ENVIRONMENTAL FACTORS AND RESERVOIR SHIFTS CONTRIBUTE TO THE SEASONALITY OF PATHOGENIC *VIBRIO* SPECIES

by

JEFF W. TURNER

(Under the Direction of Erin K. Lipp)

ABSTRACT

Members of the *Vibrio* genus are autochthonous inhabitants of marine coastal and estuarine ecosystems worldwide. Although *Vibrio* exist in the marine environment as common heterotrophic bacteria, a small percentage of environmental strains carry the genetic determinants for human pathogenesis. In particular, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are significant pathogens of humans worldwide. In a series of studies, we investigated the potential drivers of *Vibrio* prevalence along the coastal reaches of Georgia, USA. Surface water temperature, salinity and dissolved oxygen (DO) were strongly associated with the prevalence of total *Vibrio* (cultured on thiosulfate citrate bile sucrose agar, TCBS) and the prevalence of virulent and avirulent *V. cholerae* and *V. vulnificus* (PCR detection of species-specific and virulence-associated gene targets) ($P < 0.05$ for each). However, each species responded differently to seasonal changes in environmental parameters. For example, *V. parahaemolyticus* (species-specific gene target) was detected year round even when water temperatures fell below 10^{o}C. Interestingly, the prevalence of genes associated with virulent strains of *V. parahaemolyticus* was strongly associated with temperature ($P < 0.05$). Regardless, relationships with environmental parameters alone were unable to capture the complexity of *Vibrio* seasonality

in this study. The prevalence of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* was shown to be associated with the abundance of specific plankton taxa (copepods, diatoms and decapods, respectively) ($P < 0.05$ for each). Additionally, peaks in the prevalence of *V. cholerae* were associated with peaks in the abundance of detrital particulate organic matter (POM), which coincided with the periodicity of phytoplankton blooms in this region. These findings led to the application of a GFP-tagged *V. cholerae* strain to investigate and document associations between *V. cholerae* and the copepod (an important environmental host). Overall, these investigations confirm temperature and salinity as *Vibrio* drivers. Furthermore, these investigations support the hypothesis that shifts in the abundance of specific plankton taxa and detrital POM are associated with the seasonal distribution of pathogenic *Vibrio* species.

INDEX WORDS: *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, plankton, seasonality, copepods*

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JEFF W. TURNER

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JEFF W. TURNER

Major Professor: Erin K. Lipp

Committee: Dana Cole
James Porter
Mary Ann Moran
John J. Maurer

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my mother and father, who believed and sacrificed and gave me this opportunity.

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CHAPTER 1

INTRODUCTION

Members of the *Vibrio* genus are gram-negative, halophilic bacteria indigenous to coastal marine systems (Thompson *et al.*, 2003). While these common bacteria persist as a natural component of the marine microbial flora, a small percentage of environmental isolates carry the genetic determinants for human pathogenesis (Nishibuchi and Kaper, 1995; Chakraborty *et al.*, 2000; Rivera *et al.*, 2001). Currently, *Vibrio* infections are the leading cause of seafood-borne bacterial gastroenteritis in the United States and together, *Vibrio cholerae* (non-O1 and non-O139), *V. parahaemolyticus* and *V. vulnificus* account for the majority of those infections (Mead *et al*., 1999). Among the 4,754 *Vibrio* infections reported to the Centers for Disease Control and Prevention (CDC) from 1997-2006, 3,544 (75%) of those infections were foodborne in origin and 1,210 (25%) of those infections were non-foodborne in origin (Dechet *et al*., 2008).

Although *Vibrio* species are among the most abundant culturable bacteria in coastal marine environments, the *Vibrio* population exhibits distinct seasonal variation, in that warmer water temperatures select for the growth of these bacteria (Heidelberg *et al*., 2003; Thompson *et al*., 2003). Not surprisingly, the environmental prevalence of pathogenic *Vibrio* species is directly correlated with the risk of *Vibrio*-related illness. From 1998 to 1996, the Gulf Coast *Vibrio* Surveillance investigated 422 *V. vulnificus* infections and 89% of all trace-backs implicated oysters harvested during summer months (Shapiro *et al.*, 1998). In 1599, early

historian William Butler understood that “it is unreasonable and unwholesome in all months that have not an r in their name to eat an oyster” (Potasman *et al*., 2002).

The concentration of living and non-living particulate organic matter (POM), commonly higher in coastal regions, also selects for the growth of *Vibrio*. These particles, such as plankton and marine aggregates, represent nutrient-rich microhabitats (Heidelberg *et al*., 2002; Grossart *et al*., 2005), capable of selectively enriching heterotrophic bacteria, including *Vibrio* species (Huq *et al*., 1983; Heidelberg *et al*., 2002). Ecologically, *Vibrio* species elaborate an extracellular chitinase and play an important role in the decomposition of the chitinous exoskeletons of higher plankton, such as copepods and decapods (Heidelberg *et al*., 2002; Thompson *et al*., 2003). Epidemiologically, copepods heavily colonized by *V. cholerae* are a well-documented vehicle for the transmission of cholera (Huq and Colwell, 1996; Colwell *et al*., 2003).

Historically, models aimed at predicting the prevalence of pathogenic *Vibrio* species have focused on linear relationships between prevalence and environmental conditions like temperature and salinity. These models fall short of accurately describing the complexity of these pathogens. Recently, researchers have shown that the presence of *Vibrio* species and the incidence of *Vibrio* illness can be driven by shifts in plankton abundance and described in the context of plankton blooms (Huq *et al*., 2005).

This dissertation is an investigation of how changes in the composition of the plankton community contribute to the seasonality of the *Vibrio* population, either synergistically with or independent of physical parameters such as temperature and salinity. The second chapter of this dissertation details the history, ecology and epidemiology of three *Vibrio* species (*V. cholerae, V. parahaemolyticus* and *V. vulnificus*). The third, forth and fifth chapters present the results of two environmental studies focused on synergistic relationships between *Vibrio* and plankton. The

sixth chapter, built upon hypotheses derived from our environmental studies, presents the findings of two of microcosm focused on the colonization of copepods by *V. cholerae*.

While the work presented in this dissertation confirms the role of temperature, salinity and dissolved oxygen in *Vibrio* ecology, these results show how seasonal changes in the relative abundance of specific plankton taxa contribute to the seasonality of pathogenic *Vibrio*. Further, this work shows that some plankton taxa, such as copepods, may be especially important reservoirs of pathogenic *Vibrio*. Together, these findings highlight the potentially complex relationship between seasonal shifts in plankton composition and their net effect on *Vibrio* levels and suggest that *Vibrio* prevalence should be considered within the context of bloom formation and decline as well single-point determinations of plankton composition.

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CHAPTER 2

LITERATURE REVIEW

IMPORTANCE AND HISTORICAL PERSPECTIVES

Estuarine and marine waters are known to harbor autochthonous bacteria belonging to the *Vibrio* genus. Members of the *Vibrio* genus are gram-negative, halophilic, comma-shaped and highly motile with one or more polar flagella (Thompson *et al.*, 2003). They tend to be facultatively anaerobic and can be differentiated from the *Enterobacteriaceae* by a positive oxidase test (Vila *et al.*, 1992). At least 12 *Vibrio* species are known human pathogens although together, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* account for the majority of *Vibrio*-related infections worldwide (Morris, 2003). In the United States, non-epidemic *V. cholerae* (non O1 and non O139), *V. parahaemolyticus*, and *V. vulnificus* account for 20% of seafood-borne illnesses and 99% of seafood-related mortality (Lipp and Rose, 1997).

While cholera is primarily a health concern associated with the lack of clean water and sanitation in developing countries, illnesses caused by *V. parahaemolyticus* and *V. vulnificus* are primarily food-borne, and commonly occur in developed nations (Lipp and Rose, 1997; Shapiro *et al.*, 1998; Wechsler *et al.*, 1999; Su and Liu, 2007). Food-borne infections are often associated with the consumption of raw or undercooked seafood, especially shellfish (Daniels *et al.*, 2000). In the United States, the incidence of shellfish-borne disease has significantly

increased over the past 30 years, despite a 37% decrease in bivalve consumption from 1970-1997 (Morris, 2003). Additional significant routes of infection include recreational and occupational exposure (CDC, 2006; CDC, 2008; Dechet *et al.*, 2008).

Although *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are members of the same genus, the presentation and severity of illness varies greatly (Altekruse *et al.*, 1997; Daniels *et al.*, 2000; Morris, 2003; Thompson *et al*., 2003). *V. cholerae* infections can result in fluid loss and osmotic shock that, if untreated, can cause death within hours (Cockburn and Cassanos, 1960). *V. parahaemolyticus* infections are rarely life threatening and commonly manifest as a self limiting gastroenteritus (Su and Liu, 2007). *V. vulnificus* infections can become life threatening if the bacterium enters the bloodstream – causing septicemia, septic shock and blistering skin lesions (Levin, 2005).

Historically, *V. cholerae* (the etiologic agent of cholera) has been known since antiquity. In fact, records of a cholera-like disease date to the time of Hippocrates (460 to 377 BC). In contrast *V. parahaemolyticus* and *V. vulnificus* were discovered only recently. Due to its antiquity and significance as a pathogen of global importance, *V. cholerae* is among the most intensely researched bacterial pathogen. Indeed, much of our knowledge of other *Vibrio* species centers on our understanding of the ecology, epidemiology and pathogenicity of *V. cholerae*. Italian physician Filippo Pacini first identified *V. cholerae* as the etiologic agent of cholera in 1854 (Thompson *et al.*, 2003). Although Pacini hypothesized that cholera was a contagious bacterial disease, most scientists and physicians subscribed to the miasmatic theory of disease (that disease arose directly from foul odors, vapors and ‘bad air’). During an outbreak in England between 1830 and 1850, physician John Snow challenged the miasmatic theory when he traced the source of cholera to contaminated drinking water. Shortly thereafter, during an

outbreak of cholera in India in 1883, Robert Koch concluded that *V. cholerae* was the etiologic agent of cholera and that contaminated water supplies were the origin of the bacterium (Faruque *et al.,* 1998). Koch's findings were reported globally and his work played a seminal role in the establishment of the germ theory of disease.

It is generally accepted that we are currently experiencing the 7th pandemic of cholera (Faruque *et al.,* 1998). The 1st began in 1817, and it was during the 5th pandemic that Koch isolated the comma-shaped bacilli responsible for the disease. With the exception of the 7th, most recent pandemic, each originated from the Indian subcontinent. The 7th originated on the island of Sulawesi in Indonesia. The current 7th pandemic began in 1961 and is considered the most extensive in regards to duration and geographic spread (Faruque *et al.*, 1998).

Among the approximately 200 known O serogroups of *V. cholerae*, only two serogroups (O1 and O139) have been associated with epidemic cholera. Among the O1 serogroups, strains are categorized into two biotypes (Classical and El Tor). The Classical biotypes are further distinguished as Inaba or Ogawa serotype. The International Center for Diarrheal Disease Research in Bangladesh (ICDDR,B) has systematically monitored cholera outbreaks for more than 35 years. The Classical Inaba serotype was the dominant serotype associated with epidemic cholera until 1972 when a shift occurred and the classical Ogawa serotype became dominant. Shortly thereafter, in 1973, the El Tor biotype appeared and quickly displaced the Classical biotype as the dominant strain associated with epidemic cholera. During an outbreak in 1992, a newly virulent strain appeared that did not agglutinate with O1 antisera. Since its emergence, this newly virulent biotype, recognized as O139 Bengal, has been associated with cholera outbreaks worldwide (Faruque *et al.*, 1998).

The additional 198+ O serogroups of *V. cholerae* are collectively referred to as non-O1 and non-O139 and are largely non-pathogenic, although some strains cause sporadic cases of non-epidemic cholera (Thompson *et al.*, 2003). Non-O1 and non-O139 *V. cholerae* are the third most commonly reported *Vibrio* infection in the United States (Morris, 2003). Case rates of epidemic cholera in the United States are few and most confirmed infections are acquired outside the United States. However, toxigenic *V. cholerae* O1 is known to occur naturally in coastal waters of the United States, especially in the Gulf of Mexico (Colwell *et al*., 1981, Kaper *et al*., 1979). To date, *V. cholerae* O1 remains the only pandemic strain; however, the emergence of the newly pathogenic O139 strain in 1992, causing epidemics in Peru, India and Bangladesh, continues to persist and may represent the beginning of the 8th cholera pandemic (Faruque *et al.*, 1998).

Cholera is estimated to be as prevalent today as it was 50 years ago and *Vibrio cholerae* has been identified as a reemerging disease that continues to threaten developing countries (Huq and Colwell, 1996; Morris, 2003). Between 1995 and 2004, the World Health Organization (WHO) reported 100,000 to 300,000 cholera cases. Due to poor surveillance systems and frequent under reporting, WHO estimates that approximately 99% of cholera cases go unreported. WHO estimates that actual case rates approach 3 to 5 million with more than 120,000 of those cases leading to mortality. In 2005, 95% of cholera cases originated in Africa where epidemics are commonly associated with war and natural disasters, which further tax over populated regions with few resources, poor sanitation and limited access to health care (Zuckerman *et al.*, 2007).

V. parahaemolyticus was first isolated during an outbreak of gastroenteritis following the consumption of sardines in Japan in 1950 (Daniels *et al.*, 2000). Since its identification, *V.*

parahaemolyticus has been recognized as the most common cause of seafood-borne bacterial illness in Japan, Taiwan and the United States (Su and Liu, 2007; Strom *et al.*, 2008). In the United States, between 1973 and 1998, 40 seafood-associated *V. parahaemolyticus* outbreaks were reported to the CDC (Daniels *et al.*, 2000). Moreover, between 1997 and 2006, the occurrence of 5 major outbreaks in the United States indicates that *V. parahaemolyticus* is an emerging human pathogen of increasing importance (CDC, 1999; Wechsler *et al.*, 1999; McLaughlin *et al.*, 2005; CDC, 2006; Su and Liu, 2007).

In 1996, food-borne disease surveillance agencies in India and Taiwan noted a sudden increase in disease that corresponded to an increase in *V. parahaemolyticus* infections (Okuda *et al.*, 1997b; Chiou *et al.*, 2000). *V. parahaemolyticus* case rates have since remained elevated and are attributed to the emergence of a new, highly virulent serovar (O3:K6) capable of causing larger outbreaks than classical strains (Chiou *et al*. 2000). Since its emergence in 1996, the O3:K6 serovar has been identified as the dominant serovar in Asia (Chiou *et al*., 2000; Matsumoto *et al*., 2000).

In the United States, O3:K6 appeared in 1998, and was responsible for the largest *V. parahaemolyticus* outbreak in United States history (Daniels *et al*., 2000). The O3:K6 serovar is currently classified as a pandemic strain; however, additional serovars are still locally endemic. In the Pacific Northwest, O4:K12 is the dominant serovar related to clinical infections and was responsible for major *V. parahaemolyticus* outbreaks in 1997 and 2006 (CDC, 1998; CDC, 2006). In 2004, *V. parahaemolyticus* O6:K18 was responsible for an outbreak in Alaska's Prince William Sound, expanding the northern range of this pathogen by more than 1,000 km (McLaughlin *et al.*, 2005).

V. vulnificus was first isolated by the CDC in 1964; however, this pathogen was not recognized as a new virulent *Vibrio* species until the 1970s (Strom and Paranjpye, 2000). *V. vulnificus* is commonly differentiated into two biotypes through examination of lipopolysaccharide (LPS) side chains (biotype 1 has variable side chains and biotype 2 has only one side chain type) (Strom and Paranjpye 2000). Biotype 1 is a significant cause of farmed eel mortality while biotype 2 is more commonly associated with human infections.

V. vulnificus is unique in both type of infection and severity of symptoms. Most *V. vulnificus* infections occur among persons who are immunocompromised (Lipp and Rose, 1997). In the United States, the CDC reported that of the 422 *V. vulnificus* infections reported between 1988 and 1996, 45% were wound infections, 43% leading to primary septicemia, 5% gastroenteritis and in 7% the route of exposure was not determined (Shapiro *et al.*, 1998). Of the 181 cases of septicemia, 173 (approximately 95%) of the patients reportedly consumed raw shellfish prior to the onset of illness. The severity of septic infections and the increased susceptibility of immunocompromised persons drive the high mortality rate (30-48%) and as a result *V. vulnificus* infections account for 95% of *Vibrio* related deaths in the United States (Shapiro *et al.*, 1998; Morris, 2003).

Compared to *V. cholerae* and *V. parahaemolyticus* infections, *V. vulnificus* cases are rare in the United States, and fewer than 100 infections are reported annually (Levin, 2005). Yet in spite of its rarity, *V. vulnificus* is recognized as the most invasive *Vibrio* species due to its ability to cause life-threatening septicemia (Harwood *et al.*, 2004; Levin, 2005). Individuals or groups with underlying immunocompromising conditions (such as hemochromatosis, hepatitis or

cirrhoss) are at a higher risk of severe and or septic infections (Thompson *et al.*, 2003). Among immunocompromised persons, fatality rates can exceed 50% (Shapiro *et al.*, 1998; Harwood *et al.*, 2004).

It is important to note that *Vibrio* infections are not limited to humans. At least 15 *Vibrio* species are known to be pathogens of marine animals (Austin and Austin, 1999). Among coral pathogens, *V. coralliilyticus* and *V. shiloi* are responsible for significant bleaching events (Thompson *et al.*, 2003). Among pathogens of animals reared for aquaculture, *V. anguillarum*, *V. salmonicida* and *V. vulnificus* cause mass mortalities in aquaculture facilities (Coleman *et al.*, 1996; Thompson *et al.*, 2003). As aquaculture industries attempt to meet the demand for protein by a growing population, the economic impact of pathogenic *Vibrio* species will continue to present a problem.

OCCURRENCE AND DISTRIBUTION

Members of the *Vibrio* genus often comprise <1% of the total marine bacterioplankton; however, *Vibrio* species are among the most abundant culturable bacteria in estuarine and marine waters throughout the world (Thompson *et al.*, 2003). The genus is comprised of some 64 species (although the phylogeny of the genus is under constant revision) (Morris, 2003). Survival of pathogenic species, such as *V. cholerae*, was long thought to be limited outside of the human host; however, discoveries of the past four decades have revealed that *V. cholerae* and other *Vibrio* species are a natural component of marine ecosystems worldwide (Colwell *et al.*, 1977; Colwell *et al.*, 1981; DePaola *et al.*, 1997; Bej *et al.*, 1999; Chakraborty *et al.*, 2000; Lipp *et al.*, 2003; Baffone *et al.*, 2006).

Vibrio species exist as free-living bacteria and in association with particulate organic matter (POM), phytoplankton, zooplankton, finfish and shellfish. In particular, the association with marine particles (POM and plankton) is an important aspect of *Vibrio* ecology, and numerous studies have made the distinction between free-living bacteria and particle-associated bacteria (Huq *et al.*, 1983; Tamplin *et al.*, 1990; Montanari *et al.*, 1999; Maugeri *et al.*, 2004; Nicolas *et al.*, 2004; Baffone *et al.*, 2006; Turner *et al.*, 2009). The distinction is of importance because bacteria associated with marine particles account for a large fraction of the microbial activity in the pelagic water column (Cottingham *et al.*, 2003), and bacterial populations associated with marine particles are metabolically and taxonomically distinct from free-living bacteria (Long and Azam, 2001a) (DeLong *et al*., 1993).

The addition of organic carbon and the presence of particulate organic matter have been shown to support the rapid growth of *V. cholerae* in microcosm and mesocosm studies (Mourino-Perez *et al*., 2002; Worden *et al.*, 2006). Thus, *V. cholerae* and other *Vibrio* species are often regarded as bacteria with an "opportunistic" or "r" growth strategy and are especially well adapted to a particle-associated "lifestyle" (Pernthaler and Amann, 2005). The alternation between free-living and particle-associated lifestyles may reflect an alternation between "r" and "K" growth strategies, which may vary according to substrate availability and environmental conditions (Long and Azam, 2001b).

As heterotrophic bacteria, the association with and degradation of chitin is an important aspect of *Vibrio* ecology and an important aspect of nutrient cycling in the marine environment (Meibom *et al.*, 2004). Chitin, a polymer of *N*-acetyl glucosamine residues, is the 2nd most abundant polymer in nature, and produced as the exoskeleton of many higher zooplankton and other crustaceans. All *Vibrio* species elaborate an extracellular chitinase (Huq *et al.*, 1983).

Thus, *Vibrio* are believed to play an important role in the mineralization of the chitinous exoskeletons of zooplankton and are known to be closely associated with copepods and other higher plankton (Colwell *et al*. 1977, Carman and Dobbs, 1997).

Although *Vibrio* species are ubiquitous in warm oceans around the globe, they do not exhibit spatially or temporally static characteristics. Changes in the density and diversity of the *Vibrio* community can be correlated with fluctuations in the physiochemical parameters such as temperature and salinity (Thompson *et al*., 2004). Additionally, changes in the *Vibrio* community may be correlated with shifts in the abundance or composition of *Vibrio* reservoirs such as plankton, sediment and shellfish (Maugeri *et al*., 2004, Thompson *et al*., 2004, Kaneko *et al*., 1978). In consequence to these external factors, the abundance and diversity of the *Vibrio* population is known to exhibit a complex seasonal distribution driven by fluctuations in physiochemical parameters and shifts in *Vibrio* reservoirs.

In general, *Vibrio* species prefer warmer water temperatures (>15^{o}C) and can thrive in a range of salinities (5-25) (Thompson *et al.*, 2003). *V. cholerae* alone has the ability to survive at a salinity of 0, although it has an absolute growth requirement for sodium (Thompson *et al.*, 2003). Thus, the environmental prevalence of *Vibrio* species displays a strong seasonal variation, and temperature and salinity are among the dominant physiochemical drivers (Thompson *et al.*, 2003). However, each species responds differently to seasonal fluctuations in temperature and salinity. For example *V. vulnificus* is known to exhibit a more pronounced temperature-driven seasonality compared to *V. parahaemolyticus* and *V. cholerae* (Lipp and Rose, 1997).

During a 15-month study conducted along the coastal reaches of the Northern Atlantic, Thompson *et al*. (2004) reported that the total *Vibrio* population reached a maximum

concentration of 8.0 X 10^3 ± 9.2 X 10^2 cells ml^{-1} in June 2002 and a minimum concentration of 37 ± 5 cells ml^{-1} in April 2002. Wright *et al*. (1996) showed that *V. vulnificus* comprised 0.6% to 17.4% of the total bacterial population in Chesapeake Bay during warmer months. Heidelberg *et al*. (2002) detected *V. cholerae* and *V. vulnificus* concentrations of 10^3 to 10^4 organisms ml^{-1} of seawater in Chesapeake Bay.

SURVIVAL IN THE ENVIRONMENT

Key to the seasonal prevalence of *Vibrio* species is the ability of these bacteria to survive and persist in a marine environment with fluctuations in temperature, salinity, nutrient concentration, DO and pH (Thompson *et al.*, 2003). *Vibrio* species, like other bacterioplankton, must overcome the challenges posed by viral and protozoan predation, which are significant removal processes that can limit growth and proliferation (Cole, 1999; Matz *et al.*, 2005).

Numerous mechanisms aid in survival and persistence, and one such mechanism involves entry into a dormant state characterized by a decrease in metabolic rate, loss of the flagellum, conformational change to a small sphere and an inability to grow on standard microbiological media (Huq *et al*., 2000; McDougald *et al*., 1998). It is a physiological response to conditions that are unfavorable for growth and confers resistance to those extreme conditions (Oliver, 2005). This dormant state was first described in *V. cholerae* by Xu *et al*. (1982) and coined as the viable-but-non-culturable state (VBNC). Since its discovery, the ability to enter a VBNC state has been characterized for many Gram-negative bacteria including *V. parahaemolyticus* and *V. vulnificus* (Oliver 2004).

Another mechanism for survival involves the ability to associate with biotic and abiotic surfaces such as POM, plankton and sediment (Huq *et al.*, 1983; Carman and Dobbs, 1997;

Heidelberg *et al.*, 2002; Turner *et al.*, 2009). Associations range from a simple sporadic attachment to the development of dense, highly differentiated structures called biofilms (Watnick *et al.*, 1999; Matz *et al.*, 2005). Previous studies have shown that bacteria involved in biofilm formations are more resistant to extreme environmental conditions and protozoan predation (Cottingham *et al.*, 2003; Matz *et al.*, 2005). Further, it has been suggested that the biofilm-enhanced colonization of marine particles may be the result of a strong evolutionary pressure exerted on planktonic cells by bacterivirous protozoan predators (Matz *et al.*, 2005).

Association with POM and plankton is also a means by which *Vibrio* species and other heterotrophic bacteria obtain nutrients. Plankton and POM represent nutrient-rich 'hot spots' capable of selectively enriching heterotrophic bacteria at much higher densities than the surrounding water column (Huq *et al.*, 1983; Tamplin *et al.*, 1990). Additionally, the microbial degradation of these particles releases a bloom of dissolved organic matter DOM (Mourino-Perez *et al.*, 2003; Eiler *et al.*, 2007). Mourino-Perez *et al*. (2003) showed the dissolved organic matter (DOM) produced during a phytoplankton bloom supported the growth of free-living *V. cholerae* 3 orders of magnitude higher than the known minimum infectious dose of 10^4 cells ml^{-1}. Similarly, net gains in *V. cholerae* population growth have been observed during intense phytoplankton bloom conditions, when high concentrations of DOM resulted in growth rates that overcame grazing mortality (Worden *et al.*, 2006).

Ideally, algal species and plankton taxon represent unique microhabitats for microbial colonization and as such, previous studies have shown that some algal species harbor distinct microbial assemblages (Grossart *et al.*, 2005). Substrate availability and quality may play a large role in the development of unique microbial-particle associations. Additionally, competition for substrate and antagonistic interactions with other microbiota has been shown to limit the growth

of some bacteria species (Long and Azam, 2001a). For instance, bacteria isolated and cultured from the marine particles have been shown to limit the growth and proliferation of *V. cholerae* in laboratory studies (Long *et al.*, 2005). Further, Long *et al*. (2005) showed that antagonism decreases with warmer water temperatures. Thus, bacterial-bacterial antagonism is one mechanism that contributes to the control and regulation of *V. cholerae* in the marine environment.

