10.1128/AEM.66.11.4649-4654.2000

DePaola, A., Ulaszek, J., Kaysner, C. A., Tenge, B. J., Nordstrom, J. L., Wells, J., Puhr, N., & Gendel, S. M. (2003). Molecular, serological, and virulence characteristics of Vibrio parahaemolyticus isolated from environmental, food, and clinical sources in North America and Asia. *Applied and Environmental Microbiology*, *69*(7), 3999–4005. https://doi.org/10.1128/AEM.69.7.3999-4005.2003

Destoumieux-Garzón, D., Canesi, L., Oyanedel, D., Travers, M. A., Charrière, G. M., Pruzzo, C., & Vezzulli, L. (2020). Vibrio–bivalve interactions in health and disease. *Environmental Microbiology*, *22*(10), 4323–4341. https://doi.org/10.1111/1462-2920.15055

Distefano, P., Muth, M. K., Arsenault, J. E., Cajka, J. C., Cates, S. C., Coglaiti, M. C., Karns, S. A., O', M., & Viator, N. C. (2011). *Analysis of How Post-harvest Processing Technologies for Controlling Vibrio vulnificus Can Be Implemented Final Report Prepared for*. //efaidnbmnnnibpcajpcglclefindmkaj/https://www.issc.org/Data/Sites/1/media/rti%20final%20report%20on%20php%20implementation_march%202011.pdf

Drake, S. L., Depaola, A., & Jaykus, L. A. (2007). An overview of vibrio vulnificus and vibrio parahaemolyticus. *Comprehensive Reviews in Food Science and Food Safety*, *6*(4), 120–144. https://doi.org/10.1111/j.1541-4337.2007.00022.x

Elmahdi, S., DaSilva, L. V., & Parveen, S. (2016). Antibiotic resistance of Vibrio parahaemolyticus and Vibrio vulnificus in various countries: A review. *Food Microbiology*, *57*(410), 128–134. https://doi.org/10.1016/j.fm.2016.02.008

Elmahdi, S., Parveen, S., Ossai, S., DaSilva, L. V., Jahncke, M., Bowers, J., & Jacobs, J. (2018). Vibrio parahaemolyticus and Vibrio vulnificus recovered from oysters during an oyster relay study. *Applied and Environmental Microbiology*, *84*(3). https://doi.org/10.1128/AEM.01790-17

Erken, M., Lutz, C., & McDougald, D. (2015). Interactions of Vibrio spp. with Zooplankton . *Microbiology Spectrum*, *3*(3). https://doi.org/10.1128/microbiolspec.ve-0003-2014

U.S. Federal Drug and Administration, FDA, (2020). *National Shellfish Sanitation Program (NSSP)*. FDA Archive. https://www.fda.gov/food/federalstate-food-programs/national-shellfish-sanitation-program-nssp

Ferchichi, H., St-Hilaire, A., Ouarda, T. B. M. J., & Lévesque, B. (2021). Impact of the future coastal water temperature scenarios on the risk of potential growth of pathogenic Vibrio marine bacteria. *Estuarine, Coastal and Shelf Science*, *250*(107094), 1–10. https://doi.org/10.1016/j.ecss.2020.107094

Fernández-Delgado, M., Sanz, V., Giner, S., Suárez, P., Contreras, M., Michelangeli, F., & García-Amado, M. A. (2016). Prevalence and distribution of Vibrio spp. In wild aquatic birds of the Southern Caribbean Sea, Venezuela, 2011–12. *Journal of Wildlife Diseases*, *52*(3), 621–626. https://doi.org/10.7589/2015-06-154

Fleischmann, S., Herrig, I., Wesp, J., Stiedl, J., Reifferscheid, G., Strauch, E., Alter, T., & Brennholt, N. (2022). Prevalence and Distribution of Potentially Human Pathogenic Vibrio spp. on German North and Baltic Sea Coasts. *Frontiers in Cellular and Infection Microbiology*, *12*(July), 1–15. https://doi.org/10.3389/fcimb.2022.846819

Ford, C. L., Powell, A., Lau, D. Y. L., Turner, A. D., Dhanji-Rapkova, M., Martinez-Urtaza, J., & Baker-Austin, C. (2020). Isolation and characterization of potentially pathogenic Vibrio species in a temperate, higher latitude hotspot. *Environmental Microbiology Reports*, *12*(4), 424–434. https://doi.org/10.1111/1758-2229.12858

Froelich, B. A., & Daines, D. A. (2020). In hot water: effects of climate change on Vibrio–human interactions. *Environmental Microbiology*, *22*(10), 4101–4111. https://doi.org/10.1111/1462-2920.14967

Fu, S., Hao, J., Yang, Q., Lan, R., Wang, Y., Ye, S., Liu, Y., & Li, R. (2019). Long-distance transmission of pathogenic Vibrio species by migratory waterbirds: a potential threat to the public health. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-52791-5

Gao, Q., Ma, X., Wang, Z., Chen, H., Luo, Y., Wu, B., Qi, S., Lin, M., Tian, J., Qiao, Y., Grossart, H. P., Xu, W., & Huang, L. (2022). Seasonal variation, virulence gene and antibiotic resistance of Vibrio in a semi-enclosed bay with mariculture (Dongshan Bay, Southern China). *Marine Pollution Bulletin*, *184*(August). https://doi.org/10.1016/j.marpolbul.2022.114112

Hlady, G. W. (1997). Vibrio infections associated with raw oyster consumption in Florida, 1981-1994. *Journal of Food Protection*, *60*(4), 353–357. https://doi.org/10.4315/0362-028x-60.4.353