The chitinous exoskeletons of higher plankton, such as copepods and decapods, play an especially important role in the ecology of *Vibrio* species as all *Vibrio* are known to elaborate an extracellular chitinase and can utilize the chitinous exoskeletons as a source of carbon and nitrogen (Thompson *et al.*, 2003). As a result, *Vibrio* species are commonly found in association with the exoskeletons of marine copepods and other higher plankton (Kaneko and Colwell, 1975; Huq *et al.*, 1983; Kirchner, 1995; Hansen and Bech, 1996; Rawlings *et al.*, 2007). Similarly, *Vibrio* species have been reported as the dominant bacterial component of copepod gut flora (Sochard *et al.*, 1979). In addition to copepods, planktonic organisms to which *V. cholerae* is known to attach include cyanobacteria, diatoms, dinoflagellates and cladocerans (Cottingham *et al.*, 2003).

Heidelberg *et al*. (2002a) hypothesized that *Vibrio* species may demonstrate a competitive advantage when plankton-associated (Heidelberg *et al.*, 2002a). An ecological association between *V. cholerae* and plankton was first recognized in 1960, when Cockburn and Cassanos (1960) showed a direct correlation between the incidence of cholera and the abundance of chlorophyll *a* in water samples associated with the cholera cases. Since that discovery, a series of studies have shown that dissolved organic matter (DOM), released during phytoplankton blooms, supports the explosive growth of heterotrophic bacteria including *Vibrio*

species (Eilers *et al.*, 2000; Riemann *et al.*, 2000; Mourino-Perez *et al.*, 2003; Eiler and Bertilsson, 2004; Eiler *et al.*, 2006; Eiler *et al.*, 2007).

While the association of *Vibrio* species with plankton has been a subject of interest, few studies have evaluated what environmental signals mediate adsorption (Huq *et al.*, 1983; Huq *et al.*, 1984; Tamplin *et al.*, 1990). Further, the effect of plankton-association on natural microbial populations has not been well characterized (Cottingham *et al.*, 2003). It has yet to be determined if these associations are benign or if one or more of these organisms serve as an environmental host. Additionally, changes in the abundance and diversity of the *Vibrio* community may be correlated with shifts in the abundance or composition of the plankton reservoir (Tamplin *et al.*, 1990; Maugeri *et al.*, 2004; Turner *et al.*, 2009).

LINKS BETWEEN ECOLOGY AND EPIDEMIOLOGY

Given that pathogenic *Vibrio* species live in the environment, a discussion of their ecology is key to any epidemiologic *Vibrio* investigation. For example, *V. cholerae* outbreaks follow seasonal cycles in regions where cholera is endemic (Huq and Colwell, 1996). In Bangladesh, a small outbreak precedes the early monsoon season (March-June) and a larger outbreak follows the late monsoon season (September-December) (Faruque *et al.*, 1998). Outbreaks are also known to coincide with warming sea surface temperatures associated with large-scale climate events such as the El Niño-Southern Oscillation (Colwell, 1996; Lipp *et al.*, 2002).

Since the human infectious dose of a pathogenic *Vibrio* species ranges from 10^4 to 10^6 cells (Thompson *et al.*, 2003), concentration of the pathogen in the environment is often a necessary step in primary disease transmission (Huq and Colwell, 1996). Concentration in the

environment can occur when a biotic or abiotic source of nutrients enriches the pathogenic *Vibrio* at higher concentrations than the surrounding water column. Filter-feeding shellfish can also accumulate pathogenic *Vibrio* species and serve as a reservoir for seafood-borne illnesses, especially when those shellfish are consumed raw (Lipp and Rose, 1997). Similarly, nutrient-rich plankton, colonized by pathogenic *Vibrio* species, can serve as a vehicle for the transmission of disease, as in the case of cholera (Huq and Colwell, 1996).

In Bangladesh, outbreaks of cholera have been correlated with seasonally high abundances of plankton (Huq and Colwell, 1996; Huq *et al.*, 2005). In particular, the marine copepod (especially *Acartia tonsa*) has received much attention following the discovery that copepods, colonized by *V. cholerae* O1, can serve as a vehicle for cholera transmission. The filtration of copepod and other zooplankton from drinking water was shown to significantly reduce the incidence of cholera in Bangladesh (Colwell *et al.*, 2003).

An ecological link between *Vibrio* prevalence and plankton plays a potential role in the epidemiology of these pathogens as plankton serves as a primary food source of filter-feeding bivalves (Dupuy *et al.*, 2000). Since bivalve grazing is a selective process based on size exclusion, clearance rate and plankton taxon (Fritz *et al.*, 1984; Loret *et al.*, 2000; Cognie *et al.*, 2001), it follows that plankton harboring *Vibrio* densities higher than the surrounding water column, if selected, could contribute to pathogen loading. Thus, the composition of the plankton community could be a factor in the transmission of *Vibrio* species associated with bivalves. In summer months, *V. vulnificus* and *V. parahaemolyticus* can be isolated from shellfish and just one gram of oyster meat can carry 10^4 *V. vulnificus* cells (Morris, 2003).

Given that *Vibrio* live in the environment and survival is independent of a human host, the genes involved in human pathogenesis may serve a natural purpose in the marine

environment (Matz *et al.*, 2005). In the marine environment, chitinous surfaces represent a microhabitat comprised of high concentrations of bacteria, phage and exogenous DNA and recent studies have shown that *V. cholerae* growth on chitin can induce the acquisition of exogenous DNA by natural transformation (Meibom *et al.*, 2004). Prior to this discovery, means of gene acquisition in *Vibrio* was thought to be limited to transduction and conjugation since *Vibrio* were not known to exhibit natural competence (Meibom *et al.*, 2004). In the human host, *V. cholerae* associated with chitin exhibits an increased tolerance for gastric acid and thereby reduces the minimum infectious dose. Thus, some of the genes responsible for human pathogenesis may be the consequence of a selective adaptation to an ecological setting.

MOLECULAR ASPECTS OF PATHOGENESIS

While *Vibrio* species persist as a natural component of the marine microbial flora, a small percentage of the *Vibrio* population carry the genetic determinants for human pathogenesis (Nishibuchi and Kaper, 1995; Zhang and Austin, 2005). Thus, it is important to distinguish between virulent and avirulent strains of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (Panicker *et al.*, 2004). This task can be complicated because pathogenicity in each species results from a complex combination of co-regulated virulence genes and neither species shares the same mechanism for pathogenesis or the same means of virulence regulation.

The initial steps of bacterial pathogenesis involve the attachment and colonization of host surfaces. Often, the same factors required for colonization of the human intestine are also required for the colonization of abiotic and biotic surfaces in the marine environment (Watnick *et al*., 1999; Chiavelli *et al*., 2001; Meibom *et al*., 2004). In the example of *V. cholerae*, intestinal colonization is mediated by pili such as the toxin co-regulated pilus (TCP) and the mannose

sensitive hemagglutinin (MSHA) (Hase and Mekalanos, 1998; Chiavelli *et al.*, 2001). In the marine environment, TCP and MSHA are required for biofilm formation on the chitinous surfaces of plankton (Chiavelli *et al.*, 2001; Reguera and Kolter, 2004).

Pili can also mediate motility, secretion of extracellular proteins, cell signaling and the transfer of mobile genetic elements (Strom and Paranjpye, 2000). These mobile elements include transposons, temperate bacteriophages (i.e., prophages), super-integrons and pathogenicity islands (PAIs) (Faruque *et al.*, 1998). Acquisition of new genes via lateral and horizontal gene transfer is an important source or genetic diversity and an important mechanism by which non-virulent strains can acquire the genetic determinants of virulence (Guidolin and Manning, 1987; Faruque *et al.*, 1998; Chakraborty *et al.*, 2000; Li *et al.*, 2002; Hurley *et al.*, 2006). As a result, *Vibrio* species exhibit a high degree of genome plasticity and are characterized by a high propensity for the exchange of genetic material.

Post-intestinal colonization, pathogenicity is commonly mediated via one or more extracellular virulence factor. Common *Vibrio* virulence factors include enterotoxins, haemolysins, proteases, lipases, phospholipases, siderophores, elastases, collogenases, sulfatases as well as many virulence-associated factors like pili, capsules and haemagglutinins (Levin, 2005; Zhang and Austin, 2005). However, only a small percentage of environmental strains carry the genetic determinants for human pathogenesis. In the case of *V. cholerae*, only 2 of approximately 200 O serogroups are capable of causing epidemic cholera and less than 5% of non-O1 and non-O139 strains produce cholera toxin (Rivera *et al.*, 2001; Cottingham *et al.*, 2003). Thus, environmental strains seem to constitute a reservoir for virulence genes; however, the ecologic role of virulence gene expression in the environment is poorly understood (Chakraborty *et al.*, 2000).

The major virulence genes of *V. cholerae* are organized into 2 major clusters – the *Vibrio* pathogenicity island (VPI) and the cholera toxin element (CTX) (Faruque *et al.*, 1998). The *Vibrio* pathogenicity island (VPI) encodes a number of virulence-associated genes including the gene encoding the toxin co-regulated pilus (TCP), a type IV pilin. The CTX element is comprised of the 3 major toxins (*ace*, *zot* and *ctx*AB), a core-encoded pilin (*cep*) and an open reading frame (ORF) of unknown function (*orf*U). The CTX element resembles a transposon-like element originating from a temperate filamentous bacteriophage (CTXϕ) (Faruque *et al.*, 1998). The CTXϕ can be induced in the marine environment and then the free phage particles then play a role in the emergence of newly pathogenic strains when the phage interacts with the TCP receptor of non-toxigenic *V. cholerae* strains (Faruque *et al.*, 1998).

Cholera toxin (CT) is the principle virulence factor of *V. cholerae* and this enterotoxin is responsible for the copious amounts of rice water diarrhea often associated with endemic cholera (Thompson *et al.*, 2003). Cholera toxin is an ADP-ribosylating enzyme composed of a single A subunit and 5 identical B subunits (DiRita *et al.*, 1991). However, numerous additional virulence factors contribute to human pathogenesis and these include the zonula occludens toxin (*zot*), the Ace toxin and hemolysins (*hly*A in *V. cholerae* O1 El Tor strains) (Faruque *et al.*, 1998). Regardless of the accumulated body of knowledge concerning *V. cholerae*'s mode of infectivity, 70% of human volunteers still exhibit diarrhea when inoculated with a *V. cholerae* strain lacking CT, Zot and Ace (DiRita *et al.*, 1991).

Hemolysins are primary virulence factors that are expressed in some pathogenic *Vibrio* species. Hemolysins are cytotoxins that lyse erythrocyte membranes, thereby liberating iron in the form of hemoglobin, transferrin and lactoferrin (Zhang and Austin, 2005). In addition to erythrocytes, hemolysins are known to exhibit a cytotoxic activity against mast cells, neutrophils

and polymorphonuclear cells (Zhang and Austin, 2005). The thermolabile hemolysin (TDH) is a principle virulence factor of *V. parahaemolyticus* (Okuda *et al.*, 1997; Bej *et al.*, 1999), while the El Tor hemolysin (HlyA) of *V. cholerae* and the VVH hemolysin of *V. vulnificus* contribute to human pathogenesis (Zhang and Austin, 2005). *V. vulnificus* expresses 2 different hemolysins. The first, VIIY, is responsible for hemolytic activity on a blood agar media containing rabbit erythrocytes (Strom and Paranjpye, 2000). The second, a heat-liable cytolysin encoded by the *vvh*A gene of *V. vulnificus* awaits further characterization since *vvh*A mutants do not exhibit attenuated virulence (Strom and Paranjpye, 2000).

Virulent strains of *V. parahaemolyticus* have historically been identified as those capable of eliciting the Kanagawa phenomena (KP), which occurs when the thermostable direct hemolysin (TDH) causes the lysis of erythrocytes on a special blood agar medium (Nishibuchi *et al.*, 1992; Zhang and Austin, 2005). Prior to an outbreak in 1985, the majority of clinical isolates were known to exhibit the KP phenomena; however, some clinical isolates from this outbreak were non-KP strains (Matsumoto *et al.*, 2000). Rather than elaborate KP, these clinical strains produced a hemolysin related to TDH, called the thermolabile related hemolysin (TRH) (Matsumoto *et al.*, 2000). Studies highlighting differences between classical KP-positive strains and O3:K6 strains revealed no significant differences in expression levels of *tdh*, antibiotic susceptibility or survival rate (Okuda and Nishibuchi, 1998). Rather, some O3:K6 strains share a filamentous phage (f237) encoding an adhesive protein that may increase the ability of this pathogen to adhere to and colonize the human gut (Yeung *et al.*, 2003).

All virulent strains of *V. vulnificus* elaborate a capsular polysaccharide (CPS) (Levin, 2005). Encapsulated cells appear opaque when cultured whereas non-encapsulated cells appear transparent. The capsule is an acidic polysaccharide coating and an antiphagocytic surface

antigen known to protect the pathogen from host immune responses (Lipp and Rose, 1997). The CPS also contributes to septic shock through the induction of cytokines (Strom and Paranjpye, 2000). LPS also contributes to septic shock through the induction of pyrogenic responses. To date colony morphology remains an effective means of distinguishing between virulent and avirulent strains (Harwood *et al.*, 2004).

DETECTION AND ISOLATION OF *VIBRIO* SPECIES

The *Vibrio* population exhibits a high degree of inter and intra-species variability, characterized by a diverse range of serovars and genotypes within the same species (Thompson *et al.*, 2003). Further, there exists significant genetic variation between virulent and avirulent strains (Chiou *et al.*, 2000; Su and Liu, 2007). These genetic differences can be used to screen samples for potentially virulent *Vibrio* strains. For example, the thermolabile direct hemolysin (*tdh*) of *V. parahaemolyticus* is more commonly found in clinical isolates and this gene is often used to mark virulence in this species (Okuda *et al.*, 1997; Bej *et al.*, 1999). Indeed, the control and prevention of *Vibrio* infections is largely based on the screening of water and seafood for the presence or absence of pathogenic *Vibrio* species and the genes responsible for their pathogenesis. Unfortunately, the prevalence of these pathogens is not spatially or temporally static and can vary greatly between localities. Additionally, methods of detection can be difficult, time consuming and expensive. Further, not all clinical isolates elaborate the more common virulence genes (Okuda *et al.*, 1997; Bej *et al.*, 1999).

Traditional microbiology commonly employs two types of media to enrich and isolate pathogenic *Vibrio* species. Alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.5) is a common *Vibrio* enrichment broth (Levin, 2005) and thiosulfate citrate bile salts sucrose (TCBS)

agar is routinely used to selectively isolate and enumerate pathogenic *Vibrio* species (Pfeffer *et al.*, 2003). These media use an alkaline pH, ox bile and a moderate salinity to encourage *Vibrio* growth and discourage growth of non-*Vibrio* species (Harwood *et al.*, 2004). Unfortunately, both APW and TCBS lack selectivity in that other Gram-negative bacteria including *Flavobacterium, Pseudoalteromonas* and *Shewanella* may present growth as well (Thompson *et al.*, 2003).

Originally, APW and TCBS were developed to isolate pathogenic *Vibrio* species from clinical sources which are often less complex than environmental samples (Kobayashi *et al.*, 1963). Recently, more selective media have been developed for the isolation of specific *Vibrio* species from environmental samples. Many of these alternative media, such as colistin-polymyxin-B-cellobiose (CPC) agar (Massad and Oliver, 1987) and sodium dodecyl sulfate-polymyxin-sucrose (SPS) agar (Massad and Oliver, 1987; Donovan and Van Netten, 1995), were developed for the selection of *V. vulnificus* due to this pathogen's high mortality rate and importance to the shellfish industry (Levin, 2005). However, regardless of more recent developments in culture media, APW and TCBS remain the preferred choice for isolating a broad range of potentially pathogenic *Vibrio* species.

Historically, a combination of biochemical and physiological characteristics has been employed to confirm the identity of a pathogenic *Vibrio* species post isolation from clinical or environmental samples (Thompson *et al.*, 2003). Such assays typically require long assays times and are not practical for high throughput analysis. Alternately, the use molecular techniques such as polymerase chain reaction (PCR) can preclude the use of culture-based and biochemical-based techniques (Lyon, 2001; Panicker *et al.*, 2004; Raghunath *et al.*, 2007). In addition, the distinction between virulent and avirulent strains can be accomplished by using PCR to first

target species-specific genes followed by the targeting of multiple virulence-associated genes (Bej *et al.*, 1999; Rivera *et al.*, 2001; Lipp *et al.*, 2003). Additionally, PCR based detection allows the rapid analysis of large numbers of environmental samples (Lipp *et al.*, 2003).

Environmental detection can be complicated when physiochemical stress initiates a viable-but-non-culturable state (VBNC), which can contribute to an underestimation of the environmental prevalence of pathogenic *Vibrio* species (Lebaron *et al.*, 1999; Halpern *et al.*, 2007). Since public health determinations are often based on the environmental prevalence of a pathogen, the failure to consider VBNC cells could result in an underestimation of risk (Cottingham *et al.*, 2003). Although the infectivity of VBNC cells is still controversial, Colwell *et al*. (1996) demonstrated that VBNC *V. cholerae* cells were still capable of causing infection in the rabbit-loop assay. Therefore, to date, the most accurate detection methods are based upon direct detection by PCR, which has proven a highly sensitive approach to detect both viable and non-culturable cells (Lipp *et al.*, 2003).

CURRENT STATUS AND FUTURE DIRECTIONS

Currently, bacteriological standards (fecal coliform bacteria and enterococci) are used as indicators of marine water quality associated with human sewage contamination (e.g., Noble *et al.*, 2003; Jin *et al.*, 2004). While these enteric indicators serve as a proxy for fecal contamination, they are not predictive of non-enteric microbial threats such as pathogenic *Vibrio* species (Lipp and Rose, 1997). Thus, current public health guidelines for marine waters fail to predict the prevalence of naturally occurring microbial pathogens, of which *Vibrio* species represent the greatest percentage of seafood related illness and death.

To date, no isolation media can reliably identify and quantify the presence of pathogenic *Vibrio* species without further confirmation using molecular techniques. As a result, the sensitivity of said assays underestimate the actual number of pathogens in food and environmental samples. Molecular techniques, such as real-time PCR assays, can return more accurate data in shorter time. However, problems inherent to PCR include false positives due to the presence of dead cells, small sample size and the inhibition of DNA polymerase in complex environmental samples (Lipp and Rose, 1997; Harwood *et al.*, 2004). Given the growing number of infections caused by *Vibrio* species, a more comprehensive approach is needed to safeguard the public from these pathogens.

A greater knowledge of the ecology of these pathogens can be combined with environmental conditions and environmental factors to develop predictive models that alert risk prior to the outcome. Key to the development of accurate predictive models is a greater understanding of the ecology of these environmental pathogens. Furthermore, accurate models will require a clearer understanding of how complex factors such as climate change, anthropogenic disturbances and global transport may result in changes in the prevalence, diversity and distribution of pathogenic *Vibrio* species.

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CHAPTER 3

PLANKTON COMPOSITION AND ENVIRONMENTAL FACTORS CONTRIBUTE TO *VIBRIO* SEASONALITY

Turner JW, Good B, Cole D, Lipp EK (2009). Plankton composition and environmental factors contribute to *Vibrio* seasonality. *The International Society of Microbial Ecology (ISME) Journal* **3**: 1082-1092.[1]

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ABSTRACT

Plankton represents a nutrient-rich reservoir capable of enriching *Vibrio* species, which can include human pathogens, at higher densities than the surrounding water column. To better understand the relationship between vibrios and plankton, the partitioning of culturable vibrios on TCBS between free-living and plankton-associated (63-200 and >200 μm size fractions) was monitored over a one-year period in coastal waters of Georgia, U. S. A.. Seasonal changes in *Vibrio* concentration were then compared to changes in environmental parameters as well as changes in the composition of the plankton community. *Vibrio* concentrations were strongly associated with temperature, especially when those vibrios were plankton-associated ($R^2 = 0.69$ and 0.88 for the water and both plankton fractions; respectively) ($p < 0.01$). Multivariate general linear models revealed that *Vibrio* concentrations in the plankton fractions were also correlated to shifts in the relative abundance of specific plankton taxa. In the 63-200 μm fraction, every 1% increase in the relative abundance of diatoms corresponded to a 16.19-fold decrease in *Vibrio* concentration. In the >200 μm fraction, every 1% increase in the relative abundance of copepods corresponded to a 16.68-fold increase in *Vibrio* concentration. Our results confirm the role of temperature in *Vibrio* seasonality and highlight an important and independent role for plankton composition in explaining seasonal changes in *Vibrio* concentration.

INTRODUCTION

Vibrio species are autochthonous members of the bacterial community in warm estuarine and coastal waters worldwide (Thompson *et al.*, 2003a). They are among the most abundant bacteria readily cultured from the marine environment and exhibit distinct seasonal variation in population density and diversity (Thompson *et al.*, 2003a). Ecologically, vibrios play an important role in the degradation of organic matter and act as a link that transfers dissolved organic carbon (DOC) to higher trophic levels of the marine food web (Grossart *et al.*, 2005). Epidemiologically, at least 12 *Vibrio* species are known to be important pathogens of humans and marine animals (Colwell and Grimes, 1984; Morris, 2003; Panicker *et al.*, 2004).

The association of vibrios with planktonic organisms, especially copepods, has been suggested as an important component of *Vibrio* ecology, especially for *V. cholerae* (Sochard *et al.*, 1979; Huq *et al.*, 1983; Huq *et al.*, 2005). Plankton represent organic-rich microenvironments (Long and Azam, 2001; Grossart *et al.*, 2005), and the high nutrient concentrations of the plankton microhabitat can selectively enrich heterotrophic bacteria, including vibrios (Huq *et al.*, 1983; Tamplin *et al.*, 1990; Lipp *et al.*, 2003; Long *et al.*, 2005). The production of an extracellular chitinase allows vibrios to utilize the chitinous exoskeletons of some plankton taxa as a source of carbon and nitrogen (Thompson *et al.*, 2003a), leading to the hypothesis that some vibrios may demonstrate a competitive advantage when plankton-associated (Heidelberg *et al.*, 2002a).

Plankton colonized by pathogenic *Vibrio* species can potentially act as a vehicle of disease transmission, as in the case of cholera (Huq and Colwell, 1996); however, the details of the interactions between members of the *Vibrio* genus and the plankton community remain largely unknown. Recent work indicates that the structure and composition of the heterotrophic

bacterial community may be dependent upon the structure and composition of the plankton community (Riemann *et al.*, 2000a; Fandino *et al.*, 2001; Pinhassi *et al.*, 2004). It has also been hypothesized that individual plankton species may harbor specific bacterial communities (Grossart *et al.*, 2005).

The objective of this study was to determine if changes in the composition of plankton community contribute to the seasonality of the *Vibrio* population, either synergistically with or independent of temperature. Several studies have described the relationship between vibrios and plankton by characterizing the *Vibrio* population as either free-living or plankton-associated (Baffone *et al.*, 2006), but few studies have addressed the relationship between the structure and composition of the plankton community and that of the *Vibrio* population (Heidelberg *et al.*, 2002a; Grossart *et al.*, 2005; Huq *et al.*, 2005). We hypothesized that shifts in the composition of the plankton reservoir could account for some of the observed seasonality in the *Vibrio* population.

MATERIALS AND METHODS

Sampling sites. This study included 12 sampling sites along the coast of Georgia, U.S.A. (Fig. 3.1). The study sites were representative of two distinct estuaries, Sapelo Sound and Wassaw Sound. Typical of the South Atlantic Bight (southeastern USA) and coastal Georgia in particular, mixing in these estuaries is dominated by tidal exchange (Verity *et al.*, 2006). Furthermore, these two estuaries are unique in that they receive little direct river input.

Sampling sites were selected in coordination with an on-going water quality program administered by the Georgia Department of Natural Resources. Sites were selected to represent a range of environments and hydrologic conditions including tidal creeks and open-water sounds.

Each site was part of a network of oyster beds open for commercial or public harvest. The 12 stations were divided equally between Wassaw Sound (Chatham County) and Sapelo Sound (McIntosh County), and were sampled bi-monthly beginning January 2006 and ending in February 2007 (Fig. 3.1).

Sample collection. Monthly sample collection coincided with the ebb tide of neap tide events. Three fractions were collected from each site: surface water and 2 plankton fractions (63-200 μm and >200 μm). Briefly, approximately 1-liter of surface water was collected in a sterile polypropylene bottle. Plankton fractions were collected by a 5-minute horizontal tow of 63 μm and 200 μm plankton nets (Sea-Gear Corp., model 9000, Melbourne, FL, U.S.A.) at < 1 m depth. The 63 μm fraction was subsequently filtered through a 200 μm mesh. Plankton samples were then washed and resuspended to a volume of 1 l in a sterile polypropylene bottle using sterile phosphate buffered saline (PBS). Sampling was completed within a 6-hour period, beginning early in the morning. Samples were stored in a cooler filled with ambient seawater and transported to the lab, and processed on the same day. Temperature variability during transit was monitored using a max/min thermometer (SPER Scientific, #736690, Scottsdale, AZ, USA).

Plankton identification. A 25 ml aliquot of each plankton sample was fixed (4% v/v, formalin, final concentration) and stored at 5^{o}C. The wet weights (g ml^{-1}) were determined for the fixed plankton samples. Samples were then preserved for long-term storage in 70% ethanol (v/v) and shipped to EcoAnalysts Inc. (Moscow, ID) for taxonomic identification. For each sample (63-200 and >200 μm), the relative abundance of phytoplankton and zooplankton were determined separately by identifying the first 100 phytoplankton and the first 100 zooplankton encountered to the lowest taxonomical unit (LTU).

Enumeration of culturable vibrios. Presumptive *Vibrio* species were enumerated on a selective medium (thio-citrate-bile-sucrose, TCBS cholera medium, Oxoid CM0333, Basingstoke, Hampshire, UK) (Pfeffer and Oliver, 2003; Thompson *et al.*, 2003a). Briefly, water and plankton samples were serially diluted in phosphate buffered saline prior to spread plating 100 µl on duplicate TCBS plates. At least 2 to 3 dilutions were plated to ensure that the plates were countable. Prior to dilution, the plankton samples were homogenized for 1 minute at 12 000 rpm using a PRO homogenizer (PRO Scientific Inc., model 200, Oxford, CT, USA). Plates were incubated in the dark at 30^{o}C for 16-20 hours and all yellow and green colonies were counted as presumptive vibrios, and reported as colony forming units (CFU) ml^{-1} of water and CFU g^{-1} of plankton.

Environmental parameters. Surface temperature (^{o}C), salinity and dissolved oxygen (% saturation) were recorded at each sampling site using a YSI Multi-meter (YSI Environmental, model 556, Yellow Springs, OH, U.S.A.).

Data analysis. Bacterial counts were log-transformed to fit a normal distribution (Anderson-Darling statistic, $\alpha = 0.10$). Pearson correlation coefficients were calculated to evaluate relationships between the log-transformed *Vibrio* concentrations (CFU ml^{-1} water and CFU g^{-1} plankton) and environmental parameters. One-way analysis of variance (ANOVA) was used to determine the significance of differences in *Vibrio* concentrations between fractions, sampling sites and collection months; significance was declared when $p < 0.05$. All univariate and bivariate analyses were carried out in MINITAB version 15.0 (MINITAB Inc.).

General linear regression models (PROC GLM, SAS Institute, Inc. vers. 9.1) were developed for each fraction (water, 63-200 and >200 μm plankton) to examine relationships between the relative abundance of each plankton taxon (in the plankton fractions), environmental variables and *Vibrio* concentrations. All variables found to be correlated ($p < 0.10$) in univariate analyses, and their appropriate interaction terms, were initially entered into the models. Maximum likelihood estimates of each variable parameter were determined, and variables were eliminated from the model by backward elimination when $p > 0.05$. All graphs were constructed in SigmaPlot version 9.0 (Systat Software Inc.).

RESULTS

Small plankton (63-200 μm). The 63-200 μm plankton samples [N= 64] were composed primarily of phytoplankton and smaller forms of zooplankton. Diatoms (mean relative abundance 55.4%), cyanobacteria (mean relative abundance 26.3%) and green algae (mean relative abundance 17.4%) accounted for 99.1% of all phytoplankton in the 63-200 μm fractions (Fig. 3.2). The relative abundance of diatoms was inversely related to temperature ($r = -0.40$) while the relative abundance of cyanobacteria varied directly with temperature ($r = 0.41$) ($p < 0.05$ for each). Copepods (mean relative abundance 77.8%), decapods (mean relative abundance 0.02%), sessilia (mean relative abundance 0.03%), foraminifera (mean relative abundance 15.7%) and rotifera (mean relative abundance 6.23%) accounted for 99.8% of all zooplankton in this fraction (Fig. 3.2). The relative abundance of copepods was directly related to temperature ($r = 0.42$) while the relative abundance of foraminiferida was inversely related to temperature ($r = -0.42$) ($p < 0.05$ for each).

Large plankton (>200 µm). The >200 µm plankton samples [N = 64] were composed primarily of zooplankton; however, some phytoplankton were also captured in this size fraction. Copepods (mean relative abundance 79.1%), decapods (mean relative abundance 16.0%), sessilia (mean relative abundance 4.8%) and foraminifera (mean relative abundance 0.08%) accounted for 99.9% of all zooplankton in this size fraction (Fig. 3.3). Diatoms (mean relative abundance 52.1%), cyanobacteria (mean relative abundance 27.7%) and green algae (mean relative abundance 19.2%) accounted for 99.0% of all phytoplankton in this size fraction (Fig. 3.3). The relative abundance of decapods and sessilia were directly related to temperature ($r = 0.78$ and 0.38 respectively) while the relative abundance of copepods was inversely related to temperature ($r = -0.72$) ($p < 0.05$ for each).

***Vibrio* concentrations.** A total of 64 water samples and 116 plankton samples (N = 58 for each size fraction) were collected during the study period. Temperature during transport of samples varied by $\leq 2^{o}$C. Fluctuations in mean monthly *Vibrio* concentration followed a strong seasonal trend (Fig. 3.4). Univariate analyses (Pearson's correlation coefficient) revealed that *Vibrio* concentrations in all fractions were directly related to temperature ($r = 0.69$ for water and $r = 0.88$ for both plankton fractions [$p < 0.01$ for each]). Conversely, *Vibrio* concentrations were inversely related to DO in all fractions ($r = -0.49$, -0.67 and -0.68 for the water, 63-200 and >200 µm fractions, respectively [$p < 0.01$ for each]). Salinity showed a positive, although weak, correlation with *Vibrio* concentrations ($r = 0.36$, 0.35 and 0.39 for the water, 63-200 and >200 µm fractions, respectively [$p < 0.01$ for each]).

The mean *Vibrio* concentration in the water fraction (500 CFU ml^{-1} [N = 64]) was up to 6 orders of magnitude less than the mean concentration of vibrios in the plankton fractions (4.91×10^{7} and 1.58×10^{8} CFU g^{-1} for the 63-200 and >200 µm fractions respectively [N = 58 for both])

(Fig. 3.4). *Vibrio* concentrations in the water fraction ranged from 20 CFU ml^{-1} in February 2007 to 6,150 CFU ml^{-1} in August 2006. Concentrations in the 63-200 μm fractions ranged from 1.83×10^3 CFU g^{-1} in February 2007 to 5.98×10^8 CFU g^{-1} in September 2006. Concentrations in the >200 μm fraction ranged from 7.36×10^2 CFU g^{-1} in January 2007 to 1.58×10^8 CFU g^{-1} in August 2006 (Fig. 3.4). *Vibrio* concentrations were not significantly different between the two plankton fractions or between the different sampling sites (ANOVA, $p < 0.001$).

General linear models. Multivariate general linear models (GLMs) were constructed for the water fraction (Table 3.1) and each plankton fraction (Tables 3.2 and 3.3). In the 63-200 μm fraction, only models incorporating copepods, diatoms and cyanobacteria were significantly predictive (Table 3.2) and in the >200 μm fraction, only models incorporating copepods and decapods were significantly predictive (Tables 3.3).

Water fraction model. Temperature and salinity were the only significant correlates of *Vibrio* concentration in the water fraction (Table 3.1). DO and the concentration of vibrios in both plankton fractions (63-200 and >200 μm), as well as their interaction terms, were included in the model as potential drivers; however, these variables were not significant and were removed from the final model. The model estimated that each 1^oC rise in temperature corresponded to a 0.043-fold increase in the log concentration of vibrios. Meanwhile, each 1.0 increase in salinity corresponded to a 0.049-fold increase in the log concentration of vibrios. Although the magnitude of these parameter estimates was small, temperature accounted for 33.36% of the model variance while salinity accounted for 66.64% of the model variance (Table 3.1).

63-200 μm fraction copepod model. This model estimated that each 1% increase in the relative abundance of copepods corresponded to a 6.41-fold decrease in the log concentration of

vibrios (Table 3.2). Copepods accounted for 16.63% of the model variation (8.11% of this variation was independent and an additional 8.52% was through interactions with DO). Each one-log increase in *Vibrio* concentration in the water fraction corresponded to a 3.80-fold increase in the 63-200 μm plankton fraction. The log concentration of vibrios in the water fraction accounted for 34.37% of the model variation (16.97% independently and an additional 8.74% and 8.66% from interactions with temperature and DO, respectively). Each 1^{o}C increase in temperature was responsible for a modest 0.28-fold increase in the log *Vibrio* concentration. Independently, temperature accounted for 48.90% of the variation. While DO was retained in the final model because it was an important interaction variable, there was no significant independent relationship between plankton-associated *Vibrio* concentrations and DO (Table 3.2).

63-200 μm fraction diatom model. This model estimated that each 1% increase in the relative abundance of diatoms corresponded to a 16.19-fold decrease in log *Vibrio* concentration (Table 3.2). Diatoms accounted for 67.22% of the model variance (18.36% independently and an additional 37.18% and 11.68% due to interactions with temperature and DO, respectively). Each one-log increase in the concentration of vibrios in the water fraction corresponded to a 0.43-fold increase in log *Vibrio* concentration in the 63-200 μm fraction, and accounted for 8.76% of the model variance. Each 1^{o}C change in temperature and each 1.0% change in DO corresponded to 0.12 and 0.093-fold decreases in log *Vibrio* concentrations, respectively. Independently, temperature and DO accounted for 5.84% and 18.19% of the model variance, respectively (Table 3.2).

63-200 μm fraction cyanobacteria model. This model estimated that each 1% increase in the relative abundance of cyanobacteria corresponded to a 28.11-fold decrease in the log concentration of vibrios (Table 3.2). The relative abundance of cyanobacteria accounted for

14.98% of the model variance in log *Vibrio* concentrations in the 63-200 μm fraction (7.36% independently and an additional 7.62% through interaction with salinity). Each one-log increase in the concentration of vibrios in the water fraction corresponded to a 1.97-fold decrease in the 63-200 μm fraction, and explained 9.01% of the model variation (4.36% independently and an additional 4.65% through interaction with DO). Each 1^{o}C change in temperature corresponded to a 0.18-fold increase in the log concentration of vibrios, and explained 65.19% of the model variance. Salinity and DO also showed significant negative associations with log *Vibrio* concentrations in this fraction (Table 3.2).

>200 μm fraction copepod model. This model estimated that each 1% increase in the relative abundance of copepods corresponded to a 16.68-fold increase in the log concentration of vibrios; however, this relationship was modified by interactions between copepods and temperature and DO that were inversely related to log *Vibrio* concentrations (parameter estimates = -0.38 and -0.08, respectively) (Table 3.3). The relative abundance of copepods accounted for 24.79% of the model variance (12.61% independently and an additional 8.12 and 4.06% through interactions with temperature and DO, respectively). Each one-log increase in the concentration of vibrios in the water fraction corresponded to a 6.40-fold decrease in the log concentration of vibrios in the >200 μm fraction, and accounted for 40.69% of the model variance (12.59% independently and additional 11.92 and 16.18% through interactions with temperature and salinity, respectively). Each 1^{o}C increase in temperature corresponded to a 0.79-fold increase in log *Vibrio* concentration, and accounted for 20.01% of the model variance. Each 1.0 increase in salinity corresponded to a 0.62-fold decrease in log Vibrio concentration, and accounted for 11.29% of the model variance (Table 3.3).

>200 μm fraction decapod model. This model estimated that each 1% increase in the relative abundance of decapods corresponded to a 1.32-fold decrease in the log concentration of vibrios (Table 3.3). The relative abundance of decapods accounted for 7.65% of the model variance. Each one-log increase in the concentration of vibrios in the water fraction corresponded to a 7.27-fold decrease in the log concentration of vibrios in the >200 μm fraction, and accounted for 37.22% of the model variance (12.68% independently and an additional 10.23 and 14.31% through interactions with temperature and salinity, respectively). Each 1^{o}C increase in temperature corresponded to a 0.38-fold increase in log *Vibrio* concentration, and accounted for 46.27% of the model variance. Each 1.0 increase in salinity corresponded to a 0.60-fold decrease in the log *Vibrio* concentration, and accounted for 8.85% of the model variance (Table 3.3).

Environmental parameters. Temperature, dissolved oxygen (DO) and salinity ranged from 9.3 to 30.8^{o}C, from 48.7 to 105.8% saturation and from 26.3 to 32.2 ppt, respectively (Fig. 3.4). Temperature and DO exhibited strong seasonal trends, while salinity varied less and showed no seasonal pattern. DO and temperature were inversely related ($r = -0.73$), while salinity and temperature were directly related ($r = 0.40$) ($p < 0.01$).

DISCUSSION

Results of this study show that seasonal variations in *Vibrio* concentrations can be modeled using changes in both environmental variables and the composition of the plankton reservoir. While these results confirm previous studies citing temperature, salinity and DO as significant drivers of *Vibrio* seasonality (Heidelberg *et al.*, 2002b; Lipp *et al.*, 2003; Thompson *et al.*, 2003b; Baffone *et al.*, 2006), this study also supports the hypothesis that plankton

composition is an important driver of *Vibrio* seasonality. Further, it appears that the effect of changes in plankton composition can be independent of or synergistic with changes in environmental parameters.

Whereas previous studies have primarily focused on the relationship between *V. cholerae* and copepods (Huq *et al.*, 1983; Huq *et al.*, 1984; Tamplin *et al.*, 1990), this study focused on associations between the larger *Vibrio* genus and the greater plankton community. For this purpose we employed a proven and effective *Vibrio*-selective media (TCBS) that, compared to alternate media like CPC, SPS, VV and TCI, is more efficient and more selective at isolating a broad range of vibrios and therefore preferred for the study of the *Vibrio* genus as a whole (Massad and Oliver, 1987; Pfeffer *et al.*, 2003). Granted, a reliance on culturable techniques lacks specificity and fails to account for viable but non-culturable (VBNC) bacteria (Lipp *et al.*, 2003). For this reason, our current efforts are focused on the coupling of culturable data and species-specific molecular data.

Univariate analyses alone, although informative, were unable to capture the complexity and synergy of the *Vibrio*-plankton relationship. A taxonomic analysis of plankton samples confirmed that the relative abundance of certain plankton taxa exhibited distinct patterns of seasonal variation, and through the use of multivariate general linear models, we were able to show that seasonal changes in the plankton community were related to changes in co-measured *Vibrio* concentrations. Although our determination of the relative abundance of plankton taxa was a strictly qualitative analysis, our data indicate that *Vibrio* concentrations were related to shifts in the relative abundance of some plankton taxa. Similarly, a previous study has described how the abundance of *V. cholerae* can be driven by shifts in plankton abundance and described in the context of plankton blooms (Huq *et al.*, 2005).

The GLM describing the water fraction showed that temperature and salinity were the only significant drivers of free-living *Vibrio* concentrations; however, salinity accounted for a greater proportion of the model variance in *Vibrio* concentration compared to temperature. This result was especially interesting given that we observed only moderate changes in salinity during the study period. It is possible that our selection of tidally dominated study sites and the timing of our collection during the ebb tide of neap tidal events may have normalized for salinity. Salinity has been reported as correlated to the occurrence of some vibrios but its significance can vary across the *Vibrio* genus (Thompson *et al.*, 2003a) and change depending on the range of temperature in the system (Lipp *et al.*, 2001; Randa *et al.*, 2004).

In both plankton size fractions (63-200 and >200 μm), the relative abundance of copepods was significantly associated with the concentration of vibrios. In particular, parameter estimates showed a strong direct relationship between *Vibrio* concentration and the relative abundance of copepods in the >200 μm fraction. Copepods have been implicated as a potential vector of *Vibrio cholerae* (Huo *et al.*, 1996; Colwell *et al.*, 2003), and an ecological relationship between *V. cholerae* and copepods has been suggested previously (Huq *et al.*, 1983; Huq *et al.*, 1984; Tamplin *et al.*, 1990; Thomas *et al.*, 2006). The inverse parameter estimate in the 63-200 μm fraction may reflect differences in copepod life stages between the fractions. The majority of copepods in the 63-200 μm fraction represented earlier life stages (i.e., naupliar and copepodite), which displayed a bimodal distribution, peaking in May and October 2006, when water temperatures were not optimal for the growth of most vibrios (Thompson *et al.*, 2003a). These earlier copepod life stages also differ physiologically in that earlier stages are continuously molting, and shedding attached vibrios in the process (Tamplin *et al.*, 1990).

The remaining models in the 63-200 μm plankton fraction incorporate two autotrophic plankton types, diatoms and cyanobacteria. In both models, an increase in the relative abundance of diatoms or cyanobacteria corresponded to a significant decrease in co-measured *Vibrio* concentration. It is possible that the inverse relationship reflects a release of nutrients that corresponds with the decay of a diatom or cyanobacterial bloom (Middelboe *et al.*, 1995; Riemann *et al.*, 2000b). The decline of the bloom could then stimulate an increase in secondary production by heterotrophic bacteria, like vibrios (Lancelot and Billen, 1984; Pomeroy and Wiebe, 2001). Cyanobacterial-derived organic matter has been reported as an important growth factor of *V. cholerae* and *V. vulnificus (*Eiler *et al., 2007).* Alternately, the increase in the abundance of cyanobacteria, which are known to produce a range of antibacterial metabolites, might have contributed to a decline in the growth of vibrios in that plankton fraction (Lam *et al.*, 2008; Singh *et al.*, 2008).

The final model describes a weak relationship between *Vibrio* concentration and the relative abundance of decapods in the >200 μm fraction. This model stands alone in that changes in parameters other than plankton abundance; namely temperature, salinity and the concentration of vibrios in the water fraction, were the primary drivers of *Vibrio* concentration. We believe the decapod association was weak for two reasons; 1) decapods comprised a relatively small proportion of this fraction, and 2) the occurrence of decapods coincided with water temperatures that were optimal for the growth of vibrios. Thus, the magnitude of change in the relative abundance of decapods was so much smaller than the magnitude of change occurring in the *Vibrio* population that decapods appeared as a non-significant factor; however, this result does not preclude the idea that a rare member of the plankton community could not have a profound affect on the *Vibrio* population.

Together, our results confirm the importance of temperature, salinity and DO as independent drivers of both free-living and plankton-associated *Vibrio* concentrations. Additionally, we have shown that plankton composition plays an important and independent role as a driver of *Vibrio* concentrations in natural estuarine systems. These findings highlight the potentially complex relationship between seasonal shifts in plankton composition and the net effect on *Vibrio* levels and suggest that *Vibrio* concentrations should be considered within the context of bloom formation and decline as well with single point determinations of plankton composition.

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TABLES

Table 3.1. General linear model describing changes in the log *Vibrio* concentrations in the water fraction.

Parameter	Estimate ± SE[a]	P value	Type III SS[b]	% variation described[c]
Temperature	0.043 ± 0.0078	<0.001	5.82	33.36%
Salinity	0.049 ± 0.0062	<0.001	11.62	66.64%

[a] Standard Error

[b] Sums of Squares

[c] The additional variability (expressed as percent) explained by each parameter in the full model based on the Type III sums of squares (SS)

Table 3.2. Taxon-specific plankton models describing changes in the log *Vibrio* concentration in the 63 – 200 µm plankton fraction.

Model	Parameter	Estimate ± SE[a]	P value	Type III SS[b]	% variation described[c]
Copepod	Copepod abundance	-6.41 ± 2.42	0.0108	3.47	8.11%
R^2 = 0.99	Water *Vibrio*[d]	3.80 ± 0.99	<0.001	7.25	16.97%
	Temperature	0.28 ± 0.043	<0.001	20.90	48.90%
	DO	0.0031 ± 0.011	0.787	0.037	0.09%
	Copepod*DO	0.083 ± 0.031	0.009	3.64	8.52%
	Water *Vibrio**Temp	-0.056 ± 0.020	0.008	4.06	8.74%
	Water *Vibrio**DO	-0.032 ± 0.011	0.0008	3.70	8.66%
Diatom	Diatom abundance	-16.19 ± 5.42	0.004	3.63	18.36%
R^2 = 0.85	Water *Vibrio*	0.043 ± 0.21	0.044	1.73	8.76%
	Temperature	-0.12 ± 3.57	0.098	1.15	5.84%
	DO	-0.093 ± 0.031	0.004	3.59	18.19%
	Diatom*Temp	0.042 ± 0.098	<0.001	7.35	37.18%
	Diatom*DO	0.012 ± 0.049	0.021	2.31	11.68%
Cyanobacteria	Cyanobacteria abundance	-28.11 ± 3.90	0.008	3.90	7.36%
R^2 = 0.81	Water *Vibrio*	-1.97 ± 0.93	0.040	2.31	4.36%
	Temperature	0.18 ± 0.022	<0.001	34.55	65.19%
	Salinity	-0.29 ± 0.11	0.012	3.49	6.58%
	DO	-0.067 ± 0.032	0.042	2.25	4.24%
	Cyanobacteria*Salinity	1.02 ± 0.36	0.007	4.04	7.62%
	Water *Vibrio**DO	0.028 ± 0.013	0.034	2.46	4.65%

[a] Standard Error
[b] Sums of Squares
[c] The additional variability (expressed as percent) explained by each parameter in the full model based on the Type III sums of squares (SS)
[d] Log concentration of vibrios in the water fraction

Table 3.3. Taxon-specific plankton models describing changes in the log *Vibrio* concentration in the >200 µm plankton fraction.

Model	Parameter	Estimate ± SE[a]	P value	Type III SS[b]	% variation described[c]
Copepod	Copepod abundance	16.69 ± 4.61	<0.001	5.20	12.61%
R^2 = 0.99	Water *Vibrio*[d]	-6.40 ± 1.77	<0.001	5.19	12.59%
	Temperature	0.79 ± 0.17	<0.001	8.25	20.01%
	Salinity	-0.62 ± 0.18	0.001	4.65	11.29%
	DO	0.065 ± 0.035	0.074	1.33	3.22%
	Copepod*Temperature	-0.38 ± 0.13	0.005	3.34	8.12%
	Copepod*DO	-0.08 ± 0.040	0.045	1.67	4.06%
	Water *Vibrio**Temperature	-0.11 ± 0.032	<0.001	4.91	11.92%
	Water *Vibrio**Salinity	0.31 ± 0.075	<0.001	6.67	16.18%
Decapod	Decapod abundance	-1.32 ± 0.53	0.017	2.41	7.65%
R^2 = 0.84	Water *Vibrio*	-7.27 ± 2.30	0.0026	3.99	12.68%
	Temperature	0.38 ± 0.063	<0.001	14.54	46.27%
	Salinity	-0.60 ± 0.23	0.011	2.78	8.85%
	Water *Vibrio**Temperature	-0.08 ± 0.029	0.0064	3.22	10.23%
	Water *Vibrio**Salinity	0.31 ± 0.093	0.0015	4.50	14.31%

[a] Standard Error
[b] Sums of Squares
[c] The additional variability (expressed as percent) explained by each parameter in the full model based on the Type III sums of squares (SS)
[d] Log concentration of vibrios in the water fraction

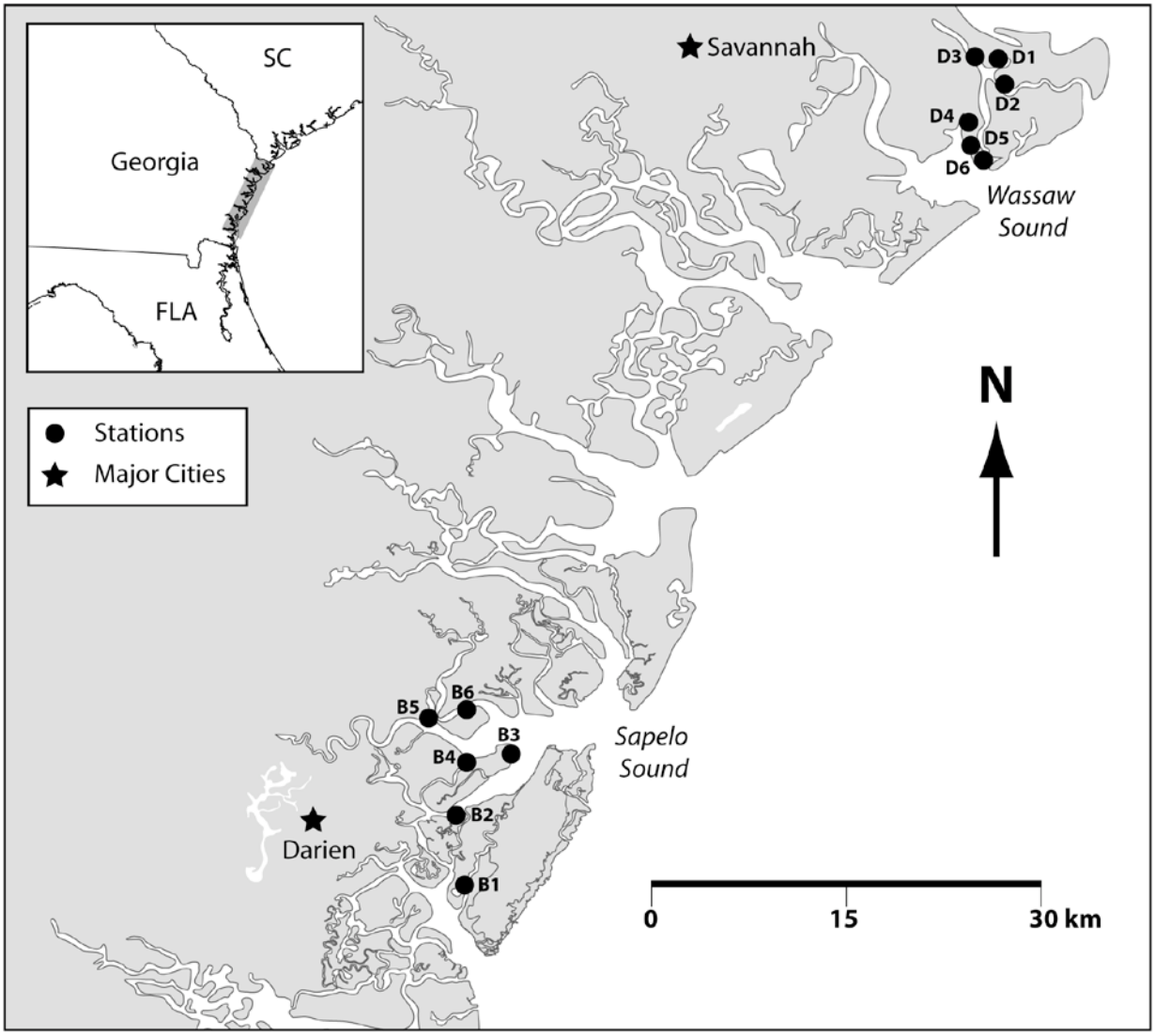

Figure 3.1. Map of study sites.