Gennari, M., Ghidini, V., Caburlotto, G., & Lleo, M. M. (2012). Virulence genes and pathogenicity islands in environmental Vibrio strains nonpathogenic to humans. *FEMS Microbiology Ecology*, *82*(3), 563–573. https://doi.org/10.1111/j.1574-6941.2012.01427.x

Graczyk, T. K., & Schwab, K. J. (2000). Foodborne infections vectored by molluscan shellfish. *Current Gastroenterology Reports*, *2*(4), 305–309. https://doi.org/10.1007/s11894-000-0023-y

Grimes, D. J. (2020). The Vibrios: Scavengers, Symbionts, and Pathogens from the Sea. *Microbial Ecology*, *80*, 501–506. https://doi.org/10.1007/s00248-020-01524-7/Published

Håkonsholm, F., Lunestad, B. T., Aguirre Sánchez, J. R., Martinez-Urtaza, J., Marathe, N. P., & Svanevik, C. S. (2020). Vibrios from the Norwegian marine environment: Characterization of associated antibiotic resistance and virulence genes. *MicrobiologyOpen*, *9*(9), 1–19. https://doi.org/10.1002/mbo3.1093

Halpern, M., & Izhaki, I. (2017). Fish as hosts of Vibrio cholerae. In *Frontiers in Microbiology* (Vol. 8, Issue FEB). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2017.00282

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W., & Ge, B. (2007). Antimicrobial susceptibilities of Vibrio parahaemolyticus and Vibrio vulnificus isolates from Louisiana Gulf and retail raw oysters. *Applied and Environmental Microbiology*, *73*(21), 7096–7098. https://doi.org/10.1128/AEM.01116-07

Heidelberg, J. F., Heidelberg, K. B., & Colwell, R. R. (2002). Bacteria of the γ-subclass Proteobacteria associated with zooplankton in Chesapeake Bay. *Applied and Environmental Microbiology*, *68*(11), 5498–5507. https://doi.org/10.1128/AEM.68.11.5498-5507.2002

Heng, S. P., Letchumanan, V., Deng, C. Y., Ab Mutalib, N. S., Khan, T. M., Chuah, L. H., Chan, K. G., Goh, B. H., Pusparajah, P., & Lee, L. H. (2017). Vibrio vulnificus: An environmental and clinical burden. *Frontiers in Microbiology*, *8*(MAY). https://doi.org/10.3389/fmicb.2017.00997

Hiyoshi, H., Kodama, T., Iida, T., & Honda, T. (2010). Contribution of Vibrio parahaemolyticus virulence factors to cytotoxicity, enterotoxicity, and lethality in mice. *Infection and Immunity*, *78*(4), 1772–1780. https://doi.org/10.1128/IAI.01051-09

Horseman, M. A., & Surani, S. (2011). A comprehensive review of Vibrio vulnificus: An important cause of severe sepsis and skin and soft-tissue infection. In *International Journal of Infectious Diseases* (Vol. 15, Issue 3). https://doi.org/10.1016/j.ijid.2010.11.003

Huang, K.-C., Wen, R., & Hsu, W. (2005). Vibrio fluvialis Hemorrhagic Cellulitis and Cerebritis. *Clinical Infectious Diseases*, *40*, 75–82.

Huq, A., Small, E. B., West,', P. A., Imdadul Huq, M., Rahman, R., & Colwell', R. R. (1983). Ecological Relationships Between Vibrio cholerae and Planktonic Crustacean Copepods. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *45*(1), 275–283. http://aem.asm.org/

Igbinosa, E. O., & Okoh, A. I. (2010). Vibrio fluvialis: An unusual enteric pathogen of increasing public health concern. *International Journal of Environmental Research and Public Health*, *7*(10), 3628–3643. https://doi.org/10.3390/ijerph7103628

Iwamoto, M., Ayers, T., Mahon, B. E., & Swerdlow, D. L. (2010). Epidemiology of seafood-associated infections in the United States. *Clinical Microbiology Reviews*, *23*(2), 399–411. https://doi.org/10.1128/CMR.00059-09

Jacobs, J. M., Rhodes, M., Brown, C. W., Hood, R. R., Leight, A., Long, W., & Wood, R. (2014). Modeling and forecasting the distribution of Vibrio vulnificus in Chesapeake Bay. *Journal of Applied Microbiology*, *117*(5), 1312–1327. https://doi.org/10.1111/jam.12624

Jiang, Y., Yao, L., Li, F., Tan, Z., Zhai, Y., & Wang, L. (2014). Characterization of antimicrobial resistance of Vibrio parahaemolyticus from cultured sea cucumbers (Apostichopus japonicas). *Letters in Applied Microbiology*, *59*(2), 147–154. https://doi.org/10.1111/lam.12258

Johnson, C. N., Bowers, J. C., Griffitt, K. J., Molina, V., Clostio, R. W., Pei, S., Laws, E., Paranjpye, R. N., Strom, M. S., Chen, A., Hasan, N. A., Huq, A., Noriea, N. F., Grimes, D. J., & Colwell, R. R. (2012). Ecology of vibrio parahaemolyticus and vibrio vulnificus in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). *Applied and Environmental Microbiology*, *78*(20), 7249–7257. https://doi.org/10.1128/AEM.01296-12

Johnson, C. N., Flowers, A. R., Noriea, N. F., Zimmerman, A. M., Bowers, J. C., DePaola, A., & Grimes, D. J. (2010). Relationships between environmental factors and pathogenic vibrios in the northern Gulf of Mexico. *Applied and Environmental Microbiology*, *76*(21), 7076–7084. https://doi.org/10.1128/AEM.00697-10

Jones, E. H., Feldman, K. A., Palmer, A., Butler, E., Blythe, D., & Mitchell, C. (2013). Vibrio Infections and Surveillance in Maryland, 2002–2008. *Public Health Reports*, *128*(November-December), 537–545.

Jones, J. L., Lüdeke, C. H. M., Bowers, J. C., Garrett, N., Fischer, M., Parsons, M. B., Bopp, C. A., & DePaola, A. (2012). Biochemical, serological, and virulence characterization of clinical and oyster Vibrio parahaemolyticus isolates. *Journal of Clinical Microbiology*, *50*(7), 2343–2352. https://doi.org/10.1128/JCM.00196-12

Jones, T. F., Scallan, E., & Angulo, F. J. (2007). FoodNet: Overview of a decade of achievement. *Foodborne Pathogens and Disease*, *4*(1), 60–66. https://doi.org/10.1089/fpd.2006.63

Julie, D., Solen, L., Antoine, V., Jaufrey, C., Annick, D., & Dominique, H. H. (2010). Ecology of pathogenic and non-pathogenic Vibrio parahaemolyticus on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. *Environmental Microbiology*, *12*(4), 929–937. https://doi.org/10.1111/j.1462-2920.2009.02136.x

Jutla, A., Whitcombe, E., Hasan, N., Haley, B., Akanda, A., Huq, A., Alam, M., Sack, R. B., & Colwell, R. (2013). Environmental factors influencing epidemic cholera. *American Journal of Tropical Medicine and Hygiene*, *89*(3), 597–607. https://doi.org/10.4269/ajtmh.12-0721

Kaneko, T., & Colwell, R. R. (1973). Ecology of Vibrio parahaemolyticus in Chesapeake Bay. *JOURNAL OF BACTERIOLOGY*, *113*(1), 24–32. http://jb.asm.org/

Kaspar, C. W., & Tamplin, M. L. (1993). Effects of temperature and salinity on the survival of Vibrio vulnificus in seawater and shellfish. *Applied and Environmental Microbiology*, *59*(8), 2425–2429. https://doi.org/10.1128/aem.59.8.2425-2429.1993

Kaysner, C. A., DePaola, A., & Jones, J. L. (2019). *BAM Chapter 9: Vibrio*. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-9-vibrio

Kelly, M. T. (1982). Effect of Temperature and Salinity on Vibrio (Beneckea) vulnificus Occurrence in a Gulf Coast Environment. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *44*(4), 820–824.

Kim, J. S., Lee, E. G., & Chun, B. C. (2022). Epidemiologic Characteristics and Case Fatality Rate of Vibrio vulnificus Infection: Analysis of 761 Cases From 2003 to 2016 in Korea. *Journal of Korean Medical Science*, *37*(9), 1–12. https://doi.org/10.3346/jkms.2022.37.e79

Kim, Y. R., Lee, S. E., Kook, H., Yeom, J. A., Na, H. S., Kim, S. Y., Chung, S. S., Choy, H. E., & Rhee, J. H. (2008). Vibrio vulnificus RTX toxin kills host cells only after contact of the bacteria with host cells. *Cellular Microbiology*, *10*(4), 848–862. https://doi.org/10.1111/j.1462-5822.2007.01088.x

Klontz, K. C., & Desenclos, J.-C. A. (1990). CLINICAL AND EPIDEMIOLOGICAL FEATURES OF SPORADIC INFECTIONS WITH VIBRIO. *Source: Journal of Diarrhoeal Diseases Research*, *8*(1), 24–26. https://www.jstor.org/stable/23498047

Krantz, G. E., Colwell, R. R., & Lovelace, E. (1969). Vibrio parahaemolyticus from the Blue Crab Callinectes sapidus in Chesapeake Bay. *Science, New Series*, *164*(3885), 1286–1287. https://www.jstor.org/stable/1726891

Krumperman, P. H. (1983). Multiple Antibiotic Resistance Indexing of Escherichia coli to Identify High-Risk Sources of Fecal Contamination of Foods. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *46*(1), 165–170. https://journals.asm.org/journal/aem

Kumarage, P. M., De Silva, L. A. D. S., & Heo, G. J. (2022). Aquatic environments: A potential source of antimicrobial-resistant Vibrio spp. *Journal of Applied Microbiology*, *133*(4), 2267–2279. https://doi.org/10.1111/jam.15702

Lai, C. H., Hwang, C. K., Chin, C., Lin, H. H., Wong, W. W., & Liu, C. Y. (2006). Severe watery diarrhoea and bacteraemia caused by Vibrio fluvialis. *Journal of Infection*, *52*(3). https://doi.org/10.1016/j.jinf.2005.05.023

Laviad-Shitrit, S., Izhaki, I., Arakawa, E., & Halpern, M. (2018). Wild waterfowl as potential vectors of Vibrio cholerae and Aeromonas species. *Tropical Medicine and International Health*, *23*(7), 758–764. https://doi.org/10.1111/tmi.13069

Laviad-Shitrit, S., Izhaki, I., & Halpern, M. (2019). Accumulating evidence suggests that some waterbird species are potential vectors of Vibrio cholerae. *PLoS Pathogens*, *15*(8). https://doi.org/10.1371/journal.ppat.1007814