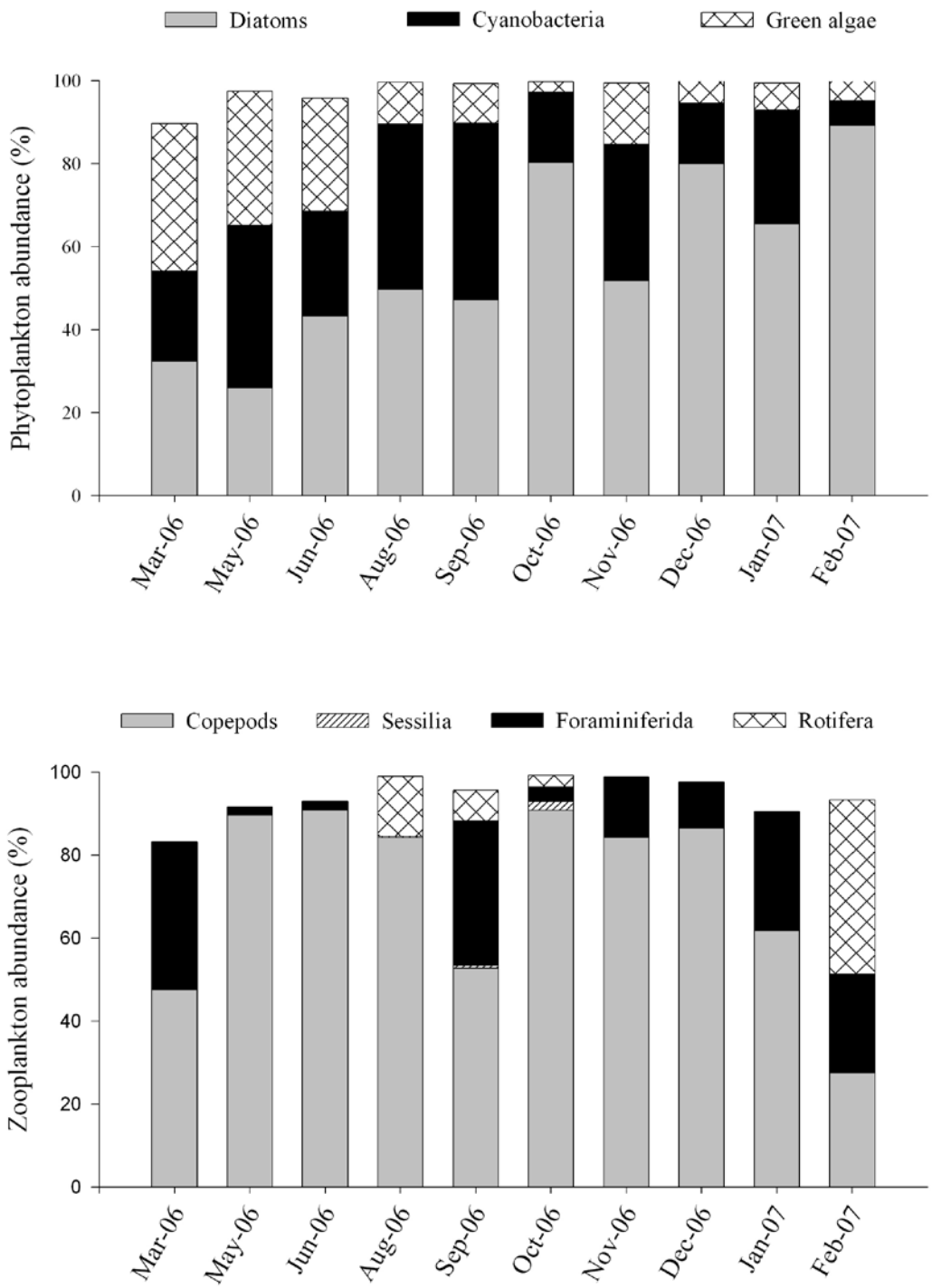

Figure 3.2. Seasonal changes in the relative abundance of phytoplankton taxa in the 63-200 µm fraction (A) and the >200 µm fraction (B).

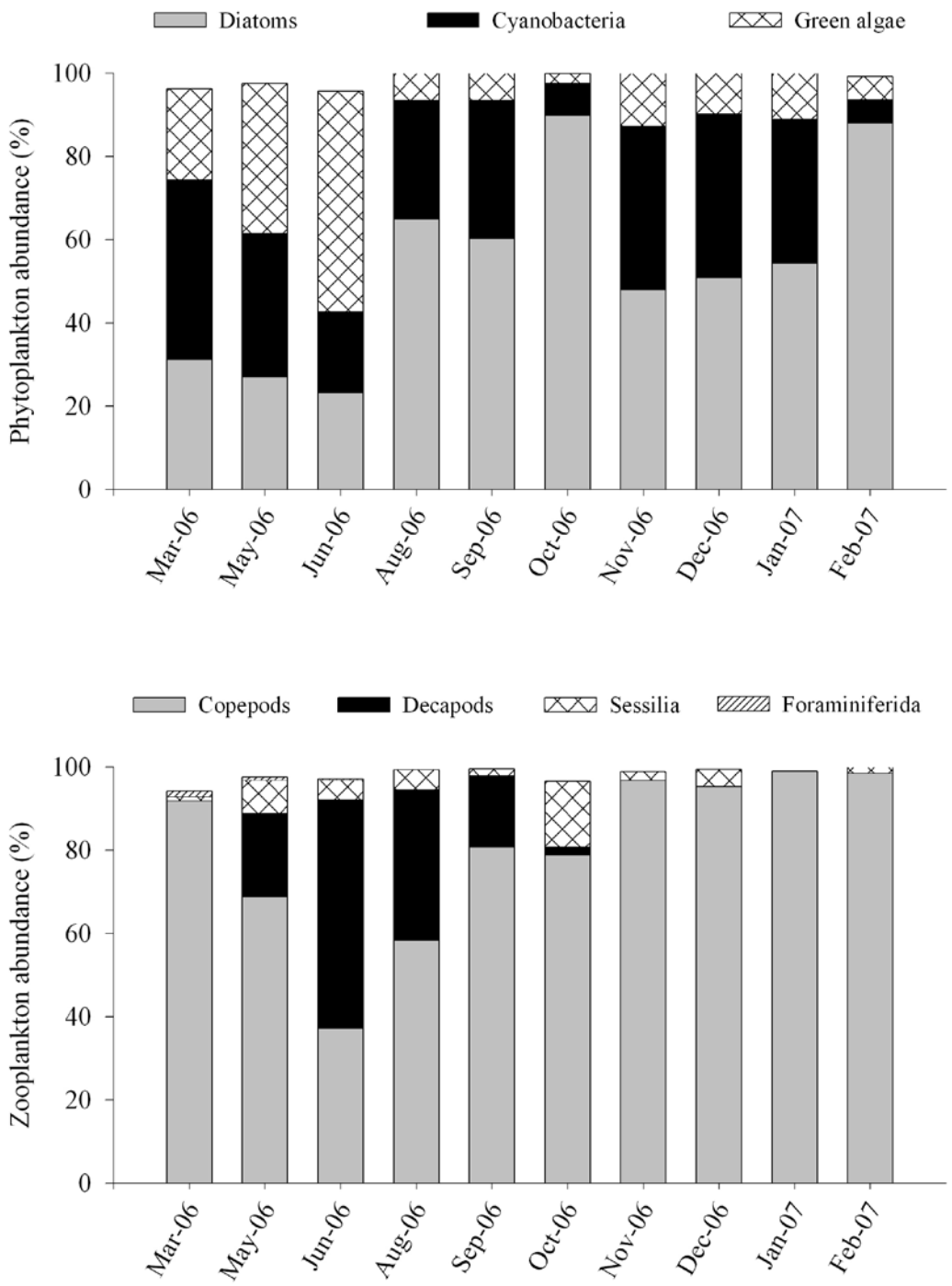

Figure 3.3. Seasonal changes in the relative abundance of zooplankton taxa in the 63-200 µm fraction (A) and the >200 µm fraction (B).

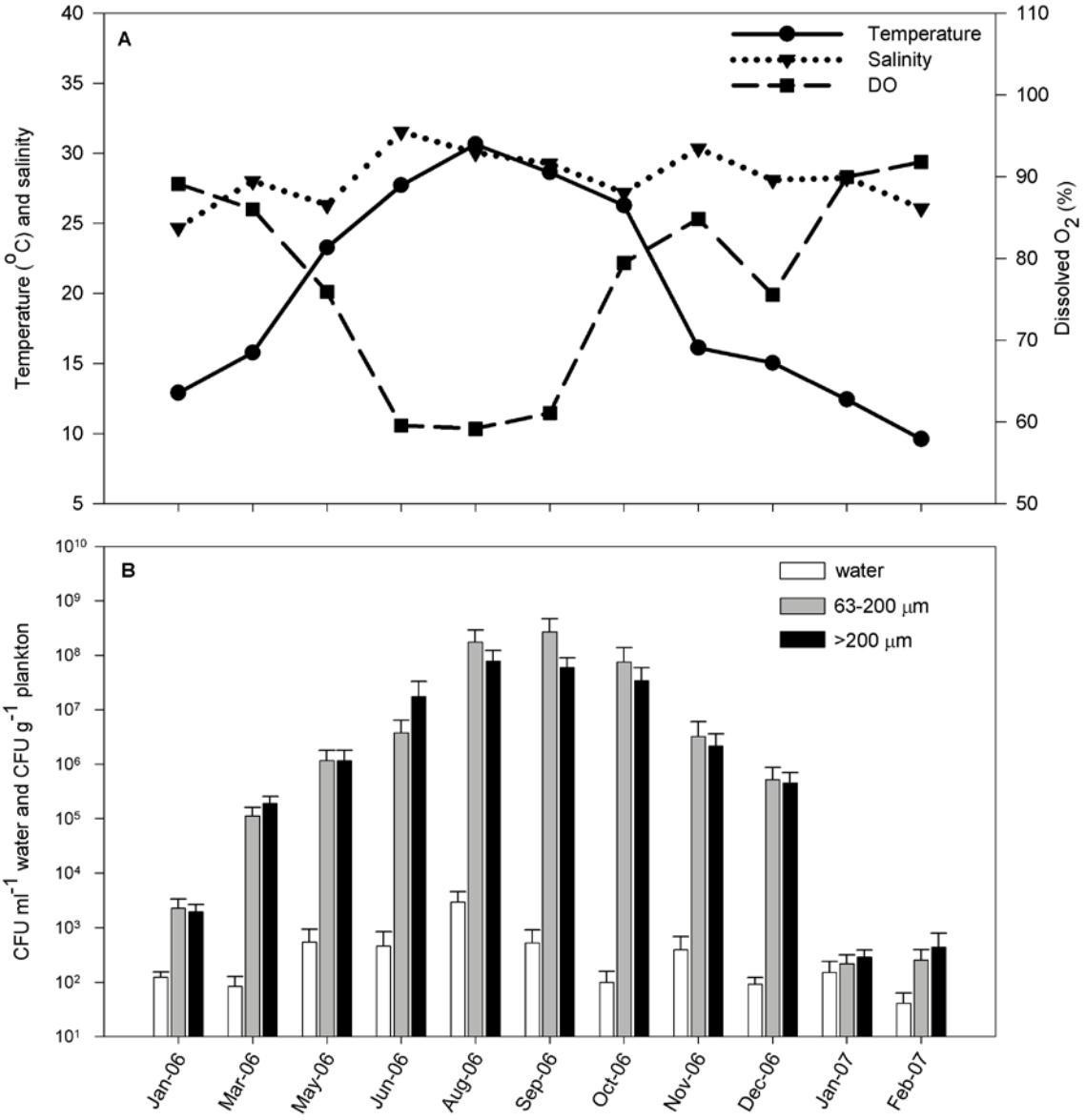

Figure 3.4. Seasonal fluctuations in mean monthly environmental conditions (temperature, salinity and DO) (A) and seasonal fluctuations in mean monthly *Vibrio* concentrations (CFU ml^{-1} in the water column and CFU g^{-1} for the small [63-200 μm] and large [>200 μm] plankton fractions) (B).

CHAPTER 4

TEMPERATURE AND RESERVOIR SHIFTS CORRELATE WITH THE SEASONAL DISTRIBUTION PATHOGENIC *VIBRIO* SPECIES IN SHELLFISH HARVESTING WATERS

Turner JW, Malayil L, Guadognoli D, Cole D, Lipp EK. To be submitted to *International Society of Microbial Ecology (ISME) Journal.*

ABSTRACT

Vibrio cholerae, *V. vulnificus* and *V. parahaemolyticus* are important human pathogens and natural inhabitants of marine and estuarine systems worldwide. Over a one-year period, water and plankton (63-200 and >200 µm size fractions) were collected from shellfish harvesting waters and analyzed by PCR for the presence of these pathogens and their virulence-associated genes. Trends in prevalence and distribution were examined for relationships with changes in environmental parameters as well as shifts in the relative abundance of specific plankton taxa. The prevalence of *V. vulnificus* increased significantly with warming surface water temperatures in all fractions (water, 63-200 and >200 µm) ($P < 0.001$ for each). Similarly, the prevalence of *V. cholerae* increased significantly with warming surface water temperatures in the water and 63-200 µm fractions ($P < 0.05$ for each). In contrast, *V. parahaemolyticus* was detected year round even when water temperatures dropped below 10^{o}C; however, the prevalence of the pandemic-specific phage-encoded region (ORF8) of *V. parahaemolyticus* increased significantly with warming surface water temperature in all fractions ($P < 0.05$ for each). In regards to shifts in the abundance of plankton taxa, the prevalence of *V. parahaemolyticus* increased significantly with increasing abundance of diatoms in the 63-200 µm fraction ($P < 0.05$). A significant direct relationship was also observed between *V. vulnificus* prevalence and decapod abundance (63-200 µm fraction) ($P < 0.0001$) and *V. cholerae* prevalence and copepod abundance (>200 µm fraction) ($P < 0.05$). Together, these results stress the importance of temperature and plankton composition in defining the ecology of co-occurring pathogenic *Vibrio* species. Further, this study demonstrates that *V. parahaemolyticus*, *V. vulnificus* and *V. vulnificus* respond uniquely to seasonal changes in environmental conditions and shifts in the composition of the plankton

community. Thus, the inclusion of plankton data could improve the predictive power of *Vibrio* models and *Vibrio* risk assessments.

INTRODUCTION

Members of the *Vibrio* genus are Gram-negative, halophilic bacteria indigenous to coastal marine systems (Thompson *et al*., 2003a). These common heterotrophic bacteria persist as a natural component of the microbial flora, although a small percentage of environmental isolates carry the genetic determinants of human pathogenesis (Nishibuchi and Kaper, 1995; Chakraborty *et al.*, 2000; Rivera *et al.*, 2001). Currently, *Vibrio* infections are the leading cause of seafood-borne bacterial gastroenteritis in the United States (Mead *et al*., 1999); and together, *Vibrio parahaemolyticus, V. vulnificus* and *V. cholerae* (non-O1 and non-O139) account for the majority of those infections (Morris, 2003).

Previous studies have characterized the distribution of *Vibrio* species as both free-living and plankton-associated (Heidelberg *et al*., 2002a; Lipp *et al*., 2003; Maugeri *et al*., 2004; Baffone *et al*., 2006; Turner *et al*., 2009). The nutrient-rich surfaces of plankton, especially the chitinous exoskeletons of copepods, can selectively enrich pathogenic *Vibrio* species at higher densities than the surrounding water column (Huq *et al*., 1983; Tamplin *et al*., 1990; Maugeri *et al*., 2004). When heavily colonized by *Vibrio cholerae*, copepods are a well-documented vehicle for the transmission of cholera (Huq and Colwell, 1996; Colwell *et al*., 2003; Huq *et al*., 2005). Additional plankton taxa such as diatoms (Kaneko *et al*., 1977; Kumazawa *et al*., 1991), cyanobacteria (Islam *et al*., 1999; Islam *et al*., 2004) and decapods (Krantz *et al*., 1969) have also been identified as *Vibrio* reservoirs.

Association with plankton is thought to provide a protective microhabitat from extremes in environmental conditions and nutrient concentrations (Huq *et al*., 1983). Protection from these extremes is also afforded by the ability to revert to a “viable but non-culturable” (VBNC) state (Colwell *et al*., 1985; Lebaron *et al*., 1999; Oliver, 2005). Although VBNC cells cannot be

cultured, a change to more favorable conditions has been shown to restore metabolic activity and pathogenicity in some *Vibrio* species (Oliver, 1995; Colwell *et al*., 1996; Oliver, 2005). Due to the VBNC phenomenon, a reliance on culture-dependent techniques can underestimate the prevalence of pathogenic *Vibrio* species (Lipp *et al*., 2003; Oliver, 2005).

In this study, we used polymerase chain reaction (PCR) for the direct detection of species-specific and virulence-associated gene targets of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* from environmental samples. The primary objective of this study was to determine the seasonal prevalence of these three pathogens and their virulence-associated genes in water and plankton collected from shellfish harvesting areas off the coast of Georgia, USA. Second, by focusing on the plankton community as a group of distinct organisms, each representing a different microhabitat for bacterial association, we set out to determine if the prevalence of these pathogens and select virulence-associated genes could be correlated with the relative abundance of specific plankton taxon. We hypothesized that changes in the prevalence and distribution of pathogenic *Vibrio* species would be correlated to seasonal changes in environmental parameters as well as shifts in the composition of the plankton community.

MATERIALS AND METHODS

Sample collection. Sampling sites and collection procedures were described previously (Turner *et al*., 2009). Briefly, twelve commercial and public oyster harvesting sites, selected in coordination with the Georgia Department of Natural Resources (GA DNR), were divided equally between Wassaw Sound and Sapelo Sound, along the Atlantic Bight of coastal Georgia, USA. Beginning in January 2006 and ending in February 2007, surface water and plankton samples (63-200 and >200 µm sized plankton fractions) were collected bi-monthly from each

station. Surface raw water samples (1 L) were collected in sterile 1 L polypropylene bottles and plankton samples were collected by a 5 minute horizontal tow of 63 µm and 200 µm nets at a depth of less than 1 m. The 63-200 µm fractions were obtained by passing the catch from the 63 µm net through a 200 µm net. Each plankton fraction was resuspended in 1 L of phosphate buffered saline (PBS). Water temperature (^{o}C), salinity and dissolved oxygen (DO) (% saturation) were recorded at each sampling site using a YSI Multi-meter (YSI Environmental, model 556, Yellow Springs, OH, USA).

Plankton taxonomy. A 25 ml aliquot of each plankton sample was fixed in formaldehyde (4% v/v final concentration) and then preserved for long-term storage in 70% ethanol. Samples were shipped to EcoAnalysts Inc. (Moscow, ID) where the relative abundance of phytoplankton and zooplankton were determined separately by identifying the first 100 phytoplankton and the first 100 zooplankton encountered to the lowest taxonomical unit (LTU). Plankton types were then grouped into common categories as described previously (Turner *et al*. 2009).

Enrichment and DNA purification. All samples were enriched in alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.6) prior to DNA extraction. Briefly, 100 ml of each water sample and 25 ml of each plankton sample were filtered onto sterile 0.22 µm polycarbonate membranes (Millipore, GTTP04700, Billerica, Massachusetts) and washed twice with 5 ml of phosphate-buffered saline (PBS). Filters were added to sterile 50 ml tubes containing 30 ml of APW and incubated at 30^{o}C with shaking (100 rpm) (New Brunswick Scientific, model R2, Edison, NJ, USA) for 16-20 hours.

Total DNA was extracted from 5 ml aliquots of the APW enrichments using a DNeasy tissue kit (Qiagen Sciences, Valencia, CA, USA). Briefly, cells were pelleted by centrifugation

(2,450 x *g* for 10 minutes at 4^{o}C) (Thermo IEC, Marathon 21000R, Needham Heights, MA, USA) washed with PBS (1 ml), and then transferred to 2.0 ml microcentrifuge tubes and suspended in 180 µl of tissue lysis buffer (DNeasy tissue kit). Purification of DNA was performed according to the manufacturer's protocol. DNA was eluted with 50 µl of sterile nuclease-free PCR water (Fisher Scientific, Pittsburg, PA, USA). DNA was quantified with a UV spectrophotometer (Eppendorf BioPhotometer, model 6131, Hamburg, Germany), diluted to ~100 ng ml^{-1} and stored at -80^{o}C.

Bacterial strains and media. *V. cholerae* O1 ATCC 14035 (*ctx*$^{+}$, *tox*R^{+}, *tcp*$^{+}$), *V. cholerae* O139 AI1877 (*ctx*$^{+}$, *tox*R^{+}, *tcp*$^{+}$), *V. parahaemolyticus* ATCC 17803 (*tl*$^{+}$, *trh*$^{+}$, *tdh*$^{-}$), *V. parahaemolyticus* ATCC 43996 (*tl*$^{+}$, *trh*$^{-}$, *tdh*$^{+}$), *V. parahaemolyticus* ATCC BA-238 (*tl*$^{+}$, *trh*$^{+}$, *tdh*$^{+}$, ORF8^{+}) and *V. vulnificus* ATCC 27562 (*viu*B^{+}) were used as positive controls. Controls were grown in Luria-Bertani (LB) broth supplemented with NaCl (2% final conc.) at 30^{o}C overnight with shaking (100 rpm) (New Brunswick Scientific, model R2, Edison, NJ, USA) for 16-20 hours. Bacterial strains were preserved in glycerol (10% final concentration) at -80^{o}C.

PCR detection. Primers for the detection of all species-specific and virulence-associated gene targets are described in Table 1. For all reactions, 1 µl of sample DNA (~100 ng) was added to a 24 µl master mix prepared using an Eppendorf 5-PRIME kit (Hamburg, Germany). All reactions contained 1 X PCR buffer (providing 1.5 mM Mg^{2+}), and 1 X Taq-Master PCR enhancer.

For assays targeting the species-specific thermolabile hemolysin (*tl)* and the virulence-associated thermolabile related and thermolabile direct (*trh* and *tdh*) hemolysin genes of *V. parahaemolyticus*, reaction mixtures included 0.2 µM deoxynucleoside triphosphates (dNTPs), 1.0 µM of each primer and 1.25 units of *Taq* polymerase (Bej *et al*., 1999). For assays targeting

the pandemic-specific ORF8 region of *V. parahaemolyticus*, reaction mixtures included 0.1 µM dNTPs, 1.0 µM of each primer and 1.0 unit of *Taq* polymerase (Yeung *et al*., 2003).

For assays targeting the region flanking the cytolysin/hemolysin (*hly*) gene, unique to *V. vulnificus*, reaction mixtures included 0.2 µM dNTPs, 1.0 µM of each primer and 1.25 units of *Taq* polymerase (Pfeffer *et al*., 2003). For assays targeting the siderophore-related *viu*B gene of *V. vulnificus* (a putative virulence gene), reaction mixtures included 0.2 µM dNTPs, 2.0 µM of each primer and 1.25 units of *Taq* polymerase (Panicker *et al*., 2004).

For assays targeting the species-specific 16S-23S intragenic spacer region (ITS) of *V. cholerae*, the *wbe*O and *rfb* genes specific to *V. cholerae* O1 and O139 and the virulence associated *ctx*A (encoding the A subunit of cholera toxin), *tox*R (a global virulence regulatory gene) and *tcp*A (encoding the toxin co-regulated pili), reaction mixtures included 0.2 µM dNTPs, 1.25 µM of each primer and 0.625 units of *Taq* polymerase (Lipp *et al*., 2003).

The necessary volume of sterile nuclease-free PCR water (Fisher Scientific, Pittsburg, PA, USA) was added to produce a 25 µl reaction mixture. A no-template negative control composed of 24 µl of reaction mixture amended with 1 µl of sterile nuclease free PCR water was used in all experiments. Positive controls were composed of 24 µl of reaction mixture amended with 1 µl of purified DNA (~100 ng) from a control culture known to harbor the desired gene target. Amplified products (5 µl) were separated by agarose gel electrophoresis (1.5% [wt/vol] agarose) in 1X tris-acetate EDTA (TAE) electrophoresis buffer. Gels were stained with ethidium bromide (EtBr) and visualized using a KODAK Gel Logic 200 UV trans-illuminator (KODAK, Inc.). All post-amplification work was conducted in a separate dedicated laboratory with a separate air handling system to avoid cross contamination.

PCR was performed sequentially such that all samples were first tested for the presence of species-specific targets and only species-positive samples were subsequently analyzed for the presence of their respective virulence genes. For each sample, the presence of PCR inhibitors was addressed by testing three dilutions (undiluted, 1:10 and 1:20). Detection limits were determined by spiking autoclave-sterilized water and plankton samples with a known concentration of cell stocks (*Vibrio cholerae* O1 ATCC 14035, *V. parahaemolyticus* ATCC 17803 and *V. vulnificus* ATCC 27562). For the species-specific targets, the limit of detection for *V. cholerae* (ITS region) was 10 cells per PCR reaction in the water enrichment and 65 cells per reaction in the plankton enrichments. The limit of detection for *V. parahaemolyticus* (*tl* gene) was 5 cells per PCR reaction in the water enrichment and 10 cells per reaction in the plankton enrichments. The limit of detection for *V. vulnificus* (*hly* gene) was 20 cells per PCR reaction in the water enrichment and 30 cells per reaction in the plankton enrichments.

Data analysis. Samples were recorded as positive for the presence of target genes if any dilution was found positive by PCR. A Tukey's multiple proportions test (MULTPROP.MAC, macro for Minitab) (MINITAB Inc., State College, PA, USA) was used to compare the detection frequencies of target genes between species, fractions and sampling stations. Environmental conditions (temperature, salinity and DO) and the relative abundance of different plankton taxa (copepoda, diatoms and decapoda) were categorized and then the linearity of relationships between the prevalence of gene targets and environmental conditions and the relative abundance of different plankton taxa was evaluated using the Cochran-Armitage Trend Test (PROC FREQ) (SAS Institute Inc., Cary, NC, USA). Environmental conditions were assigned categories based on natural breakpoints in the data [temperature (<15, 15-25 and >25^{o}C), salinity (<27.8 and >27.8) and DO (<71.0, 71.0-86.85, >86.85 % saturation)]. The relative abundances of various

plankton taxa were described in a related study (Turner *et al*., 2009) and that data was also organized into categorical groups based on natural breakpoints in the data [copepoda and diatoms (63-200 µm fraction) (<25, 25-50, 50-75 and >75%), and decapoda (<10, 10-30 and >30%)]. For all tests, significance was declared at $P < 0.05$. All graphs were created in SigmaPlot (Systat Software Inc., San Jose, CA, USA).

RESULTS

Among all samples (water, 63-200 and >200 µm size fractions; n = 210 for all samples combined and n = 70 samples for each fraction), *Vibrio parahaemolyticus* (species-specific thermolabile hemolysin, *tl*-gene) (Bej *et al*., 1999), *V. vulnificus* (species-specific region flanking the cytolysin-hemolysin genes, *hly*-gene) (Coleman and Oliver, 1996) and *V. cholerae* (species-specific 16S-23S rRNA intragenic spacer, ITS region) (Chun *et al*., 1999) exhibited different rates of detection. The prevalence of *V. parahaemolyticus* (81.4%, 171/210) was significantly greater than the prevalence of *V. vulnificus* (37.1%, 78/210), and the prevalence of both *V. parahaemolyticus* and *V. vulnificus* was significantly greater than *V. cholerae* (12.9%, 27/210) (Tukey's test, $P < 0.05$).

V. parahaemolyticus was detected in 97.1% (68/70) of the water samples, 72.9% (51/70) of the 63-200 µm plankton samples and 74.3% (52/70) of the >200 µm plankton samples (Table 4.2). Detection of *V. parahaemolyticus* was significantly greater in the water fraction compared to the two plankton fractions (Tukey's test, $P < 0.05$). Among *V. parahaemolyticus* positive samples, the *trh* gene (thermolabile-related hemolysin) (Bej *et al*., 1999) was detected in 92.6% (63/68) of the water samples, 96.1% (49/51) of the 63-200 µm plankton samples and 84.6% (44/52) of the >200 µm plankton samples (Table 4.2). Among the same samples, the *tdh* gene

(thermolabile-direct hemolysin) (Bej *et al*., 1999) was detected in 88.2% (60/68) of the water samples, 86.3% (44/51) of the 63-200 µm plankton samples and 86.5% (45/52) of the >200 µm plankton samples (Table 4.2). Additionally, the pandemic specific phage-encoded region (ORF8) (Yeung *et al*., 2003) was detected in 57.4% (39/68) the water samples, 45.1% (23/51) of the 63-200 µm plankton samples and 50.0% (26/52) of the >200 µm plankton samples (Table 4.2).

V. vulnificus was detected in 50% (35/70) of the water samples, 38.6% (27/70) of the 63-200 µm plankton samples and 22.9% (16/70) of the >200 µm plankton samples (Table 4.3). Detection of *V. vulnificus* varied significantly between the fractions (Tukey's test, $P < 0.05$). Among *V. vulnificus* positive samples, the siderophore-related *viu*B gene (a putative virulence gene) (Panicker et al., 2004) was detected in 62.9% (22/35) of the water samples, 77.8% (21/27) of the 63-200 µm plankton samples and 81.2% (13/16) of the >200 µm plankton samples (Table 4.3). Detection of the *viu*B gene was significantly higher in the plankton fractions (63-200 and >200 µm) compared to the water fraction (Tukey's test, $P < 0.05$).

V. cholerae was detected in 15.7% (11/70) of the water samples, 18.6% (13/70) of the 63-200 µm plankton samples and 4.3% (3/70) of the >200 µm plankton samples (Table 4.4). Detection of *V. cholerae* was significantly greater in the water and 63-200 µm plankton fraction compared to the >200 µm plankton fraction (Tukey's test, $P < 0.05$). Among *V. cholerae* positive samples, 3 water samples (27.3%, 3/11) were positive for both *ctx*A (encoding the A subunit of cholera toxin) (Fields *et al*., 1992) and *tox*R (a virulence-related global regulatory gene) (Rivera *et al*., 2001) (Table 4.4). Detection of *ctx*A occurred once (7.7%, 1/13), in the 63-200 µm plankton fraction (August 2006); and detection of *tox*R occurred once (33.3%, 1/3), in the >200 µm plankton fraction (June 2006) (Table 4.4). Neither the *tcp*A (toxin co-regulated

pilus) (Rivera *et al*., 2001), *wbe*O (O1 specific protein) (Hoshino *et al*., 1998) or *rfb* genes (O1 and O139-specific proteins) (Hoshino *et al*., 1998; Rivera *et al*., 2003) were detected.

Seasonality. Seasonal changes in physio-chemical parameters (temperature, salinity and DO) were described in a co-occurring study (Turner *et al*. 2009). Temperature was not a significant correlate for the prevalence of total *V. parahaemolyticus* (*tl* gene), which was detected year round, including when water temperatures dropped below 10^{o}C (February 2006) (Table 4.2). In contrast, the prevalence of the virulence associated *trh* and *tdh* genes were directly related to increasing temperatures in the water fraction (Cochran-Armitage, $P < 0.05$), such that 24% of *trh* positive samples were detected when temperatures were <15^{o}C and 76% were detected when temperatures exceeded 25^{o}C (Figure 4.1). Likewise, 20% of *tdh* positive samples were detected when temperatures were < 15^{o}C and 80% were detected when temperatures exceeded 25^{o}C. Additionally, the prevalence of the pandemic-associated ORF8 gene target also increased with temperature in all fractions (water, 63-200 and >200 µm) (Cochran-Armitage, $P < 0.05$) (Figure 4.1). The prevalence of the *trh* and *tdh* genes increased with salinity in the water fraction, such that 44% of *trh* positive samples were detected in the low salinity group (<27.8) and 56% in the high salinity group (>27.8) (Cochran-Armitage, $P < 0.05$). Likewise, 43% of the *tdh* positive samples were detected in the low salinity group and 57% in the high salinity group. The high salinity group also included a higher prevalence of the ORF8 gene target in the water (33% and 67% for low and high salinity, respectively) and the >200 µm fractions (31% and 69% for low and high salinity, respectively) (Cochran-Armitage, $P < 0.05$).

Unlike *V. parahaemolyticus*, *V. vulnificus* was only detected between May 2006 and November 2006, when water temperatures exceeded 15^{o}C (Table 4.3). The prevalence of total *V. vulnificus* (*hly* gene) was greatest when temperatures exceeded 25^{o}C for all fractions (water,

63-200 and >200 µm) (Cochran-Armitage, $P < 0.01$) (Figure 4.2). Likewise, the prevalence of the virulence-associated *viu*B gene was also greatest at higher temperature ranges in all fractions (water, 63-200 and >200 µm) (Cochran-Armitage, $P < 0.01$) (Figure 4.2). The prevalence of both *V. vulnificus* gene targets was similar between the low and high salinity groups but their prevalence was greatest at low DO levels in all fractions (water, 63-200 and >200 µm) (Cochran-Armitage, $P < 0.05$).

Like *V. vulnificus*, *V. cholerae* was only detected between May 2006 and November 2006, when water temperatures exceeded 15^{o}C (Table 4.4). The prevalence of total *V. cholerae* (ITS region) increased with temperature in the water fraction, reaching 23% when temperatures exceeded 25^{o}C (Cochran-Armitage, $P < 0.05$). The prevalence of *V. cholerae* in the 63-200 µm plankton fraction was significantly greater when salinity was >28.7 (9% vs 29%) (Cochran-Armitage, $P < 0.05$), but there was no difference in prevalence between salinities in the other fractions. The prevalence of *V. cholerae* in the 63-200 µm plankton fraction was also highest in among samples with low DO (Cochran-Armitage, $P < 0.05$), but no affect of DO was noted for the other fractions.

Plankton. The prevalence of *V. parahaemolyticus* in the 63-200 µm plankton fraction was found at significantly higher rates with increasing relative abundance of diatoms in the same fraction (Cochran-Armitage, $P < 0.05$), such that prevalence was only 9% among samples with a relative diatom abundance of <25% and 91% when diatom abundance exceeded 75% (Figure 4.4). There was no significant association between prevalence of *V. parahaemolyticus* and other plankton taxa nor where there any significant associations between any plankton taxa in the >200 µm fraction.

Among the 63 – 200 µm plankton fraction, there was no significant association between *V. vulnificus* and plankton taxa. In the >200 µm plankton fraction, *V. vulnificus* (*hly* gene) detection coincided with an increase in the relative abundance of decapods in the >200 µm plankton fraction, such that 8% of *hly* positive samples were detected when decapod abundance was <10% and 59% when abundance exceeded 30% (Cochran-Armitage, $P < 0.05$) (Figure 4.4). There was no association with other taxa in this fraction.

V. cholerae (ITS region) prevalence in the 63 – 200 µm fraction increased with the relative abundance of copepods (Cochran-Armitage, $P < 0.05$) (Figure 4.4). Prevalence ranged from 0% when copepod relative abundance was <25% to 30% when the relative abundance exceeded 75%. CTX was detected to infrequently to discern any trend with plankton taxa or abundance.

DISCUSSION

V. parahaemolyticus, V. vulnificus and *V. cholerae* were prevalent in the shellfish harvesting waters off the coast of Georgia, USA. In general, these species and especially the genes associated with their virulence exhibited distinct seasonal variation attributed to changes in temperature, salinity and DO. Furthermore, increases in the prevalence of *V. parahaemolyticus* and *V. cholerae* coincided with shifts in the composition of the plankton community, cited as a primary reservoir for *Vibrio* species (Huq *et al*., 1983; Lipp *et al*., 2003; Baffone *et al*., 2006).

Consistent with the ecology of many *Vibrio* species, *V. vulnificus* and *V. cholerae* were only detected when mean monthly water temperatures exceeded 15^{o}C (Heidelberg *et al*., 2002b; Thompson *et al*., 2003b; Baffone *et al*., 2006). In contrast, *V. parahaemolyticus* and its virulence-associated genes (*trh, tdh* and ORF8) were detected year-round, even when water

temperatures dropped below 15°C. Although these virulence genes were present year-round, the prevalence of the *trh* gene, *tdh* gene and the phage-encoded ORF8 region (commonly present in pandemic strains) was significantly higher during warmer months, suggesting that warmer temperatures were more selective for virulent strains of this pathogen.

Interestingly, *V. parahaemolyticus* and *V. vulnificus* responded differently to fluctuations in temperature depending upon whether they were free-living or plankton-associated. When temperatures exceeded 15°C, *V. parahaemolyticus* was detected in a larger proportion of water samples compared to plankton samples. Additionally, when water temperatures exceeded 25°C, *V. vulnificus* was detected in a larger proportion of water samples compared to plankton samples. Thus, lower temperatures seem to encourage plankton-association and this finding is supportive of the hypothesis that plankton may constitute a microenvironment protective against extremes in temperature (Thompson *et al*., 2003).

Relationships between salinity and the prevalence of *V. parahaemolyticus* (Kaneko and Colwell, 1977), *V. vulnificus* (Lipp *et al*., 2001; Pfeffer *et al*., 2003) and *V. cholerae* (Louis *et al*., 2003) and have been reported previously; however, these relationships are variable and may be system specific. In this study, the prevalence of the virulence-associated gene targets of *V. parahaemolyticus* and total *V. cholerae* was greater at high salinities, although the analysis was based on only two categories of 'low' (<28.7) and "high" (>28.7) salinity. Significant associations with these salinity categories varied between fractions (water, 63-200 and >200 µm size plankton) and among gene targets within the same species. Stronger or more uniform relationships with salinity may have been precluded by the very narrow salinity range in sampling area, a result of the hydrology of these well-mixed tidal estuaries (Verity *et al*., 2006) and the restriction of sampling to Spring tide events (Turner *et al*. 2009).

While the prevalence of our *Vibrio* gene targets were often related to temperature, salinity and DO, these parameters alone could not account for the complexity of *Vibrio* seasonality in this study. Previous studies have included plankton composition as a variable in order to account for variability among *Vibrio* species that could not be explained by environmental parameters alone (Huq *et al*., 2005; Turner *et al*., 2009).

The inclusion of plankton data revealed that the prevalence of *V. parahaemolyticus* (in the 63-200 µm size plankton) was associated with a high abundance of diatoms in the same fraction, which helped to explain the occurrence of this species especially during cooler months. The association of *V. parahaemolyticus* with diatoms has been reported previously (Kaneko *et al*., 1977; Kumazawa *et al*., 1991) and the formation of diatom blooms can play an important role in the ecology of heterotrophic bacteria, like *Vibrio* species (Cooksey and Wigglesworth-Cooksey, 1995; Riemann *et al*., 2000). Specifically, the production of chitin by some diatoms (Blackwell *et al*., 1967; Herth, 1979) may select for the attachment of *Vibrio* species.

Although temperature was a strong correlate for *V. vulnificus,* a high relative abundance of decapods (>30%) in the >200 µm plankton fraction was also significantly associated with a high prevalence of *V. vulnificus* and in the same fraction. It remains unclear whether the increase in the prevalence of *V. vulnificus* resulted from an increase in temperature or an increase in decapods, given that decapods abundance increases in warm months (Turner et al. 2009). We suggest that temperature, known to be a strong driver of *V. vulnificus* populations (Shapiro *et al.*, 1998; Pfeffer *et al.*, 2003; Randa *et al.*, 2004), was the primary driver in this system. Nonetheless, we hypothesize that decapods could serve as an important reservoir of this pathogen (Krantz *et al.*, 1969; Nogami and Maeda, 1992).

In this study *V. cholerae* prevalence in the 63-200 µm plankton fraction showed a bimodal distribution (peaking in June 2006 and November 2006). The relative maxima in *V. cholerae* prevalence coincided with peaks in the relative abundance of copepods in the 63-200 µm plankton. A natural association between *V. cholerae* and copepods has been reported previously (e.g., Huq *et al*., 1983; Tamplin *et al*., 1990). Chitinous plankton represent potentially effaceable substrates for *Vibrio* colonization (Kumazawa *et al*., 1991; Kirchner *et al*., 1995; Heidelberg *et al*., 2002a; Huq *et al*., 2005), and copepods have received special attention due to their role in the primary transmission of cholera (Colwell *et al*., 2003; Huq *et al*., 2005). The present work suggests that in coastal Georgia, smaller copepods in particular (likely juvenile stages in the 63 – 200 µm fraction) are a likely reservoir for *V. cholerae*. Larger (possibly adult stages in the >200 µm fraction) appear to be less associated with *V. cholerae* populations in these estuaries.

An ecological link between *Vibrio* prevalence and plankton composition could play a potential role in the human exposure and epidemiology of these pathogens. Given that bivalve grazing is a selective process based on size exclusion, clearance rate and plankton taxon (Fritz *et al*., 1984; Dupuy *et al*., 2000; Loret *et al*., 2000; Cognie *et al*., 2001), it follows that plankton harboring *Vibrio* densities higher than the surrounding water column, if selected, could contribute to increased pathogen loading. Thus, the composition of the plankton community could be a factor in the transmission of *Vibrio* species associated with bivalves, such as oysters.

While this study confirms the role of temperature, salinity and DO in *Vibrio* ecology, these results show how warmer temperatures can specifically select for potentially pathogenic *Vibrio* strains. Furthermore, this study suggests that some plankton taxa, such as diatoms and copepods, may be especially important *Vibrio* reservoirs. Together, these results highlight the

complexity of *Vibrio* seasonality and stress both the role of seasonal changes in environmental parameters and seasonal shifts in the composition of the plankton community.

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TABLES

Table 4.1. Primers used for the PCR detection of *Vibrio* species (*V. parahaemolyticus, V. vulnificus* and *V. cholerae*) and select virulence genes associated with each species.

Gene targets	Primers and sequences (5' to 3')	Amplicon Length (bp)	PCR Conditions[a]	References
V. parahaemolyticus tl	thermolabile hemolysin AAACGCGATTATGCAGAAGCACTG GCTACTTTCTAGCATTTTCTCTGC	450	58-2	(Bej et al., 1999)
V. parahaemolyticus trh	thermolabile related hemolysin TTGGCTTCGATATTTTCAGTATCT CATAACAAACATATGCCCATTTCCG	500	58-1	(Bej et al., 1999)
V. parahaemolyticus tdh	thermolabile direct hemolysin GTAAAGGTCTCTGACTTTTGGAC TGGAATAGAACCTTCATCTTCACC	269	58-1	(Bej et al., 1999)
V. parahaemolyticus ORF8	pandemic strain open reading frame GTTCGCATACAGTTGAGG AAGTACAGCAGGAGTGAG	746	50-0.5	(Yeung et al., 2003)
V. vulnificus hly	region flanking the cytolysin/hemolysin genes CGCCGCTCACTGGGGCAGTGGCTG GCGGGTGGTTCGGTTAACGGCTGG	340	64.5-0.5	(Coleman and Oliver, 1996)
*V. vulnificus viu*B	siderophore related gene GGTTGGGCACTAAAGGCAGATATA CGGCAGTGGACTAATACGCAGC	504	56-1	(Panicker et al., 2004)
V. cholerae ITS	16S-23S intragenic spacer TTAAGCSTTTTCRCTGAGAATG AGTCACTTAACCATACAACCCG	310	60-1	(Colwell et al., 1981)
*V. cholerae ctx*A	CT subunit A CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	564	60-1	(Fields et al., 1992)
*V. cholerae tox*R	regulatory operon CCTTCGATCCCCTAAGCAATAC AGGGTTAGCAACGATGCGTAAG	779	60-1	(Rivera et al., 2001)

*V. cholerae tcp*A	toxin co-regulated pilus CACGATAAGAAAACCGGTCAAGAG CGAAAGCACCTTCTTTCACGTTG TTACCAAATGCAACGCCGAATG	451 El Tor 620 Classical	60-1	(Rivera et al., 2001)
V. cholerae O1 *wbe*O	O1 specific protein GTTTCACTGAACAGATGGG GGTCATCTGTAAGTACAAC	192	55-1	(Hoshino et al., 1998)
V. cholerae O1 *rfb*	O1 specific protein TATCTTCTGATACTTTTCTAC CAACAGAATAGACTCAAGAA	647	55-1	(Rivera et al., 2003)
V. cholerae O139 *rfb*	O139 specific protein AGCCTCTTTATTACGGGTGG GTCAAACCCGATCGTAAAGG	449	55-1	(Hoshino et al., 1998)
V. cholerae O139 *rfb*	O139 specific protein CGTTTCGGTAGTTTTTCTGG TTACCAGTCTACATTGCC	741	55-1	(Rivera et al., 2003)

[a]Annealing temperature (^{o}C) and annealing time (minutes)

Table 4.2. The ratio of environmental samples (water, 63-200 and >200 µm plankton) PCR positive for species-specific *tl* gene of *V. parahaemolyticus* and virulence-associated (*trh*, *tdh* and ORF8) gene targets of *V. parahaemolyticus*.

	Number of samples positive for *V. parahaemolyticus* gene targets[a]											
	tl			*trh*			*tdh*			ORF8		
	water	63-200[b]	>200[b]	water	63-200[b]	>200[b]	water	63-200[b]	>200[b]	water	63-200[b]	>200[b]
Jan-06	6/6	6/6	6/6	6/6	6/6	5/6	4/6	6/6	5/6	2/4	1/6	1/6
Mar-06	6/6	2/6	2/6	5/6	2/2	2/2	5/6	2/2	2/2	1/5	0/2	0/2
May-06	5/6	1/6	1/6	4/5	1/1	1/1	5/5	1/1	1/1	2/5	1/1	1/1
June-06	6/6	4/6	6/6	6/6	4/4	6/6	6/6	4/4	6/6	6/6	4/4	5/6
July-06	6/6	4/6	4/6	6/6	4/4	3/4	6/6	4/4	4/4	3/6	2/4	1/4
Aug-06	6/6	4/6	5/6	6/6	4/4	5/5	6/6	4/4	5/5	6/6	3/4	4/5
Sept-06	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	6/6	6/6	3/6	4/6
Oct-06	6/6	6/6	4/6	6/6	6/6	4/4	5/6	5/6	4/4	4/6	3/6	4/4
Nov-06	6/6	6/6	4/6	6/6	6/6	1/4	6/6	5/6	4/4	3/6	3/6	4/4
Dec-06	5/6	5/6	5/6	5/6	5/5	4/5	5/6	4/5	1/5	2/6	1/5	1/5
Jan-07	4/4	2/4	1/4	4/4	2/2	1/1	4/4	2/2	1/1	4/4	1/2	1/1
Feb-07	6/6	5/6	6/6	3/6	3/5	6/6	2/6	3/5	6/6	0/6	1/5	0/6
Total	68/70	51/70	52/70	63/68	49/51	44/52	60/68	44/51	45/52	39/68	23/51	26/52

[a]Species-specific positive samples were subsequently tested for the presence of virulence genes
[b]Size specific plankton fractions in µm

Table 4.3. The ratio of environmental samples (water, 63-200 and >200 µm plankton) PCR positive for species-specific *hly* gene of *V. vulnificus* and virulence-associated *viu*B gene of *V. vulnificus.*

	Number of samples positive for *V. vulnificus* gene targets[a]					
	hly			*viuB*		
	water	63-200[b]	>200[b]	water	63-200[b]	>200[b]
Jan-06	0/6	0/6	0/6	–	–	–
Mar-06	0/6	0/6	0/6	–	–	–
May-06	6/6	6/6	0/6	3/6	4/6	–
June-06	6/6	4/6	3/6	2/6	4/4	1/3
July-06	6/6	5/6	3/6	4/6	3/5	2/3
Aug-06	6/6	5/6	5/6	5/6	4/5	5/5
Sept-06	5/6	3/6	2/6	4/5	2/3	2/2
Oct-06	6/6	2/6	3/6	4/6	2/2	3/3
Nov-06	0/6	2/6	0/6	–	2/2	–
Dec-06	0/6	0/6	0/6	–	–	–
Jan-07	0/6	0/6	0/6	–	–	–
Feb-07	0/6	0/6	0/6	–	–	–
Total	35/70	27/70	16/70	22/35	21/27	13/16

[a]Species-specific positive samples were subsequently tested for the presence of virulence genes
[b]Size specific plankton fractions in µm
– not tested

Table 4.4. The ratio of environmental samples (water, 63-200 and >200 µm plankton) PCR positive for the species-specific ITS (16S-23S rRNA intragenic spacer region) of *V. cholerae* and the virulence-associated (*ctx*A and *tox*R) gene targets of *V. cholerae*.

	Number of samples positive for *V. cholerae* gene targets[a]								
	ITS			*ctx*A			*tox*R		
	water	63-200[b]	>200[b]	water	63-200[b]	>200[b]	water	63-200[b]	>200[b]
Jan-06	0/6	0/6	0/6	–	–	–	–	–	–
Mar-06	0/6	0/6	0/6	–	–	–	–	–	–
May-06	2/6	1/6	0/6	1/2	0/1	–	1/2	0/1	–
June-06	2/6	5/6	1/6	0/2	0/5	0/1	0/2	0/5	1/1
July-06	2/6	2/6	1/6	0/2	0/2	0/1	0/2	0/2	0/1
Aug-06	2/6	2/6	1/6	0/2	1/2	0/1	0/2	0/2	0/1
Sept-06	0/6	1/6	0/6	–	0/1	–	–	0/1	–
Oct-06	1/6	0/6	0/6	1/1	–	–	1/1	–	–
Nov-06	2/6	2/6	0/6	1/2	0/2	–	1/2	0/2	–
Dec-06	0/6	0/6	0/6	–	–	–	–	–	–
Jan-07	0/6	0/6	0/6	–	–	–	–	–	–
Feb-07	0/6	0/6	0/6	–	–	–	–	–	–
Total	11/70	13/70	3/70	3/11	1/13	0/3	3/11	0/13	1/3

[a]Species-specific positive samples were subsequently tested for the presence of virulence genes
[b]Size specific plankton fractions in µm
– not tested

FIGURES

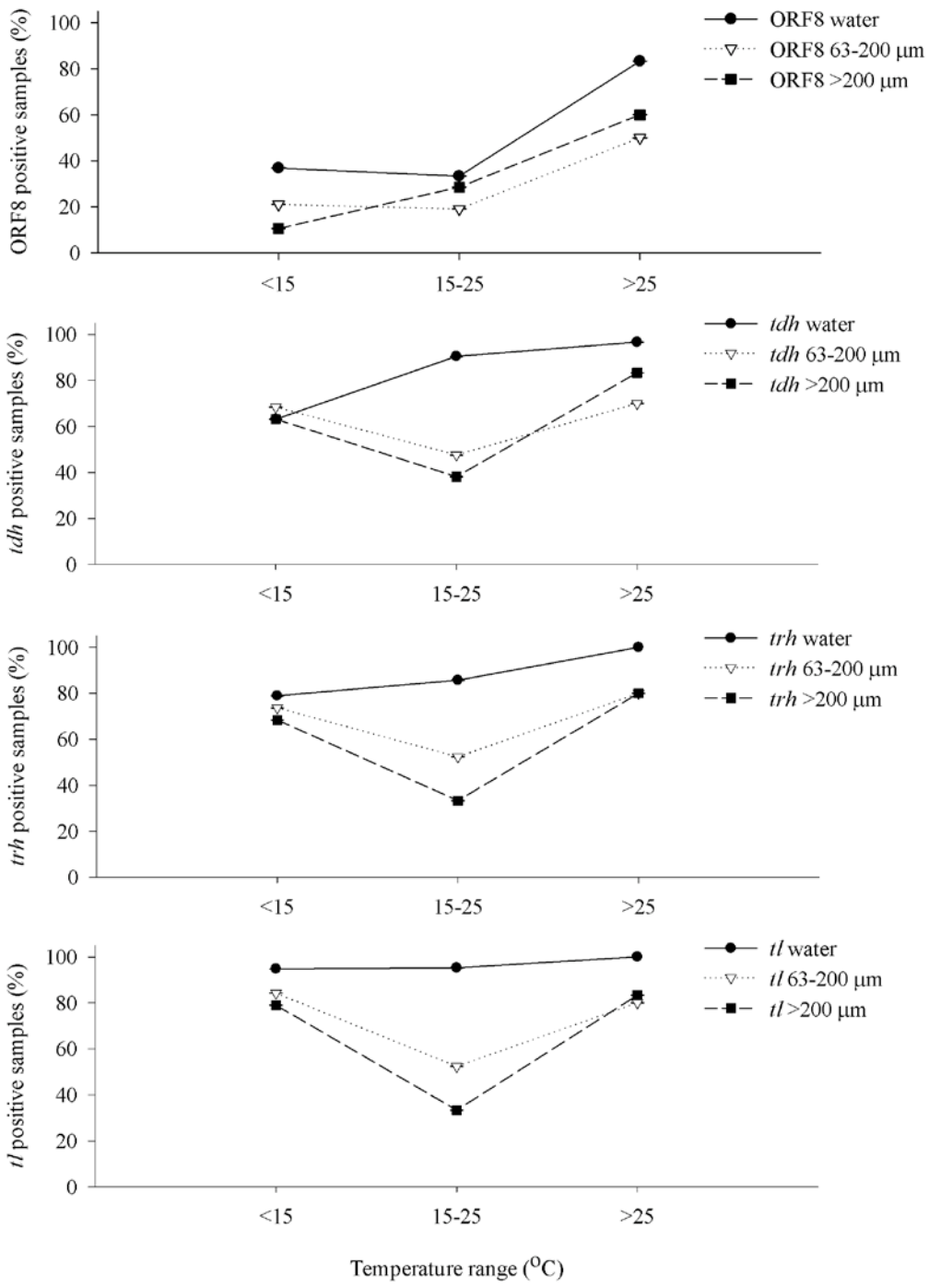

Figure 4.1. Association between the prevalence of *V. parahaemolyticus* (ORF8, *tdh*, *trh* and *tl* gene targets) and surface water temperature ranges.