Lee, B. C., Choi, S. H., & Kim, T. S. (2008). Vibrio vulnificus RTX toxin plays an important role in the apoptotic death of human intestinal epithelial cells exposed to Vibrio vulnificus. *Microbes and Infection*, *10*(14–15), 1504–1513. https://doi.org/10.1016/j.micinf.2008.09.006

Lee, J. Y., Park, J. S., Oh, S. H., Kim, H. R., Lee, J. N., & Shin, J. H. (2008). Acute infectious peritonitis caused by Vibrio fluvialis. *Diagnostic Microbiology and Infectious Disease*, *62*(2), 216–218. https://doi.org/10.1016/j.diagmicrobio.2008.05.012

Le Roux, F., Wegner, K. M., Baker-Austin, C., Vezzulli, L., Osorio, C. R., Amaro, C., Ritchie, J. M., Defoirdt, T., Destoumieux-Garzón, D., Blokesch, M., Mazel, D., Jacq, A., Cava, F., Gram, L., Wendling, C. C., Strauch, E., Kirschner, A., & Huehn, S. (2015). The emergence of Vibrio pathogens in Europe: Ecology, evolution and pathogenesis (Paris, 11-12 March 2015). *Frontiers in Microbiology*, *6*(JUL), 1–8. https://doi.org/10.3389/fmicb.2015.00830

Li, G., & Wang, M. Y. (2020). The role of Vibrio vulnificus virulence factors and regulators in its infection-induced sepsis. *Folia Microbiologica*, *65*(2), 265–274. https://doi.org/10.1007/s12223-019-00763-7

Liu, M., & Crosa, J. H. (2012). The regulator HlyU, the repeat-in-toxin gene rtxA1, and their roles in the pathogenesis of Vibrio vulnificus infections. *MicrobiologyOpen*, *1*(4), 502–513. https://doi.org/10.1002/mbo3.48

Lobitz, B., Beck, L., Huq, A., Wood, B., Fuchs, G., Faruque, A. S. G., Colwell, R., & Stanley, S. M. (2000). Climate and infectious disease: Use of remote sensing for detection of Vibrio cholerae by indirect measurement. *PNAS*, *97*(4), 1438–1443. www.csr.utexas.edu

Lopatek, M., Wieczorek, K., & Osek, J. (2018). Antimicrobial Resistance, Virulence Factors, and Genetic Profiles of Vibrio parahaemolyticus from Seafood. *Applied and Environmental Microbiology*, *84*(16), 1–10. https://doi.org/10.1128/AEM.00537-18

Lovelace, T. E., Tubiash, H., & Colwell, R. R. (1968). Quantitative and Qualitative Commensal Bacterial Flora of Crassostrea virginica in Chesapeake Bay. *Proceedings of the National Shellfisheries Association*, 82–87.

Mahoney, J. C., Gerding, M. J., Jones, S. H., & Whistler, C. A. (2010). Comparison of the pathogenic potentials of environmental and clinical Vibrio parahaemolyticus strains indicates a role for temperature regulation in virulence. *Applied and Environmental Microbiology*, *76*(22), 7459–7465. https://doi.org/10.1128/AEM.01450-10

Mandel, M. J., & Dunn, A. K. (2016). Impact and influence of the natural vibrio-squid symbiosis in understanding bacterial-animal interactions. In *Frontiers in Microbiology* (Vol. 7, Issue DEC). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2016.01982

Martinez-Urtaza, J., Blanco-Abad, V., Rodriguez-Castro, A., Ansede-Bermejo, J., Miranda, A., & Rodriguez-Alvarez, M. X. (2012). Ecological determinants of the occurrence and dynamics of Vibrio parahaemolyticus in offshore areas. *ISME Journal*, *6*(5), 994–1006. https://doi.org/10.1038/ismej.2011.156

Maryland Department of the Environment (MDE). (2023, May 31). *Get the Facts on Vibrio, https://mde.maryland.gov/publichealth/pages/vibrio.aspx*. Maryland.Gov, Public Health Home, Https://Mde.Maryland.Gov/Publichealth/Pages/Vibrio.Aspx.

Massicotte, P., & South, A. (2023). *rnaturalearth: World Map Data from Natural Earth.*

Morris, G. J., & Black, R. E. (1985). Cholera and Other Vibrioses in the United States. *The New England Journal of Medicine*, *312*(6), 343–350.

Najjar, R. G., Pyke, C. R., Adams, M. B., Breitburg, D., Hershner, C., Kemp, M., Howarth, R., Mulholland, M. R., Paolisso, M., Secor, D., Sellner, K., Wardrop, D., & Wood, R. (2010). Potential climate-change impacts on the Chesapeake Bay. *Estuarine, Coastal and Shelf Science*, *86*(1), 1–20. https://doi.org/10.1016/j.ecss.2009.09.026

Namadi, P., & Deng, Z. (2023). Optimum environmental conditions controlling prevalence of vibrio parahaemolyticus in marine environment. In *Marine Environmental Research* (Vol. 183). Elsevier Ltd. https://doi.org/10.1016/j.marenvres.2022.105828

Natividad-Bonifacio, I., Fernández, F. J., Quiñones-Ramírez, E. I., Curiel-Quesada, E., & Vázquez-Salinas, C. (2013). Presence of virulence markers in environmental Vibrio vulnificus strains. *Journal of Applied Microbiology*, *114*(5), 1539–1546. https://doi.org/10.1111/jam.12149

Ndraha, N., & Hsiao, H. I. (2021). Influence of climatic factors on the temporal occurrence and distribution of total and pathogenic Vibrio parahaemolyticus in oyster culture environments in Taiwan. *Food Microbiology, 98*(November 2020), 103765. https://doi.org/10.1016/j.fm.2021.103765