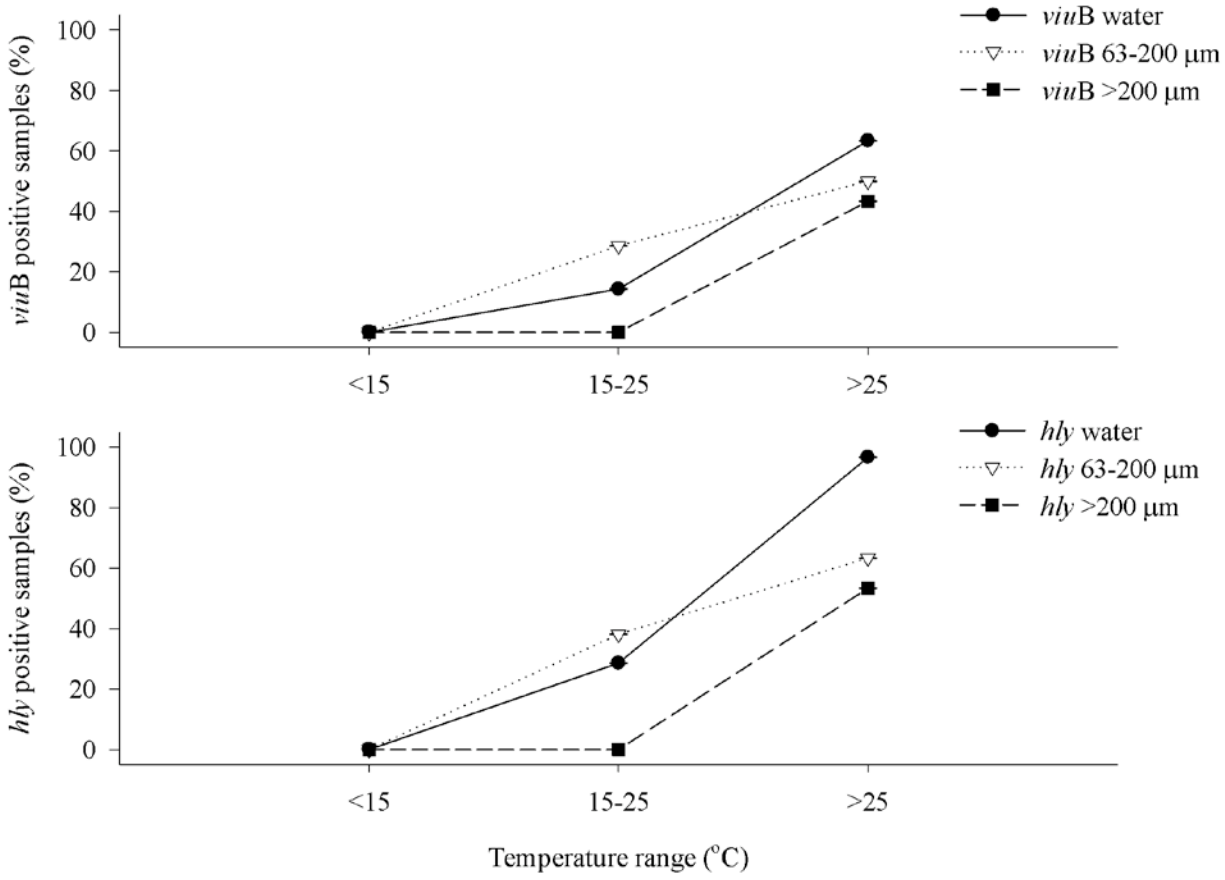

Figure 4.2. Association between the prevalence of *V. vulnificus* (*viu*B and *hly* gene targets) and surface water temperature ranges.

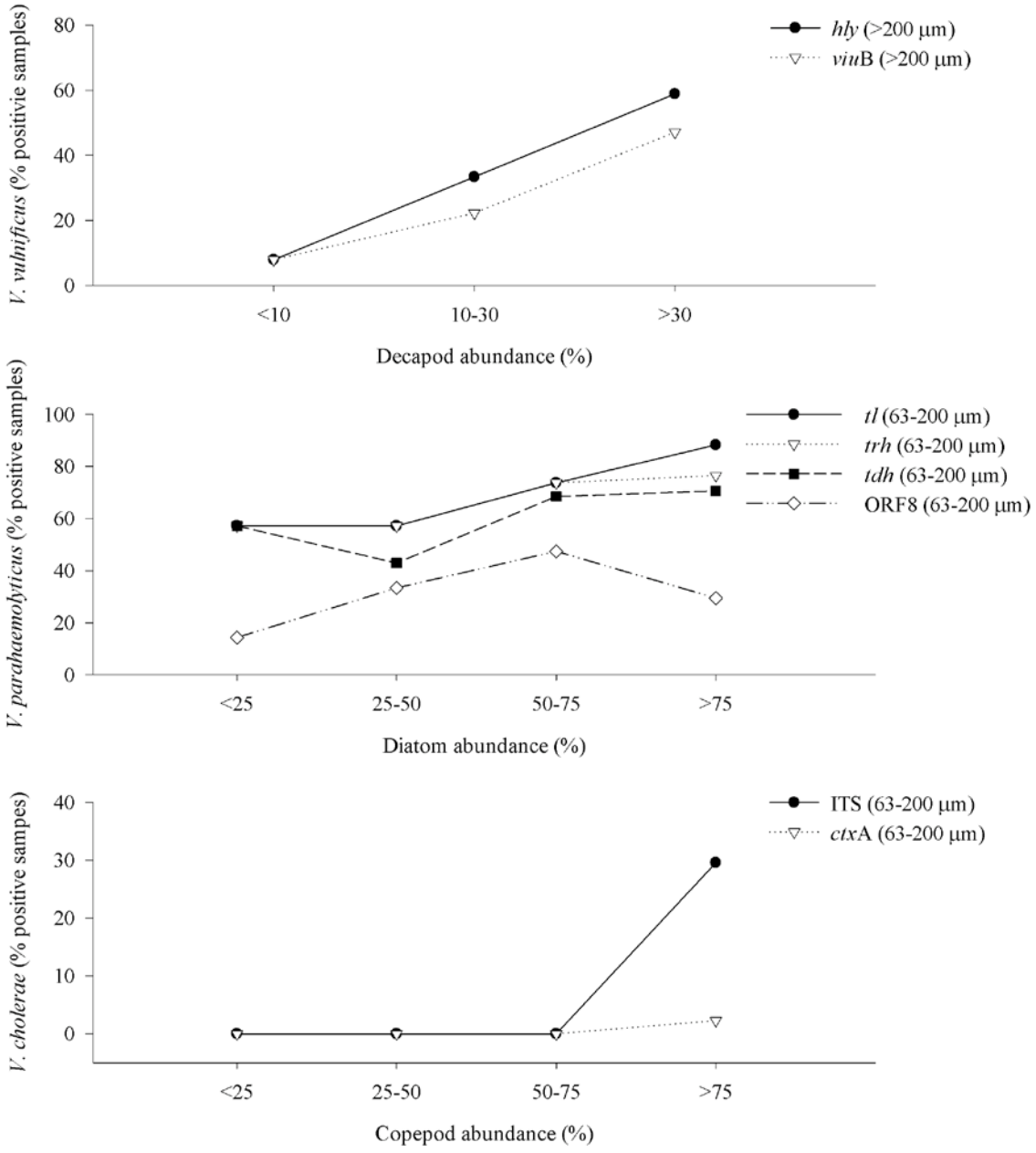

Figure 4.3. Relationship between the detection frequency of, *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* and ranges in the mean relative abundance of decapods, diatoms and copepods, respectively.

CHAPTER 5

DISTRIBUTION OF PATHOGENIC *VIBRIO* SPECIES IN AN ESTUARY DOMINATED BY AN ABUNDANCE OF DETRITAL PARTICULATE ORGANIC MATTER

Turner JW, Malayil L, Mote B, Peck A, Cole D, Lipp EK. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Vibrio is a genus of heterotrophic bacteria, which occur naturally in marine coastal and estuarine systems. Several members of the genus are opportunistic pathogens of humans and in particular, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are significant causes of water-borne and seafood-borne illness worldwide. Among these pathogens, the prevalence of *V. cholerae* and the incidence of cholera in endemic regions demonstrate periodicity with phytoplankton blooms and the abundance of copepods. In a 6-month environmental study, we investigated the prevalence of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in water samples and plankton samples collected from Skidaway River along the tidal reaches of Georgia, USA. We investigated relationships between *Vibrio* prevalence and the abundance of living and non-living particulate organic matter (POM), including the taxonomic composition of plankton among multiple size fractions. A significant proportion of *Vibrio* were detected from bulk water, suggesting that free-living (i.e., not specifically particle-attached) were an important source of pathogenic species.. Detection of pathogenic species by polymerase chain reaction showed that *V. cholerae* exhibited a bimodal distribution, which co-occurred with increases in detrital POM in April and September. This study contrasts the free-living and particle-associated strategies of pathogenic *Vibrio* in an estuary characterized by a high detrital load. We propose that detrital POM is critical to *Vibrio* dynamics and suggest that the further characterization of *Vibrio*-detrital dynamics could lead to the improvement of future *Vibrio* predictive models.

INTRODUCTION

Bacteria of the *Vibrio* genus exist as a natural heterotrophic component of marine and estuarine systems worldwide (Colwell *et al.*, 1977; Thompson *et al.*, 2003a). Although most *Vibrio* species persist as innocuous environmental microbes, at least 12 species, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are significant pathogens of humans and marine animals (Colwell and Grimes, 1984; Daniels *et al.*, 2000; Cottingham *et al.*, 2003; Levin, 2005). Key to the prevalence and distribution of *Vibrio* species is the ability to attach to and associate with suspended particulate organic matter (POM; living and non-living) (Montanari *et al.*, 1999; Maugeri *et al.*, 2004; Turner *et al.*, 2009). Association with POM is hypothesized to afford access to nutrient-rich microenvironments and protection from extreme fluctuations in environmental conditions (Huq *et al.*, 1983; Thompson *et al.*, 2003a; Huq *et al.*, 2005). To date, much work has focused on the association of pathogenic *Vibrio* species with pelagic plankton – especially copepods; however, the complex dynamics of how these pathogens proliferate in the marine environment remains poorly understood.

During periods of temperature and nutrient limitation, *Vibrio* often comprise less than 1% of the marine bacterioplankton (Thompson *et al.*, 2003a; Thompson *et al.*, 2003b); however, during periods of optimal growth conditions, *Vibrio* can comprise a significant proportion of the bacterioplankton community (Jiang and Fu, 2001; Worden *et al.*, 2006). Commonly, the abundance of *Vibrio* and the risk of *Vibrio*-related illness are linked to the temperature-driven seasonal prevalence of pathogenic *Vibrio* species (Huq *et al.*, 1984; Kaspar and Tamplin, 1993). Additionally, periods of intense nutrient availability, such as phytoplankton blooms, can support explosive *Vibrio* growth and have also been linked to outbreaks of *Vibrio*-related illness (Huq and Colwell, 1996; Huq *et al.*, 2005). For example, cholera outbreaks in endemic regions are

known to exhibit periodicity with phytoplankton blooms (Huq *et al.*, 2005; Worden *et al.*, 2006). Chitinous zooplankton, such as copepods, which are especially abundant during late phytoplankton blooms, when heavily colonized by pathogenic *V. cholerae*, can then serve as a vector for the primary transmission of cholera (Tamplin *et al.*, 1990; Huq *et al.*, 2005).

Phytoplankton blooms commonly result in a significant localized increase in dissolved organic matter (DOM) and POM (Pomeroy and Wiebe, 2001). Often, the mass of detrital or non-living particulate organic matter can be significantly greater than the mass of living matter (plankton) suspended in the marine water column (Pomeroy and Wiebe, 2001; Verity, 2002). Heterotrophic bacteria, such as *Vibrio* species, play an important role in the degradation and cycling of detrital POM (Fukami *et al.*, 1981; Fukami *et al.*, 1985; Azam *et al.*, 1994; Riemann *et al.*, 2000). Further, a direct link has been observed between phytoplankton blooms and the rapid growth of pathogenic *Vibrio* species (Mourino-Perez *et al.*, 2003; Rehnstam-Holm *et al.*2010;).

The occurrence of phytoplankton blooms and environmental concentrations of DOC and POM can serve as useful predictors of *Vibrio*-related outbreaks (Mourino-Perez *et al.*, 2003), and models describing bacteria-phytoplankton bloom or POM interactions are needed to better understand the complex dynamics and conditions that give rise to *Vibrio* outbreaks (Rehnstam-Holm *et al*., 2010). The objective of this study was to characterize the growth and distribution of co-occurring *Vibrio* species in a coastal estuary characterized by an abundance of detrital POM.

MATERIALS AND METHODS

Sample collection. Monthly surface water and plankton samples were collected from tidal reaches of the Skidaway River at the Skidaway Institute of Oceanography (SKIO), Savannah, Georgia, USA (Figure 5.1). The collection period (April 2008 to November 2008) was limited to months when environmental conditions were sufficient for the growth and proliferation of *Vibrio* species ($\geq 15^{o}$C). Briefly, 5 size-specific plankton samples were collected by pumping up to 100-liters of surface water (< 1-m depth) through a series of nylon plankton nets (30-63, 63-105, 105-150, 150-200 and 200-250 µm) (Aquatic Ecosystems, Apopka, FL, USA) using a low-capacity positive-displacement pump (Teledyne ISCO, Lincoln, NE, USA). The size fractionation was an attempt to simplify the complexity of our plankton catches and better isolate plankton types and life stages. A 1-liter bulk surface water sample was also collected from the same location. Size-fractionated plankton samples were transferred into sterile 200-ml tall-form beakers and suspended in 200 ml of phosphate-buffered saline (PBS). Water temperature (^{o}C), salinity, dissolved oxygen (DO) (% saturation) were recorded for each sampling date using a YSI Multi-meter (YSI Environmental, Model 556, Yellow Springs, OH, USA).

Plankton identification. One 25-ml aliquot of each plankton sample was preserved in 4% (v/v) formaldehyde (final concentration) and stored at 4^{o}C in a 50-ml conical tube. Within 2 weeks of collection, plankton were removed from the fixative solution and suspended in 70% (v/v) ethanol for long-term storage. For each sample, the concentration of plankton (ml^{-1} of resuspended fraction) was determined to a lowest taxonomical unit (LTU). For phytoplankton, organisms were identified by class (diatoms [*Bacillariophyceae*]) and phylum (green algae [*Chlorophyta]*). For zooplankton, organisms were identified by subclass (copepods [*Copepoda*])

and barnacle larvae [*Theconstraca*]); class (ostracods [*Ostracoda*]); order (cladocerans [*Cladocera*] and amphipods [*Amphipoda*]); and phylum (forams [*Foraminifera*] and segmented worms [*Annelida*]). All non-living particulate organic matter, such as fecal pellets, zooplankton molts, and non-identifiable organic particles were characterized as detritus.

Enumeration of presumptive *Vibrio* species. Culturable *Vibrio* species were grown on a *Vibrio*-selective medium (thiosulfate citrate bile sucrose agar [TCBS], OXIOD CM0333, Basingstoke, Hampshire, UK), as described previously (Turner *et al*., 2009). Briefly, a 10 ml aliquot (collected with a 10 ml Stimple pipette) of each bulk water and resuspended plankton sample (suspended in PBS) was utilized for enumeration. Plankton samples were homogenized for 1 min at 12,000 rpm (PRO Homogenizer, PRO Scientific, Model 200, Oxford, CT, USA). Water and plankton samples were then serially diluted in PBS before spread plating (100 µl) in duplicate on TCBS plates (100 mm). Plates were grown in the dark at 37^{o}C for 16-20 h and all yellow and green colonies were reported as colony-forming units (CFUs) of presumptive *Vibrio* species ml^{-1} of water and ml^{-1} of concentrated and resuspended plankton catch.

Enrichment and DNA purification. A 100-ml aliquot of each water sample was filtered onto a sterile 0.22 µm polycarbonate membrane (Millipore, GTTP04700, Billerica, MA, USA) and washed twice with 5 ml of PBS. Filters were then added to sterile 50 ml tubes containing 30 ml of alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.4). A 25-ml aliquot of each resuspended plankton sample (collected with a 25 ml Stimple pipette) was added directly to 25 ml of 2 X APW (1X final concentration). Water and plankton enrichments were then incubated in the dark with shaking (100 rpm) at 37^{o}C for 16-20 hours.

Post enrichment, 5 ml of each enrichment was pelleted by centrifugation (2,450 x *g*, 10 minutes at 4^{o}C) (Thermo IEC, Marathon 2100R, Needham, MA, USA) and washed twice with 1

ml of PBS. Total DNA was then extracted from the pelleted cells using a DNeasy Tissue kit (Qiagen Sciences, Valenica, CA, USA) as per the manufacturer's protocols. DNA was eluted with 50 µl of sterile nuclease-free PCR water (Fisher Scientific, Pittsburg, PA, USA), quantified with a UV spectrophotometer (Eppendorf BioPhotometer, Model 6131, Hamburg, Germany), diluted to ~100 ng μl^{-1} and stored at –80^{o}C.

PCR detection. Primers targeting species-specific and virulence-associated genes of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were described previously (Chapter 4 of this dissertation). PCR was performed using master mix concentrations and reaction conditions described previously (Chapter 4 of this dissertation).

Data analysis. Concentrations of culturable *Vibrio* species and concentrations of POM (living and non-living) were log-transformed to fit a normal distribution (Anderson-Darling statistic, $\alpha = 0.1$). Relationships between physico-chemical paramaters (temperature, salinity and dissolved oxygen [DO]), culturable *Vibrio* counts and concentrations of plankton taxa (also referred to generally as living POM with concentrations determined separately for each LTU described above) and detrital POM were evaluated using the Pearson correlation coefficient. Relationships between environmental conditions and culturable *Vibrio* counts were also analyzed using multiple linear regression models incorporating temperature, salinity and DO (with backwards elimination, $\alpha = 0.1$, $F = 4$). One-way analysis of variance (ANOVA) was used to determine differences between mean concentrations of *Vibrio*, plankton concentration and detrital POM among each fraction or sampling date ($P < 0.05$). For all tests, significance was declared when $P < 0.05$. All analyses were carried out in MINITAB (MINITAB Inc., Version 15.0, State College, PA, USA).

RESULTS

Environmental parameters. Among the 6 sampling events, the temperature, salinity and DO (% saturation) of surface water samples ranged from 16.2 to 29.7^{o}C, 24.3 to 30.6, 51.8 to 102.4%, respectively (Figure 5.2). Temperature was directly correlated with salinity ($r = 0.56$) and indirectly correlated with DO ($r = -0.92$). DO was likewise indirectly related to salinity ($r = -0.73$) ($P < 0.001$ for each).

Analysis of plankton composition among fractions. Plankton fractions were comprised of both living and non-living particulate organic matter. The mean concentration (per size fraction) of living and non-living POM showed considerable variation; however, detritus was the primary component of each plankton tow (Table 5.1). In general, the mean concentration of POM in each fraction decreased with increasing size (µm) of fraction (Table 5.1). The mean concentration of detritus peaked at 4,696 particles ml^{-1} in the 30-63 µm size fraction and 175 particles ml^{-1} in the 63-105 µm size fraction. Similarly, the mean concentration of diatoms peaked at 608 ml^{-1} in the 30-63 µm fraction and 280 ml^{-1} in the 63-150 µm size fraction (Table 5.1). The mean concentration of copepod nauplii peaked at 33 ml^{-1} in the 63-105 µm fraction and the mean concentration of copepodite and adult copepods peaked at 5 copepods ml^{-1} in the 64-105, 105-150 and 150-200 µm fractions (Table 5.1). There was no correlation between plankton taxa or detritus concentrations in any fraction and the measured physico-chemical parameters (temperature, salinity and DO).

Analysis of culturable *Vibrio*. Concentrations of presumptive *Vibrio* species by culture on TCBS showed considerable variation between sampling dates and between plankton size fractions (Figure 5.3). *Vibrio* concentrations in the water fraction ranged from 10 CFU ml^{-1} (November 12, 2008) to 930 CFU ml^{-1} (July 29, 2008). In the 30-63 µm fraction, concentrations

ranged from 192 CFU ml^{-1} (July 8, 2008) to 20,500 CFU ml^{-1} (July 29, 2008). In the 63-105 µm fraction, concentrations ranged from 180 CFU ml^{-1} (November 12, 2008) to 25,000 CFU ml^{-1} (July 29, 2008). In the 105-150 µm fraction, concentrations ranged from 50 CFU ml^{-1} (June 10, 2008) to 6,500 CFU ml^{-1} (July 29, 2008). In the 150-200 µm fraction, concentrations ranged from 2 CFU ml^{-1} (July 8, 2008) to 1,780 CFU ml^{-1} (July 29, 2008). In the 200-250 µm fraction, concentrations ranged from 0 CFU ml^{-1} (July 8, 2008) to 210 CFU ml^{-1} (July 29, 2008). In general, concentrations remained below 1,000 CFU ml^{-1} throughout the study period; however, concentrations exhibited a significant increase in all fractions in late July when concentrations peaked in all fractions (ANOVA, $P < 0.05$) (Figure 5.3). *Vibrio* concentrations were not significantly related to surface water temperature, salinity or DO over this 6 month period (Pearson's correlation coefficient and multiple linear regression).

Among the plankton fractions, mean *Vibrio* concentrations showed an approximately decreasing linear trend with increasing size fraction (4,364 CFU ml^{-1} in the 30-63 µm fraction, 4,500 CFU ml^{-1} in the 63-105 µm fraction, 1,269 CFU ml^{-1} in the 105-150 µm fraction, 411 CFU ml^{-1} in the 150-200 µm fraction and 99 CFU ml^{-1} in the 200-250 µm fraction) (Figure 5.4). Mean *Vibrio* concentrations (per size fraction) were directly correlated with the mean abundance (per size fraction) of detritus ($r = 0.93$) and diatoms ($r = 0.97$) ($P < 0.01$ for each). Since detritus and diatoms were the primary component of the plankton tows, *Vibrio* concentrations (per size fraction) were also directly related to the mean concentrations of total living POM (plankton) ($r = 0.99$) and total POM (living and non-living) ($r = 0.97$) (per size fraction) (Pearson's correlation coefficient, $P < 0.01$ for each).

Analysis of prevalence of pathogenic *Vibrio* species. Among all fractions during the study period (N = 36, 6 environmental fractions and 6 months), the detection frequency of *V.*

parahaemolyticus (*tl* gene) (34/36 samples, 94.44%) was significantly greater than *V. vulnificus* (*hly* gene) (15/36 samples, 41.67%) and *V. cholerae* (ITS region) (13/36 samples, 36.11%); however, there was no significant difference in the detection frequency between *V. vulnificus* and *V. cholerae* (Tukey's test, $P < 0.01$). The virulence-associated *trh* (23/36 samples, 63.89%), *tdh* (28/36 samples, 77.78%) and ORF8 (19/36 samples, 52.78%) genes of *V. parahaemolyticus* were detected frequently. In contrast, the virulence-associated *ctx*A (9/36 samples, 25.00%) and *tox*R (5/36 samples, 13.89%) genes of *V. cholerae* were detected infrequently. Likewise, the virulence-associated *viu*B (6/36 samples, 16.67%) gene of *V. vulnificus* was detected infrequently.

Among plankton fractions, *V. cholerae* detection ranged from 16.67% (1/6) in the 150-200 and 200-250 µm fractions to 66.67% (4/6) in the water fraction (Table 5.2). Detection of *ctx*A gene (encoding the A subunit of cholera toxin) ranged from not detectable (0/6) in the water and 200-250 µm fractions to 67.67% (4/6) in the 30-63 µm fractions (Table 5.2). The detection of *tox*R gene (encoding a global regulatory protein) ranged from not detectable (0/6) in the 30-63, 63-105, 150-200 and 200-250µm fractions to 50% (3/6) in the water fractions (Table 5.2). The mean detection frequency of *V. cholerae* by sampling date ranged from non detectable (0/6) in November 2008 to 100% (6/6) in April 2008 (Table 5.3). The mean detection frequency of *ctx*A ranged from 0% (0/6) in September and November 2008 to 50.0% (3/6) in late-July 2008 (July 29th) (Table 5.3). The mean detection frequency of *tox*R ranged from 0% (0/6) in June, late-July and November 2008) to 50% (3/6) in April 2008 (Table 5.3). Compared to other dates, the mean monthly detection frequency of *V. cholerae* was significantly higher in April 2008 (100%, 6/6) and September 2008 (67.67%, 4/6) than the other months of the study ($P < 0.05$).

Similarly, the mean monthly detection frequency of *tox*R was significantly higher in April 2008 (50%, 3/6) ($P < 0.05$).

V. parahaemolyticus (species-specific *tl* gene) detection ranged from 83.33% (5/6) in the 30-63 and 200-250 µm fractions to 100% (6/6) in the water, 63-105, 105-150 and 150-200 µm fractions (Table 5.2). Detection of the *trh* gene (thermolabile related hemolysin) ranged from 33.33% (2/6) in the 150-200 and 200-250 µm fractions to 100% (6/6) in the water and 30-63 µm fractions (Table 5.2). Detection of the *tdh* gene (thermolabile direct hemolysin) ranged from 50.0% (3/6) in the 105-150 and 150-200 µm fractions to 100% (6/6) in the water, 30-63 and 63-105 µm fractions (Table 2). Detection of the ORF8 region (pandemic specific open reading frame) ranged from 33.33% (2/6) in the 63-105 µm fractions to 67.67% (4/6) in the water and 30-63 µm fractions (Table 5.2). The mean monthly detection frequency of *V. parahaemolyticus* ranged from 83.33% (4/6) in April and early-July (July 8th) 2008 to 100% (6/6) in June, late-July, September and November 2008 (Table 5.3). The mean detection frequency of *trh* ranged from 33.33% (2/6) in June 2008 to 83.33% (4/6) in late-July and September 2008 (Table 5.3). The mean detection frequency of *tdh* ranged from 50% (3/6) in November 2008 to 100% (6/6) in June 2008 (Table 5.3). The mean monthly detection frequency of ORF8 ranged from 0% (0/6) in November 2008 to 83.33% (5/6) in April 2008 (Table 5.3).

V. vulnificus detection ranged from 0% (0/6) in the 150-200 µm fractions to 100% (6/6) in the water sample fractions (Table 5.2). Detection of the *viu*B gene (virulence related siderophore) ranged from 0% (0/6) in the 63-105 and 105-150 µm fractions to 33.33% (2/6) in the water and 30-63 µm fractions (Table 5.2). The mean detection frequency of *V. vulnificus* ranged from 33.33% (2/6) in April, early-July, September and November 2008 to 67.67% (4/6)

in late-July 2008 (Table 5.3). The mean detection frequency of *viu*B ranged from 0% (0/6) in early-July and November 2008 to 50% (3/6) late-July (Table 5.3).

DISCUSSION

By definition, investigations of seasonality typically encompass at least one annual cycle. If such an investigation takes place in a temperate region where temperatures drop below 15^{o}C for an extended period of time, *Vibrio* concentrations will often be lower than the limit of detection. Thus, seasonal trends (i.e., correlations between concentrations and temperature) can be strengthened by the absence of detection. Perhaps a more interesting question is how *Vibrio* dynamics change during periods when environmental conditions (i.e., temperature and salinity) are sufficient to support the growth of *Vibrio* species. In the temperate South Atlantic Bight, this time period begins in late March or early April and extends through November, during which surface water temperatures commonly exceed 15^{o}C (Stegmann and Yoder, 1996; Barnard *et al.*, 1997). In the estuaries along the coastal reaches of Georgia, this time period also commonly captures the occurrence of a Spring phytoplankton bloom (March and or April) and a Fall phytoplankton bloom (September and or November) (Walsh *et al.*, 1987; Dame *et al.*, 2000).

The Skidaway River is part of a tidally dominated estuary, located along the South Atlantic Bight off the coast of Georgia, USA. Due to the surrounding *Spartina alterniflora* marsh and tidal dynamics, detritus comprises a significant proportion of the suspended POM in this system (Verity *et al.*, 1998; Dame *et al.*, 2000; Odum, 2000). In this study, a significant proportion of this suspended detrital POM fell into the 30-63 and 63-105 µm size class. In general, cultuarable counts of presumptive culturable *Vibrio* species were directly related to the concentration of detrital POM in each fraction. Thus, the larger size fractions, which contained

significantly less detrital POM, contributed less to the prevalence of culturable *Vibrio* species in this system.

A difficulty in the quantification and characterization of detrital POM is the inherent complexity of POM in the marine environment. POM is a heterogeneous matrix of suspended and sinking particles both living and non-living. The non-living portion or detrital POM can consist of dead cells, dead plankton, zooplankton fecal pellets, marine snow, marine aggregates, zooplankton molts and additional various particles (Verity, 2002). These particles play a vastly important role in the rise and decline of heterotrophic bacterial populations as the biomass of bacteria attached to these particles can exceed the biomass of the detrital POM (Pomeroy and Wiebe, 1988).

In this study, we quantified POM as the concentration of particles or organisms ml^{-1} of resuspended plankton catch. The characterization of these particles was accomplished through the identification of major taxonomical groups of plankton while all non-living matter was lumped into the category of detrital POM. Future studies would benefit from the determination of the mass of both living and non-living POM and a finer scale of detrital POM identification. For this purpose, previous studies have employed nucleic acid staining followed by flow cytometry (Minor and Nallathamby, 2004).

Vibrio species are often described as free-living and POM-associated and that distinction is of importance to the ecology of the *Vibrio* genus (Heidelberg *et al.*, 2002; Maugeri *et al.*, 2004; Baffone *et al.*, 2006; Turner *et al.*, 2009). Previous work has suggested that the ability to associate with POM is a primary mechanism by which *Vibrio* persist and survive in the marine environment (Thompson *et al.*, 2003b; Worden *et al.*, 2006). However, *Vibrio* can switch easily from the free-living lifestyle to the POM-associated lifestyle and back again (Worden *et al.*,

2006). In this study, pathogenic *Vibrio* species were detected frequently as free-living. In previous studies, the occurrence of phytoplankton blooms and the prodigious quantities of DOC produced during the decline of a bloom have been shown to support a shift toward free-living heterotrophic bacteria (Eilers *et al.*, 2000; Eiler *et al.*, 2006; Eiler *et al.*, 2007). For example, Worden *et al*. (2006) demonstrated that the concentrations of DOC, produced during a bloom, supported the growth and proliferation of free-living *V. cholerae* in excess of removal processes (protozoan predation and viral lysis).

Interestingly, only *V. cholerae* exhibited a significant trend during the study period. The detection of *V. cholerae* peaked in April and again in September giving rise to a bimodal distribution. In this study, the peaks of this bimodal distribution coincided with peaks in the abundance of diatoms and detrital POM in April and September. It is probable that the increased abundance of diatoms and detritus coincided with Spring (April) and Fall (September) phytoplankton blooms. Neither *V. parahaemolyticus* or *V. vulnificus* exhibited a similar bimodal distribution associated with bloom-like conditions. Thus, compared to *V. parahaemolyticus* and *V. vulnificus, V. cholerae* may be especially well-adapted for growth during periods of intense nutrient concentrations.

This study characterized the growth and distribution of total culturable *Vibrio* and pathogenic *Vibrio* species in an estuary dominated by detrital POM. Not surprisingly, total *Vibrio* concentration was strongly associated with the mean abundance of suspended detrital POM as well as the mean abundance of diatoms. Furthermore, peaks in the prevalence of *V. cholerae* (April and November) coincided with peaks in the mean abundance of detrital POM and diatoms. Given the periodicity of phytoplankton blooms in the region of our study site, we propose the increased concentrations of total *Vibrio*, *V. cholerae*, detrital DOM and diatoms was

associated with the occurrence of a Spring and a Fall phytoplankton bloom. Thus, phytoplankton blooms and the resultant increases in detrital POM may contribute to the temporal distribution of *V. cholerae* in this ecosystem.

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TABLES

Table 5.1. The composition of plankton fractions (number of particles or organisms ml^{-1} and mean abundance %) of living and non-living POM averaged over the study period.

	Plankton Size Fraction (µm of net tow)				
Plankton type	30-63	64-105	105-150	150-200	200-250
Detritus	4696	175	19	6	3
	88	34	35	46	62
Diatoms	608	280	16	< 1	0
	11	54	30	< 1	0
Copepoda (nauplii)	5	33	12	< 1	< 1
	< 1	6	22	6	3
Copepoda	0	5	5	5	1
	0	< 1	9	38	14
Cyanobacteria	0	9	< 1	< 1	< 1
	0	2	1	3	8
Foraminfera	0	2	0	0	0
	0	< 1	0	0	0
Polychaeta	0	0	< 1	< 1	< 1
	0	0	1	1	< 1
Sessilia (barnacle larvae)	0	0	< 1	< 1	< 1
	0	0	1	3	< 1
Cladocera	0	< 1	< 1	0	< 1
	0	< 1	< 1	0	< 1
Ostracoda	0	< 1	< 1	< 1	< 1
	0	< 1	< 1	< 1	< 1
Amphipoda	0	0	0	0	< 1
	0	0	0	0	< 1
Annelida	0	0	0	0	< 1
	0	0	0	0	< 1
Other	17	16	< 1	< 1	< 1
	< 1	3	< 1	3	9
Total %	5325	521	53	13	4
	100	100	100	100	100

Table 5.2. The detection frequency of target *Vibrio* species and select virulence genes (ratio and percent of PCR positive samples) per environmental fraction (water samples and plankton size fractions).

Gene target	water	30-63 µm	63-105 µm	105-150 µm	150-200 µm	200-250 µm
V. cholerae ITS	4/6 66.67%	2/6 33.33%	2/6 33.33%	2/6 33.33%	1/6 16.67%	1/6 16.67%
*V. cholerae ctx*A	0/6 0.00%	4/6 66.67%	2/6 33.33%	2/6 33.33%	1/6 16.67%	0/6 0.00%
*V. cholerae tox*R	3/6 50.00%	0/6 0.00%	0/6 0.00%	1/6 16.67%	0/6 0.00%	0/6 0.00%
V. parahaemolyticus tl	6/6 100.00%	5/6 83.33%	6/6 100.00%	6/6 100.00%	6/6 100.00%	5/6 83.33%
V. parahaemolyticus trh	6/6 100.00%	6/6 100.00%	4/6 66.67%	3/6 50.00%	2/6 33.33%	2/6 33.33%
V. parahaemolyticus tdh	6/6 100.00%	6/6 100.00%	6/6 100.00%	3/6 50.00%	3/6 50.00%	4/6 66.67%
V. parahaemolyticus ORF8	4/6 66.67%	4/6 66.67%	2/6 33.33%	3/6 50.00%	3/6 50.00%	3/6 50.00%
V. vulnificus cty	6/6 100.00%	5/6 83.33%	3/6 50.00%	1/6 16.67%	0/6 0.00%	0/6 0.00%
*V. vulnificus viu*B	2/6 33.33%	2/6 33.33%	0/6 0.00%	0/6 0.00%	1/6 16.67%	1/6 16.67%

Table 5.3. The detection frequency of target *Vibrio* species and select virulence genes (ratio and percent of PCR positive samples) per sampling date.

Gene target	Apr-29	Jun-10	Jul-8	Jul-29	Sep-27	Nov-12
V. cholerae ITS	6/6 100.00%	1/6 16.67%	1/6 16.67%	1/6 16.67%	4/6 66.67%	0/6 0.00%
*V. cholerae ctx*A	2/6 33.33%	2/6 33.33%	2/6 33.33%	3/6 50.00%	0/6 0.00%	0/6 0.00%
*V. cholerae tox*R	3/6 50.00%	0/6 0.00%	1/6 16.67%	0/6 0.00%	1/6 16.67%	0/6 0.00%
V. parahaemolyticus tl	5/6 83.33%	6/6 100.00%	5/6 83.33%	6/6 100.00%	6/6 100.00%	6/6 100.00%
V. parahaemolyticus trh	4/6 66.67%	2/6 33.33%	4/6 66.67%	5/6 83.33%	5/6 83.33%	3/6 50.00%
V. parahaemolyticus tdh	4/6 66.67%	6/6 100.00%	5/6 83.33%	5/6 83.33%	5/6 83.33%	3/6 50.00%
V. parahaemolyticus ORF8	5/6 83.33%	4/6 66.67%	4/6 66.67%	4/6 66.67%	2/6 33.33%	0/6 0.00%
V. vulnificus cty	2/6 33.33%	3/6 50.00%	2/6 33.33%	4/6 66.67%	2/6 33.33%	2/6 33.33%
*V. vulnificus viu*B	1/6 16.67%	1/6 16.67%	0/6 0.00%	3/6 50.00%	1/6 16.67%	0/6 0.00%

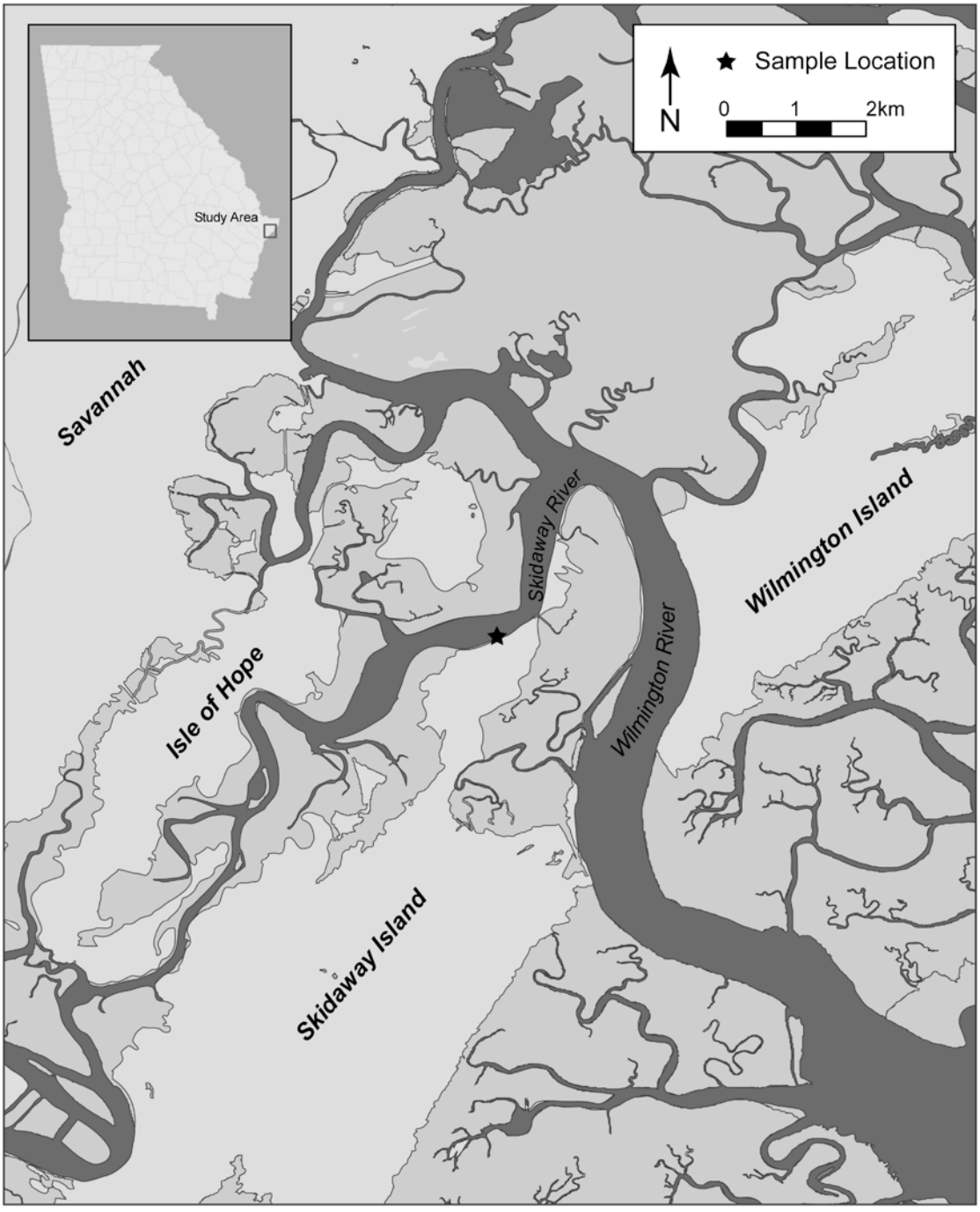

Figure 5.1. Map of study site along the tidal reaches of the Skidaway River.

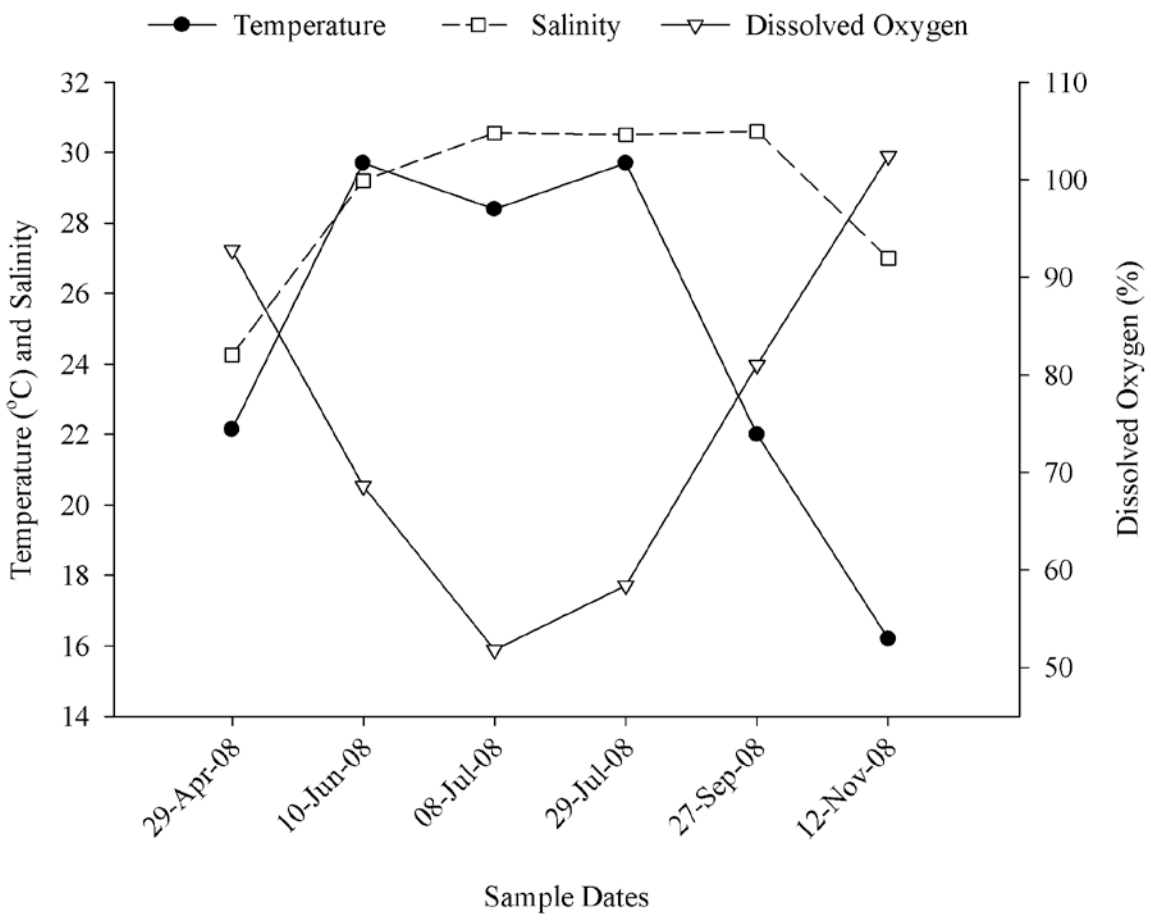

Figure 5.2. Physico-chemical parameters (temperature, salinity and dissolved oxygen (DO) by sampling date.

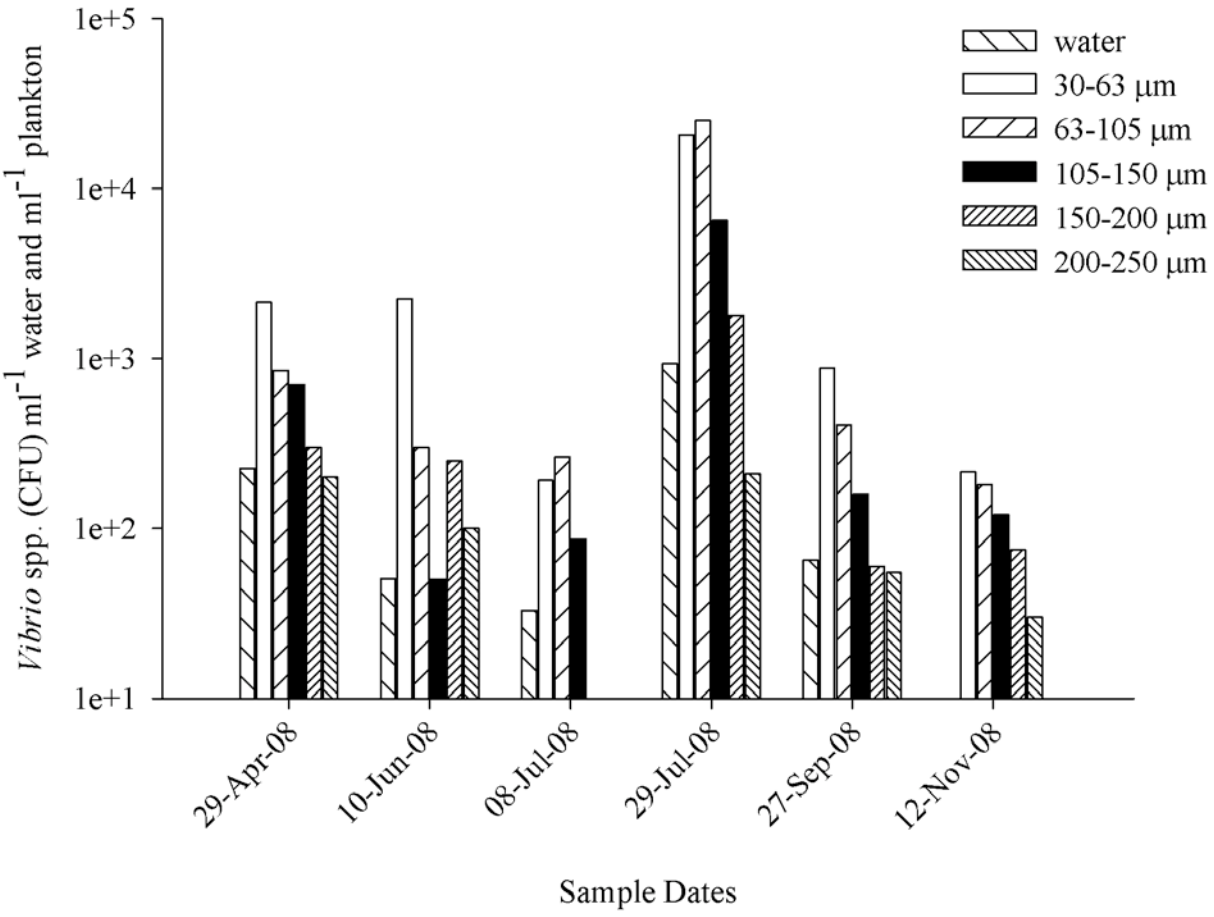

Figure 5.3. Concentrations of culturable *Vibrio* species (CFU ml^{-1} water and resuspended plankton) in all environmental fractions (water and 30-63, 63-105, 105-150, 150-200 and 200-250 µm size fractions) by sample date.