Ndraha, N., Wong, H. chung, & Hsiao, H. I. (2020). Managing the risk of Vibrio parahaemolyticus infections associated with oyster consumption: A review. *Comprehensive Reviews in Food Science and Food Safety*, *19*(3), 1187–1217. https://doi.org/10.1111/1541-4337.12557

Newton, A. E., Garrett, N., Stroika, S. G., Halpin, J. L., Turnsek, M., & Mody, R. K. (2014). *Increase in Vibrio parahaemolyticus Infections Associated with Consumption of Atlantic Coast Shellfish — 2013. Morbidity and Mortality Weekly Report, Vol. 63, No. 15 (April 18, 2014)*. www.jstor.or/stable/10.2307/24855000

Newton, A., Kendall, M., Vugia, D. J., Henao, O. L., & Mahon, B. E. (2012). Increasing rates of vibriosis in the United States, 1996-2010: Review of surveillance data from 2 systems. *Clinical Infectious Diseases*, *54*(SUPPL.5), 391–395. https://doi.org/10.1093/cid/cis243

Ngasotter, S., Mukherjee, S., Singh, S. K., Bharti, D., Ramjanul, H., Varshney, S., Nanda, C., Waikhom, D., Devi, M. S., & Singh, A. S. (2022). Prevalence, Virulence, and Antibtiotic Resistance of Vibrio Parahaemolyticus from Seafood and its Environment: An Updated Review. *Mediterranean Journal of Infection, Microbes and Antimicrobials*, *11*(January). https://doi.org/10.4274/mjima.galenos.2021.2021.1

Nishibuchi, M. (2006). Miscellaneous Human Pathogens. In F. L. Thompson, B. Austin, & J. Swings (Eds.), *The Biology of Vibrios* (pp. 367–384). https://doi.org/https://doi.org/10.1016/S1473-3099(07)70133-0

Nishibuchi, M., Fasano, A., Russell, R. G., & Kaper, J. B. (1992). Enterotoxigenicity of Vibrio parahaemolyticus with and without genes encoding thermostable direct hemolysin. *Infection and Immunity*, *60*(9), 3539–3545. https://doi.org/10.1128/iai.60.9.3539-3545.1992

Nordstrom, J. L., Kaysner, C. A., Blackstone, G. M., Vickery, M. C. L., Bowers, J. C., & DePaola, A. (2004). Effect of intertidal exposure on Vibrio parahaemolyticus levels in Pacific Northwest oysters. *Journal of Food Protection*, *67*(10), 2178–2182. https://doi.org/10.4315/0362-028X-67.10.2178

Nowakowska, J., & Oliver, J. D. (2013). Resistance to environmental stresses by Vibrio vulnificus in the viable but nonculturable state. *FEMS Microbiology Ecology*, *84*(1), 213–222. https://doi.org/10.1111/1574-6941.12052

Oliver, J. D. (2005). Wound infections caused by Vibrio vulnificus and other marine bacteria. *Epidemiology and Infection*, *133*(3), 383–391. https://doi.org/10.1017/S0950268805003894

Oliver, J. D. (2013). Vibrio vulnificus: Death on the Half Shell. A Personal Journey with the Pathogen and its Ecology. *Microbial Ecology*, *65*(4), 793–799. https://doi.org/10.1007/s00248-012-0140-9

Oliver, J. D., Hite, F., Mcdougald, D., Andon, N. L., & Simpson, L. M. (1995). Entry into, and Resuscitation from, the Viable but Nonculturable State by Vibrio vulnificus in an Estuarine Environment. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *61*(7), 2624–2630. https://journals.asm.org/journal/aem

Panicker, G., & Bej, A. K. (2005). Real-time PCR detection of Vibrio vulnificus in oysters: comparison of oligonucleotide primers and probes targeting vvhA. *Applied and Environmental Microbiology*, *71*(10), 5702–5709. https://doi.org/10.1128/AEM.71.10.5702-5709.2005

Panicker, G., Myers, M. L., & Bej, A. K. (2004). Rapid Detection of Vibrio vulnificus in Shellfish and Gulf of Mexico Water by Real-Time PCR. *Applied and Environmental Microbiology*, *70*(1), 498–507. https://doi.org/10.1128/AEM.70.1.498-507.2004

Paolisso, M. (2002). Blue Crabs and Controversy on the Chesapeake Bay: A Cultural Model for Understanding Watermen's Reasoning about Blue Crab Management. *Source: Human Organization*, *61*(3), 226–239.

Paranjpye, R. N., & Strom, M. S. (2005). A Vibrio vulnificus type IV pilin contributes to biofilm formation, adherence to epithelial cells, and virulence. *Infection and Immunity*, *73*(3), 1411–1422. https://doi.org/10.1128/IAI.73.3.1411-1422.2005

Parveen, S., Hettiarachchi, K. A., Bowers, J. C., Jones, J. L., Tamplin, M. L., McKay, R., Beatty, W., Brohawn, K., DaSilva, L. V., & DePaola, A. (2008). Seasonal distribution of total and pathogenic Vibrio parahaemolyticus in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology*, *128*(2), 354–361. https://doi.org/10.1016/j.ijfoodmicro.2008.09.019

Parveen, S., Jacobs, J. M., Ozbay, G., Chintapenta, L. K., Almuhaibed, E., Meredith, J., Ossai, S., Abbott, A., Grant, A., Brohawn, K., Chigbu, P., & Richards, G. P. (2020). Seasonal and Geographical Differences in Total and Pathogenic Vibrio parahaemolyticus and Vibrio vulnificus Levels in Seawater and Oysters from the Delaware and Chesapeake Bays Determined Using Several Methods. *Applied and Environmental Microbiology*, *86*(23), 1–19. https://doi.org/10.1128/AEM.01581-20

Pérez-Duque, A., Gonzalez-Muñoz, A., Arboleda-Valencia, J., Vivas-Aguas, L. J., Córdoba-Meza, T., Rodriguez-Rey, G. T., Díaz-Guevara, P., Martinez-Urtaza, J., & Wiesner-Reyes, M. (2021). Comparative genomics of clinical and environmental isolates of vibrio spp. Of colombia: Implications of traits associated with virulence and resistance. *Pathogens*, *10*(12). https://doi.org/10.3390/pathogens10121605

Potasman, I., Paz, A., & Odeh, M. (2002). Infectious outbreaks associated with bivalve shellfish consumption: A worldwide perspective. *Clinical Infectious Diseases*, *35*(8), 921–928. https://doi.org/10.1086/342330

Pruzzo, C., Vezzulli, L., & Colwell, R. R. (2008). Global impact of Vibrio cholerae interactions with chitin. In *Environmental Microbiology* (Vol. 10, Issue 6, pp. 1400–1410). https://doi.org/10.1111/j.1462-2920.2007.01559.x

Raghunath, P. (2014). Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in Vibrio parahaemolyticus. *Frontiers in Microbiology*, *5*(DEC), 2010–2013. https://doi.org/10.3389/fmicb.2014.00805

Ramamurthy, T., Chowdhury, G., Pazhani, G. P., & Shinoda, S. (2014). Vibrio fluvialis: An emerging human pathogen. In *Frontiers in Microbiology* (Vol. 5, Issue MAR). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2014.00091

Rippey, S. R. (1994). Infectious diseases associated with molluscan shellfish consumption. *Clinical Microbiology Reviews*, *7*(4), 419–425. https://doi.org/10.1128/CMR.7.4.419

Rizvi, A. V., & Bej, A. K. (2010). Multiplexed real-time PCR amplification of tlh, tdh and trh genes in Vibrio parahaemolyticus and its rapid detection in shellfish and Gulf of Mexico water. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *98*(3), 279–290. https://doi.org/10.1007/s10482-010-9436-2

Rizzo, L., Fraschetti, S., Alifano, P., Tredici, M. S., & Stabili, L. (2016). Association of Vibrio community with the Atlantic Mediterranean invasive alga Caulerpa cylindracea. *Journal of Experimental Marine Biology and Ecology*, *475*, 129–136. https://doi.org/10.1016/j.jembe.2015.11.013

Rodgers, C., Parveen, S., Chigbu, P., Jacobs, J., Rhodes, M., & Harter-Dennis, J. (2014). Prevalence of Vibrio parahaemolyticus, and Vibrio vulnificus in blue crabs (Callinectes sapidus), seawater and sediments of the Maryland Coastal Bays. *Journal of Applied Microbiology*, *117*(4), 1198–1209. https://doi.org/10.1111/jam.12608

Roman, M., Zhang, X., McGilliard, C., & Boicourt, W. (2005). Seasonal and annual variability in the spatial patterns of plankton biomass in Chesapeake Bay. *Limnology and Oceanography*, *50*(2), 480–492. https://doi.org/10.4319/lo.2005.50.2.0480

Rosche, T. M., Binder, E. A., & Oliver, J. D. (2010). Vibrio vulnificus genome suggests two distinct ecotypes. *Environmental Microbiology Reports*, *2*(1), 128–132. https://doi.org/10.1111/j.1758-2229.2009.00119.x

Rosche, T. M., Yano, Y., & Oliver, J. D. (2005). A rapid and simple PCR analysis indicates there are two subgroups of Vibrio vulnificus which correlate with clinical or environmental isolation. *Microbiology and Immunology*, *49*(4), 381–389. https://doi.org/10.1111/j.1348-0421.2005.tb03731.x

Sangaramoorthy, T., & Guevara, E. M. (2017). Immigrant Health in Rural Maryland: A Qualitative Study of Major Barriers to Health Care Access. *Journal of Immigrant and Minority Health*, *19*(4), 939–946. https://doi.org/10.1007/s10903-016-0417-z

Senderovich, Y., Izhaki, I., & Halpern, M. (2010). Fish as reservoirs and vectors of Vibrio cholerae. *PLoS ONE*, *5*(1). https://doi.org/10.1371/journal.pone.0008607

Shapiro, R. L., Altekruse, S., Hutwagner, L., Bishop, R., Hammond, R., Wilson, S., Ray, B., Thompson, S., Tauxe, R. V, Griffin, P. M., & Vibrio, T. (1998). The Role of Gulf Coast Oysters Harvested in Warmer Months in Vibrio vulnificus Infections in the United States, 1988-1996. *THE JOURNAL OF INFECTIOUS DISEASES*, *178*, 752–759. https://academic.oup.com/jid/article-abstract/178/3/752/928032

Shaw, K. S., Jacobs, J. M., & Crump, B. C. (2014). Impact of hurricane irene on Vibrio vulnificus and Vibrio parahaemolyticus concentrations in surface water, sediment, and cultured oysters in the Chesapeake Bay, MD, USA. *Frontiers in Microbiology*, *5*(MAY). https://doi.org/10.3389/fmicb.2014.00204

Shaw, K. S., Rosenberg Goldstein, R. E., He, X., Jacobs, J. M., Crump, B. C., & Sapkota, A. R. (2014). Antimicrobial susceptibility of Vibrio vulnificus and Vibrio parahaemolyticus recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. *PLoS ONE*, *9*(2), 1–11. https://doi.org/10.1371/journal.pone.0089616

Shaw, K. S., Sapkota, A. R., Jacobs, J. M., He, X., & Crump, B. C. (2015). Recreational swimmers' exposure to Vibrio vulnificus and Vibrio parahaemolyticus in the Chesapeake Bay, Maryland, USA. *Environment International*, *74*, 99–105. https://doi.org/10.1016/j.envint.2014.09.016

Sheahan, M., Gould, C. A., Neumann, J. E., Kinney, P. L., Hoffmann, S., Fant, C., Wang, X., & Kolian, M. (2022). Examining the Relationship between Climate Change and Vibriosis in the United States: Projected Health and Economic Impacts for the 21st Century. *Environmental Health Perspectives*, *130*(12), 1–13. https://doi.org/10.1289/EHP12437

Shravan, Y., Gill, R., Vaswani, V., Lakhani, S., & Lakhani, J. (2021). Vibrio fluvialis – Unusual Case of Cellulitis Leading to Sepsis. *Asian Journal of Research in Infectious Diseases*, 1–8. https://doi.org/10.9734/ajrid/2021/v6i430200

Sims, J. N., Isokpehi, R. D., Cooper, G. A., Bass, M. P., Brown, S. D., St John, A. L., Gulig, P. A., & Cohly, H. P. (2011). Visual Analytics of Surveillance Data on Foodborne Vibriosis , United States , 1973 – 2010. *Environmental Health Insights*, *5*(1), 71–85. https://doi.org/10.1177/EHI.S7806

Singleton, F. L., Attwell, R., Jangi, S., & Colwell, R. R. (1982). Effects of Temperature and Salinity on Vibrio cholerae Growth. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *44*(5), 1047–1058.

Sony, M., Sumithra, T. G., Anusree, V. N., Amala, P. V., Reshma, K. J., Alex, S., & Sanil, N. K. (2021). Antimicrobial resistance and virulence characteristics of Vibrio vulnificus, Vibrio parahaemolyticus and Vibrio harveyi from natural disease outbreaks of marine/estuarine fishes. *Aquaculture*, *539*(December 2020), 736608. https://doi.org/10.1016/j.aquaculture.2021.736608

Spaur, M., Davis, B. J. K., Kivitz, S., DePaola, A., Bowers, J. C., Curriero, F. C., & Nachman, K. E. (2020). A systematic review of post-harvest interventions for Vibrio parahaemolyticus in raw oysters. *Science of the Total Environment*, *745*(July), 140795. https://doi.org/10.1016/j.scitotenv.2020.140795

Sterk, A., Schets, F. M., de Roda Husman, A. M., de Nijs, T., & Schijven, J. F. (2015). Effect of Climate Change on the Concentration and Associated Risks of Vibrio Spp. in Dutch Recreational Waters. *Risk Analysis*, *35*(9), 1717–1729. https://doi.org/10.1111/risa.12365

Strom, M. S., & Paranjpye, R. N. (2000). Epidemiology and pathogenesis of Vibrio vulnificus. *Microbes and Infection*, *2*, 177–188.

Tan, C. W., Rukayadi, Y., Hasan, H., Thung, T. Y., Lee, E., Rollon, W. D., Hara, H., Kayali, A. Y., Nishibuchi, M., & Radu, S. (2020). Prevalence and antibiotic resistance patterns of Vibrio parahaemolyticus isolated from different types of seafood in Selangor, Malaysia. *Saudi Journal of Biological Sciences*, *27*(6), 1602–1608. https://doi.org/10.1016/j.sjbs.2020.01.002

Tang, H. J., Chang, M. C., Ko, W. C., Huang, K. Y., Lee, C. L., & Chuang, Y. C. (2002). In vitro and in vivo activities of newer fluoroquinolones against Vibrio vulnificus. *Antimicrobial Agents and Chemotherapy*, *46*(11), 3580–3584. https://doi.org/10.1128/AAC.46.11.3580-3584.2002

Tantillo, G. M., Fontanarosa, M., Di Pinto, A., & Musti, M. (2004). Updated perspectives on emerging vibrios associated with human infections. In *Letters in Applied Microbiology* (Vol. 39, Issue 2, pp. 117–126). Blackwell Publishing Ltd. https://doi.org/10.1111/j.1472-765X.2004.01568.x

Thompson, F. L., Iida, T., & Swings, J. (2004). Biodiversity of Vibrios. *Microbiology and Molecular Biology Reviews*, *68*(3), 403–431. https://doi.org/10.1128/mmbr.68.3.403-431.2004

Tran, V. N., Nguyen, P. N. V., & Nguyen, T. T. H. (2020). Effects of pH, temperature and oxygen-limited condition on the virulence of Vibrio parahaemolyticus. *Science and Technology Development Journal*, *23*(3), First. https://doi.org/10.32508/stdj.v23i3.2057

University of Maryland Extension. (2023). *Agriculture and Food Systems*. Https://Extension.Umd.Edu/Locations/Somerset-County/Agriculture-and-Food-Systems.

Usmani, M., Brumfield, K. D., Magers, B. M., Chaves-Gonzalez, J., Ticehurst, H., Barciela, R., McBean, F., Colwell, R. R., & Jutla, A. (2023). Combating cholera by building predictive capabilities for pathogenic Vibrio cholerae in Yemen. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-022-22946-y

Valle-Levinson, A., Boicourt, W. C., & Roman, M. R. (2003). On the Linkages among Density, Flow, and Bathymetry Gradients at the Entrance to the Chesapeake Bay. *Estuaries*, *26*(6), 1437–1449. https://doi.org/10.1007/BF02803652

Venkateswaran, K., Takai, T., Navarro, I. M., Nakano, H., Hashimoto, H., & Siebeling, R. J. (1989). Zooplankton in Their Seasonal Distribution in Fukuyama Coastal Waters , Japan. *Applied and Environmental Microbiology*, *55*(6), 1591–1598.

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P. C., Colwell, R. R., Höfle, M. G., & Pruzzo, C. (2012). Long-term effects of ocean warming on the prokaryotic community: Evidence from the vibrios. *ISME Journal*, *6*(1), 21–30. https://doi.org/10.1038/ismej.2011.89

Vezzulli, L., Colwell, R. R., & Pruzzo, C. (2013). Ocean Warming and Spread of Pathogenic Vibrios in the Aquatic Environment. *Microbial Ecology*, *65*(4), 817–825. https://doi.org/10.1007/s00248-012-0163-2

Vezzulli, L., Grande, C., Reid, P. C., Hélaouët, P., Edwards, M., Höfle, M. G., Brettar, I., Colwell, R. R., & Pruzzo, C. (2016). Climate influence on Vibrio and associated human diseases during the past half-century in the coastal North Atlantic. *PNAS*, *113*(34), E5062–E5071. https://doi.org/10.1073/pnas.1609157113

Vezzulli, L., Pezzati, E., Brettar, I., Höfle, M., & Pruzzo, C. (2015). Effects of Global Warming on Vibrio Ecology. *Microbiology Spectrum*, *3*(3), 1–9. https://doi.org/10.1128/microbiolspec.ve-0004-2014

Vezzulli, L., Pruzzo, C., Huq, A., & Colwell, R. R. (2010). Environmental reservoirs of Vibrio cholerae and their role in cholera. In *Environmental Microbiology Reports* (Vol. 2, Issue 1, pp. 27–33). https://doi.org/10.1111/j.1758-2229.2009.00128.x

Vugia, D. J., Tabnak, F., Newton, A. E., Hernandez, M., & Griffin, P. M. (2013). Impact of 2003 state regulation on raw oyster-associated vibrio vulnificus illnesses and deaths, California, USA. *Emerging Infectious Diseases*, *19*(8), 1276–1280. https://doi.org/10.3201/eid1908.121861

Vu, T. T. T., Alter, T., Braun, P. G., Dittrich, A. J., & Huehn, S. (2018). Inactivation of Vibrio sp. in pure cultures and mussel homogenates using high hydrostatic pressure. *Letters in Applied Microbiology*, *67*(3), 220–225. https://doi.org/10.1111/lam.13044

Waldbusser, G. G., Voigt, E. P., Bergschneider, H., Green, M. A., & Newell, R. I. E. (2011). Biocalcification in the Eastern Oyster (Crassostrea virginica) in Relation to Long-term Trends in Chesapeake Bay pH. *Estuaries and Coasts*, *34*(2), 221–231. https://doi.org/10.1007/s12237-010-9307-0

Warner, E. B., & Oliver, J. D. (2008). Multiplex PCR assay for detection and simultaneous differentiation of genotypes of Vibrio vulnificus biotype 1. *Foodborne Pathogens and Disease*, *5*(5), 691–693. https://doi.org/10.1089/fpd.2008.0120

Weis, K. E., Hammond, R. M., Hutchinson, R., & Blackmore, C. G. M. (2011). Vibrio illness in Florida, 1998-2007. *Epidemiology and Infection*, *139*(4), 591–598. https://doi.org/10.1017/S0950268810001354

West, P. A. (1989). The human pathogenic vibrios- A public health update with environmental perspectives. *Epidemiology and Infection*, *103*, 1–34.

Williams, T. C., Froelich, B. A., Phippen, B., Fowler, P., Noble, R. T., & Oliver, J. D. (2017). Different abundance and correlational patterns exist between total and presumed pathogenic Vibrio vulnificus and V. parahaemolyticus in shellfish and waters along the North Carolina coast. *FEMS Microbiology Ecology*, *93*(6), 1–11. https://doi.org/10.1093/femsec/fix071

Wong, K. C., Brown, A. M., Luscombe, G. M., Wong, S. J., & Mendis, K. (2015). Antibiotic use for Vibrio infections: important insights from surveillance data. *BMC Infectious Diseases*, *15*(1), 1–9. https://doi.org/10.1186/s12879-015-0959-z

Xu, H.-S., Roberts, N., Singleton, F. L., Attwell, R. W., Grimes, D. J., & Colwell, R. R. (1982). Survival and Viability of Nonculturable Escherichia coli and Vibrio cholerae in the Marine Environment. *Microbial Ecology*, *8*(4), 313–323. www.jstor.org/stable/4250723

Ye, M., Huang, Y., & Chen, H. (2012). Inactivation of Vibrio parahaemolyticus and Vibrio vulnificus in oysters by high-hydrostatic pressure and mild heat. *Food Microbiology*, *32*(1), 179–184. https://doi.org/10.1016/j.fm.2012.05.009

Yun, N. R., & Kim, D. M. (2018). Vibrio vulnificus infection: A persistent threat to public health. *Korean Journal of Internal Medicine*, *33*(6), 1070–1078. https://doi.org/10.3904/kjim.2018.159