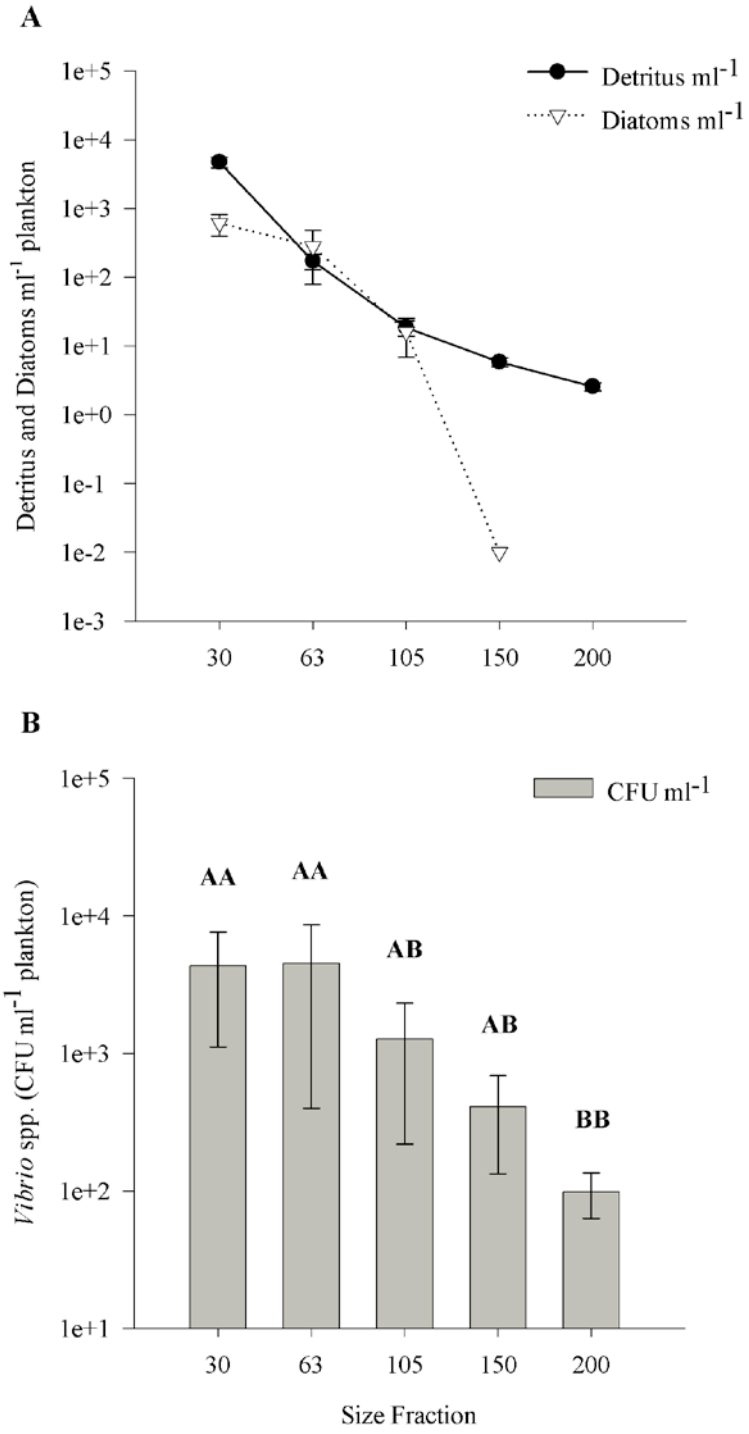

Figure 5.4. Concentration of detritus and diatoms (particles ml^{-1} and diatoms ml^{-1}) among fractionated sample sizes (A) and concentration of culturable *Vibrio* species (CFU ml^{-1} plankton) among fractionated sample sizes (B).

CHAPTER 6

APPLICATION OF A GFP-TAGGED *VIBRIO CHOLERAE* TO DOCUMENT AND VISUALIZE COLONIZATION OF AN ENVIRONMENTAL HOST – THE MARINE COPEPOD

Turner JW, Westrich J, Stabb E, Lipp EK. To be submitted to *Journal of Microbiological Methods.*

ABSTRACT

Copepods are a well-known reservoir and vector of the human bacterial pathogen, *Vibrio cholerae*, which is the etiologic agent of cholera. In this study, we investigated the potential differential attachment of *V. cholerae* to copepod developmental stages (eggs, nauplii and adult) and the exoskeleton molt. For this purpose, we utilized a *gfp*-tagged clinical strain of *V. cholerae* O1 El Tor, created by conjugative transfer, and conducted a series of microcosms focused on attachment to *Tigriopus californicus*. In-culture stability assays showed that 75.42% of cells retained the GFP plasmid after 30 generations of non-selective growth. In-host stability assays showed that 89.24% of cells retained the GFP plasmid after 4 hours of host-association under non-selective conditions. Using GFP as a qualitative tool and culture on TCBS as a means of quantification, we demonstrated that *V. cholerae* preferentially attached to egg sacs and exoskeleton molts. A comparison of copepod nauplii and adult copepods revealed no significant differences in colonization. This study utilized a means of visualizing pathogen-host interactions *in situ* and the application of this tool confirmed conclusions from previous studies. We propose the methods and techniques forwarded here could be employed to gain new insights into the ecology of *V. cholerae* and related pathogenic *Vibrio* species.

INTRODUCTION

V. cholerae, the etiologic agent of cholera, a devastating disease of global significance, is an opportunistic pathogen and a natural component of the microbial flora of marine and estuarine systems worldwide (Thompson *et al.*, 2003). Between 1995 and 2004, the World Health Organization (WHO) reported 100,000 to 300,000 annual cases of cholera worldwide (Zuckerman *et al.*, 2007). Due to poor surveillance systems and frequent under-reporting, the WHO estimates that < 5% of cases are reported. Thus, WHO estimates the actual burden of cholera approaches 3 to 5 million cases annually with an approximate mortality of 4%. (Zuckerman *et al.*, 2007).

In regions where cholera is endemic, cholera case rates can exhibit a distinct seasonality, which has been linked to monsoon periods and seasonally high abundances of plankton along the coastal reaches of these endemic regions (Colwell, 1996; Colwell *et al.*, 2003; Huq *et al.*, 2005). *Vibrio cholerae* has been shown to be closely associated with copepods (Sochard *et al.*, 1979; Colwell *et al.*, 1981; Colwell, 1996; Carman and Dobbs, 1997). In Bangladesh, copepods have been identified as a vector for the primary transmission of cholera and the filtration of copepods from drinking water has been shown to significantly reduce the incidence of disease (Colwell, 2003; Huq, 1996).

Ecologically, *V. cholerae* is a heterotrophic microbe that elaborates an extracellular chitinase and plays a role in the mineralization of the chitinous exoskeletons of higher zooplankton, such as copepods (Meibom *et al.*, 2004). The copepod, which is often the most numerically abundant zooplankton taxon (Rawlings *et al*., 2007), represents a nutrient-rich microhabitat capable of selectively enriching *V. cholerae* at densities much higher than the surrounding water column (Huq *et al.*, 1983; Huq *et al.*, 1984). One copepod can theoretically

carry an infectious dose of *V. cholerae* (10^4 – 10^6 cells) (Faruque *et al.*, 1998; Colwell *et al.*, 2003). Additionally, when associated with chitin, *V. cholerae* exhibits a higher tolerance for the stomach acids of the human host (Chiavelli *et al.*, 2001; Reguera and Kolter, 2004). Thus, the epidemiology of cholera is linked to the ecology of *V. cholerae* and the ability of this pathogen to attach to and be associated with chitinous surfaces such as the exoskeleton of the copepod (Chiavelli *et al.*, 2001; Colwell *et al.*, 2003; Cottingham *et al.*, 2003; Huq *et al.*, 2005).

The objective of this study was to determine if copepod developmental stages (eggs, nauplii and adult copepod) and molted exoskeletons are colonized equally by *V. cholerae* in laboratory-based microcosms. For this purpose, we employed a tri-parental matting technique to tag a clinical strain of *V. cholerae* O1 El Tor with a *gfp* plasmid and then attempted to visualize those differences *in situ*. To quantify differences in colonization, we employed culture on thiosulfate citrate bile salts sucrose agar (TCBS). We hypothesized that colonization would exhibit developmental stage-specificity which might translate into population-level consequences (i.e., environmental prevalence of *V. cholerae* may reflect the life history of the copepod population).

MATERIALS AND METHODS

Tri-parental mating and isolation of *gfp*-tagged *V. cholerae*. *E. coli* donor strain DH5α (pKV111) (*gfp* Cm^r) (Sawabe *et al.*, 2006) and helper strain CC118λ*pir* (pEVS104) (*tra trb* Kn^r) (Dunn *et al.*, 2006) were courteously provided by Dr. Eric Stabb at the University of Georgia, USA. The conjugative transfer and isolation of GFP-tagged *V. cholerae* proceeded as described previously (Dunn *et al.*, 2006; Sawabe *et al.*, 2006); however, growth conditions for mating and isolation (media composition, temperature and antibiotic concentration) varied

slightly. Briefly, donor and helper were grown overnight in LBS (1% tryptone, 0.5% yeast, 2% NaCl and 0.05 M Tris buffer) (amended with 40 µg ml^{-1} chloroamphenical and 40 µg ml^{-1} kanamycin, respectively) at 37^{o}C for 16-18 hours with shaking (100 rpm) (New Brunswick Scientific, model R2, Edison, NJ, USA). The *V. cholerae* recipient strain (*V. cholerae* O1 El Tor N16961 [ATCC 39315]) was grown overnight in LBS at 30^{o}C for 16-18 hours with shaking (100 rpm). Donor, helper and recipient (100 µl of each) were combined in a 1.5 ml microcentrifuge tube (Fisher Scientific, Pittsburg, PA, USA) and vortexed briefly (5 s). Cells were pelleted by centrifugation (2,450 x *g*, 5 minutes, 20^{o}C), washed twice with 1 ml of LBS and suspended in 10 µl of fresh antibiotic-free LBS. The mating mixture was spotted on an LBS plate (LBS amended with 1.5% bacto agar) using a 10 µl sterile loop. The mating mixture was then incubated in the dark for 16-18 hours and growth was streaked for isolation. For this purpose, we tested the isolation efficiency of LBS agar, ASW agar (artificial sea water, 1% peptone, 1.5% bacto agar) and thiosulfate citrate bile sucrose agar (TCBS); each amended with 40 µg ml^{-1} chloroamphenical. In addition, for each type of agar, we tested the efficiency of isolation at 28^{o}C and at room temperature (24^{o}C). ASW was prepared by dissolving approximately 35 g of Instant Ocean (Spectrum Brands, Madison, WI, USA) l^{-1} of Milli-Q water, followed by autoclave sterilization (121^{o}C, 15 psi, 15 minutes). Salinity was measured using a refractometer (Aquatic Ecosystems Inc, Model SR5, Apopka, FL, USA) and adjusted with the addition of Instant Ocean or Milli-Q water to achieve a final specific gravity of 1.021 to 1.023. Isolation plates were incubated in the dark for 2-4 days. Selected colonies were purified by streaking for isolation (a minimum of 3 times) on selective plates and then archived as frozen cells (-80^{o}C, 20% glycerol final volume). GFP-tagged *V. cholerae* isolates were confirmed by epifluorescence microscopy

(Olympus, Model BX41, Mirror unit U-MNG2) (Olympus America Inc., Center Valley, PA, USA).

Plasmid stability. To determine the stability of pKV111 (*gfp* Cm^r), transformed *V. cholerae*, isolated from the tri-parental mating assay (N = 10), were grown for 0, 10 and 30 generations and then tested for the presence of the plasmid using antibiotic selection plates as described previously (Dunn *et al*., 2006). Briefly, overnight cultures carrying the plasmid were grown in the dark in LBS broth (40 µg ml^{-1} Cm) at 30^{o}C for 16-18 hours. Overnight cultures were diluted 100-fold into 5 ml LBS (40 µg ml^{-1} Cm) and grown to an optical density of 0.6 (595 nm), designated as generation 0. Cells were pelleted, washed twice with PBS and diluted 10-fold into 5 ml LBS (no antibiotic) and grown to an optical density of 0.6, designated as generation 10. This process was repeated, in 10-generation increments, for a total of 30 generations. Serial dilutions of each 10-generation increment (0, 10, 20 and 30) were plated on LBS (no antibiotic) and grown overnight at 30^{o}C. Colonies (N = 96), from each generation, were patched onto LBS plates supplemented with 40 µg ml^{-1} Cm. Plasmid stability was determined as the percentage of patched colonies retaining the plasmid of interest (showing growth on the selective plates).

This assay was also performed for transformed *V. cholerae* grown in association with copepods (*Tigriopus californicus*) to determine in-host effects on plasmid stability. In the in-host assay, we inoculated copepods (~50 in 100 ml of ASW) with 10^6 cells ml^{-1} (final concentration) *gfp+ V. cholerae* (N = 3), and measured stability by plating serial dilutions (prepared from a homogenate of 5 copepods in 1 ml of sterile ASW) at different time points (0, 1, 2 and 4 hours) post-inoculation onto non-selective (no antibiotic) ASW plates (1% peptone, 1.5% bacto agar). Colonies (N = 96), from each time point, were patched onto ASW plates supplemented with 40 µg ml^{-1} Cm. As described above, plasmid stability was determined as the

percentage of patched colonies retaining the plasmid of interest (showing growth on the selective plates).

Copepods. Cultures of *Tigriopus californicus* were purchased from Reed Mariculture Inc. (Campbell, CA, USA). Cultures (~2,000 copepods per culture) were maintained under static conditions in l L of ASW (specific gravity 1.021 – 1.023) (in a 2 L beaker) at 25^{o}C with a 12 hour/12 hour light/dark cycle. Copepods were fed a commercially available mixed diet of live phytoplankton cultures including *Pavlova*, *Isochrysis*, *Thalassioria*, *Tetraselmis* and *Nannochloropsis* (2 drops every 2-3 days) (Phyto Feast, Reed Mariculture Inc., Campbell, CA, USA). Feeding was also supplemented with a high quality 1 mm sinking fish pellet (Spectrum – All-Purpose) (2-3 pellets 2-3 days^{-1}) (New Life International Inc., Homestead, FL, USA).

Live copepods and molts (exoskeletons) were harvested by gravity filtration (200 µm nylon sieve). Prior to conducting microcosms, live copepods were separated by sex (males and gravid females). Molts were collected from the bottom of the culture container with a serological pipette (25 ml) (Fisher Scientific, Pittsburg, PA, USA) and then washed of debris (fecal pellets and excess food) by rinsing with 1 liter of sterile ASW over a 200 µm nylon sieve. Copepods (male and gravid female) and molts were transferred (using a wide-bore glass-silicate pipette to avoid damage) (Fisher Scientific, Pittsburg, PA) into 100 ml of sterile ASW in a 200 ml tall-form beaker.

Age-specific cohorts of juvenile copepods (nauplii) were reared using a custom culture apparatus. The apparatus consisted of a 200 µm mesh basket (constructed of PVC and the appropriate-sized nylon mesh), which was suspended in 1 l of ASW in a 2 l beaker. Gravid female copepods were placed into the basket and cultured under the conditions described above for a period of 3 days. On the third day, the basket was removed and the non-swimming nauplii

(~10 µm in length) were collected from the bottom of the container with a wide-bore borosilicate pipette, and maintained in ASW at 25^{o}C until use in the specified microcosms.

The high nutrient concentrations resulting in the copepod culture flasks were shown to encourage the growth of background bacteria, which caused non-specific growth on LBS and TCBS plates. To remove background bacteria, copepods and molts were washed 3 times with 1 l of sterile ASW and then maintained in sterile ASW without the addition of food for 24 hours prior to use in microcosms.

Inoculum preparation for microcosms. *V. cholerae* O1 El Tor carrying the pKV111 plasmid with *gfp* was grown in 5 ml of ASW broth supplemented with 40 µg ml^{-1} Cm at 30^{o}C with shaking (100 rpm) for 18-24 hours. Cell cultures were stained with crystal violet and cell density was determined by microscopy. Cells were then pelleted by centrifugation (2,450 *g*, 5 min, 24^{o}C), washed three times with 5 ml of sterile ASW and then suspended in 50 µl of sterile ASW. A volume of this cell suspension, yielding a final concentration of approximately 10^{6} cells ml^{-1}, was then added to each microcosm described below.

Description of microcosms. Two microcosm studies were conducted, each focused on the differential colonization of copepod life stages. The first compared attachment of the *gfp* transformed *V. cholerae* O1 El Tor to adult male copepods, adult gravid female copepods and exoskeleton molts. The second compared attachment to nauplii (juvenile copepods) and adult copepods (1:1, male:female). Microcosm chambers consisted of sterile 24-well Falcon culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and each well contained 5 ml of sterile ASW (final volume) (maintained at 30^{o}C).

In the first microcosm study, copepods (adult males and adult gravid females) and molts were collected as described above. The microcosm consisted of 6 replicates of each copepod

type (adult male, gravid female and molt) representing each time point of the microcosm (0, 1, 2, 3 and 4 hours). These 6 replicates were prepared in triplicate (A, B and C) for a total of 18 replicates. Each replicate consisted of either 10 adult copepods, 10 gravid females or 10 copepod molts suspended in 5 ml of sterile ASW. An inoculum of *V. cholerae* was added to each replicate as described above. At each time point, a 5-copepod subsample was transferred to a 63 µm filter (using a wide-bore borosilicate pipette), which was then washed by submerging the filter into a series of 3 water baths (500 ml each, sterile ASW). Copepods were then transferred to a 1.5 ml centrifuge tube (facilitated by rinsing with sterile ASW), homogenized in 1 ml sterile ASW (final volume), serial-diluted and spread plated on TCBS. *V. cholerae* growth was quantified (CFU μm^{-1} copepod body length) following incubation in the dark at $30^{o}C$ for 16-18 hours.

In the second microcosm study, copepods (adults and nauplii) were collected as described above. The microcosm consisted of 7 replicates of each copepod type (nauplii and adult) representing each time point of the microcosm (0, 2, 4, 8, 16 and 24 hours). These 7 replicates were prepared in triplicate (A, B and C) for a total of 21 replicates. Each replicate consisted of either 10 adult copepods or 20 copepod nauplii suspended in 5 ml of sterile ASW. An inoculum of *V. cholerae* was added to each replicate as described above. At each time point, either 5 adult copepods or 10 copepod nauplii were removed (using a wide-bore borosilicate pipette) and transferred to a 63 µm nylon-mesh filter. Processing of copepods and growth conditions for the quantification of *V. cholerae* was conducted as described above.

Copepod body length and molt length was determined microscopically by fixing (4% formaldehyde v/v, final concentration) a 10-copepod subsample and averaging body length per 10 animals or molts (measured from the apex of the prosome to the terminus of the urosome).

To visualize patterns or spatial differences in attachment, non-homogenized subsamples of 5 copepods were examined by epiflourescence microscopy at each sampling interval.

Data analysis. Measurements of copepod body length and colony forming units (CFU) of *V. cholerae* per µm of copepod body length were checked for normality and equal variance (Anderson-Darling statistic, $\alpha = 0.1$). Differences in body length were compared using an unpaired t-test (Tukey's test). Differences in plasmid stability were compared using a one-way analysis of variance (ANOVA) and pairwise multiple comparison (Tukey's test). Differences in attachment between copepod types were evaluated using a one-way analysis of variance (ANOVA) and pairwise multiple comparison (Tukey's test). All statistical analyses were performed in MINITAB, Version 15.0 (MINITAB Inc., State College, PA, USA) and all graphs were created in SigmaPlot, Version 11.0 (Systat Software Inc., San Jose, CA, USA). For all tests, significance was declared when $P < 0.05$.

RESULTS

Efficiency of isolating GFP-tagged *V. cholerae*. Isolation of tagged *V. cholerae* proved difficult at 28^{o}C. At this temperature, the *E. coli* donor strain showed growth on all types of media (LBS, ASW and TCBS) within 2 days. Lowering the incubation temperature by 4^{o}C (to 24^{o}C), limited the growth of the *E. coli* donor on all media; however, the donor still showed growth on LBS and ASW within 4 days. Incubation on TCBS at 24^{o}C proved the most efficient means of isolating GFP-tagged *V. cholerae* as the tagged recipient presented robust yellow colonies within 2 days. Meanwhile, the donor strain did not present growth under these incubation conditions over a 4-day period.

Plasmid stability. In-culture stability assays showed the percentage of *V. cholerae* cells retaining the pKV111 plasmid decreased from 99.9% (0 generations) to 75.42% (30 generations) when grown under non-selective conditions (30 generations was equivalent to ~14 hours) (Figure 6.3A). When associated with *Tigriopus californicus*, the percentage of cells retaining the pKV111 plasmid declined from 100.0% (0 hours) to 89.2% (4 hours) when grown under non-selective conditions (Figure 6.3B). In an additional in-host assay, *V. cholerae* carrying the pKV111 plasmid was shown to retain fluorescence (determined by epifluorescence microscopy) at 24, 48 and 72 hours post-inoculation.

Copepods. In the first microcosm study, the body length of adult male copepods (range 95.0 to 110.0 µm), adult gravid female copepods (range 100.0 to 117.5 µm) and exoskeleton molts (range 115.0 to 130.0) showed little variance and a normal distribution (Anderson-Darling statistic, $\alpha = 0.1$) (Figure 6.1). Similarly, in the second microcosm study, the body length of adult male copepods (range 95.0 to 110.0 µm) and copepod nauplii (range 10.0 to 17.5 µm) showed little variance and a normal distribution (Anderson-Darling statistic, $\alpha = 0.1$) (Figure 6.2).

Association with *Tigriopus californicus*. In this study, our methods of analysis were unable to distinguish between surface associated and gut associated *V. cholerae*. In the first microcosm study, attachment was significantly different between adult male copepods (range 3 to 62 CFU μm^{-1} body length), adult gravid female copepods (range 8 to 202 CFU μm^{-1} body length) and exoskeleton molts (range 510 to 665 CFU μm^{-1} body length) (ANOVA, $P < 0.05$) (Figure 6.4). Attachment did not vary significantly between adult male and gravid female until the fourth hour of the exposure, while the exoskeleton molts were inundated with a significantly higher concentration of *V. cholerae* throughout the exposure (ANOVA, $P < 0.05$) (Figure 6.5A).

Microscopic examination of the GFP-expressing cells revealed that attachment was dense along the region of the egg sac proximal to the female copepod's abdomen (Figure 6.5B).

In the second microcosm study, attachment of *V. cholerae* to adult copepods (range 11 to 39 CFU μm^{-1} body length) and copepod nauplii (range 8 to 67 CFU μm^{-1} body length) showed a non-linear trend over time (Figure 6.6). Overall, differences in attachment between adult copepod and copepod nauplii were not significant. Noteworthy was the observation of nauplii molting events and the sloughing of *V. cholerae* attached to those molts during the microcosm (visualized by epifluorescence microscopy), which was a possible source of variability in the data (Figure 6.7).

DISCUSSION

Although copepods are known to be of general importance to *V. cholerae* ecology (Colwell, 1996; Carman and Dobbs, 1997; Colwell *et al.*, 2003; Cottingham *et al.*, 2003), information regarding how copepod reproduction and life history affects the environmental prevalence of this pathogen remains largely unknown. Previous studies have shown that copepod egg sacs and molts are more highly colonized compared to the adult copepod body (Huq *et al.*, 1983; Huq *et al.*, 1984; Tamplin *et al.*, 1990). Recently, Rawlings *et al.* (2007) demonstrated that *V. cholerae* exhibits a preference for the colonization of different copepod species. In a previous study, we described how the prevalence of *V. cholerae* in an estuarine environment was correlated to the relative abundance of juvenile copepods (63-200 μm in size) in natural plankton samples (Chapter 4 of this dissertation).

To investigate the life-stage specific association of *V. cholerae* and copepods, we utilized a tri-parental mating technique to tag *V. cholerae* with a *gfp* plasmid. The pVK11 has been

shown to successfully transform a variety of *Vibrio* species (Sawabe *et al*., 2006); but this study was the first to show it can be transformed with a moderate level of stability into *V. cholerae*. Essential to a successful tri-parental mating was an efficient means of isolating the tagged recipient from the donor strain. In this study, a low incubation temperature combined with a high pH and bile salts (characteristic selective properties of TCBS) proved highly effective. The documentation of plasmid stability, both in-culture and in-host, were critical for the proper design of experiments. Despite the continued fluorescence of transformed cells for up to 72h, stability assays demonstrated that microcosms with copepod host must be limited to less than 4 hours of incubation to avoid significant loss in plasmid.

In general, bacteria possess numerous mechanisms that play a role in plasmid maintenance and loss (Summers, 1991; Kulakauskas *et al.*, 1995; Smith and Bidochka, 1998). Given that association with chitin has been shown to increase *V. cholerae*'s competence for natural transformation and upregulate numerous genes, association with copepods in this study may have impacted the stability of the plasmid (Meibom *et al.*, 2004). Alternative strategies to tagging *V. cholerae* with a fluorescent plasmid include the construction of a GFP mutant using a plasmid that occurs naturally in *V. cholerae* or the transposon-mediated insertion of a *gfp* gene into the *V. cholerae* genome (Koch *et al.*, 2001).

In this study, we investigated the attachment of *V. cholerae* O1 El Tor to copepod developmental stages and molts. The distinct copepod developmental cycle and the ease of manipulating and harvesting different life-stages afforded the rearing and harvesting of age-specific cohorts of nauplii and adults; however, a limitation to our animal model was the molting frequency of the naupliar life stages, which depending on age, can occur more than once per day

(Miller *et al.*, 1984; Carlotti and Nival, 1992). Thus, during the course of a microcosm (< 24 hours and < 4 hours), a molting event can result in a sloughing of attached *V. cholerae*.

Our results confirmed that egg sacs and molts were preferentially colonized compared to the copepod itself and this finding supports those of previous investigations (Huq *et al.*, 1983; Tamplin *et al.*, 1990; Rawlings *et al.*, 2007). Of particular concern, molts were especially inundated with high concentrations of *V. cholerae*. As these *V. cholerae*-rich molts are further degraded into POM, they could serve as a source of pathogen loading in filter-feeding molluscan bivalves, such as oysters (a common vector of *Vibrio*-related illness in the United States) (McLaughlin, 2005; Shapiro, 1998).

In this study, our assay was unable to characterize significant differences in attachment to naupliar verses adult life stages; however, the question remains compelling and worthy of further investigation. Adults having a terminal molt stage may represent a more stable substrate in that *V. cholerae* attached at this stage will be less likely to be sloughed (Cottingham *et al.*, 2003). However, adults that have reached a terminal molt stage can be expected to exude less soluble chitin, which has been shown to promote growth, attachment and biofilm differentiation (Meibom *et al.*, 2004; Reguera and Kolter, 2004). The terminal molt also maintains an epicutical of waxes that covers the outermost surfaces and the net charge of this layer may limit adsorption (Reguera and Kolter, 2004).

This study confirmed that copepod developmental stages (eggs and adults) and exoskeleton molts are not equally colonized by *V. cholerae*. These results suggest that copepod life history may be a significant factor governing the seasonal distribution of *V. cholerae* in the marine environment. The methods presented in this study proved helpful in visualizing

differeces in attachment to various developmental stages. Future investigations of *V. cholerae*-copepod interactions or other *Vibrio*-environmental host interactions could benefit from the use of the methods described in this study.

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FIGURES

Figure 6.1. Mean body length (µm) of adult male copepods, adult gravid female copepods and copepod exoskeleton molts (N = 10 for each).

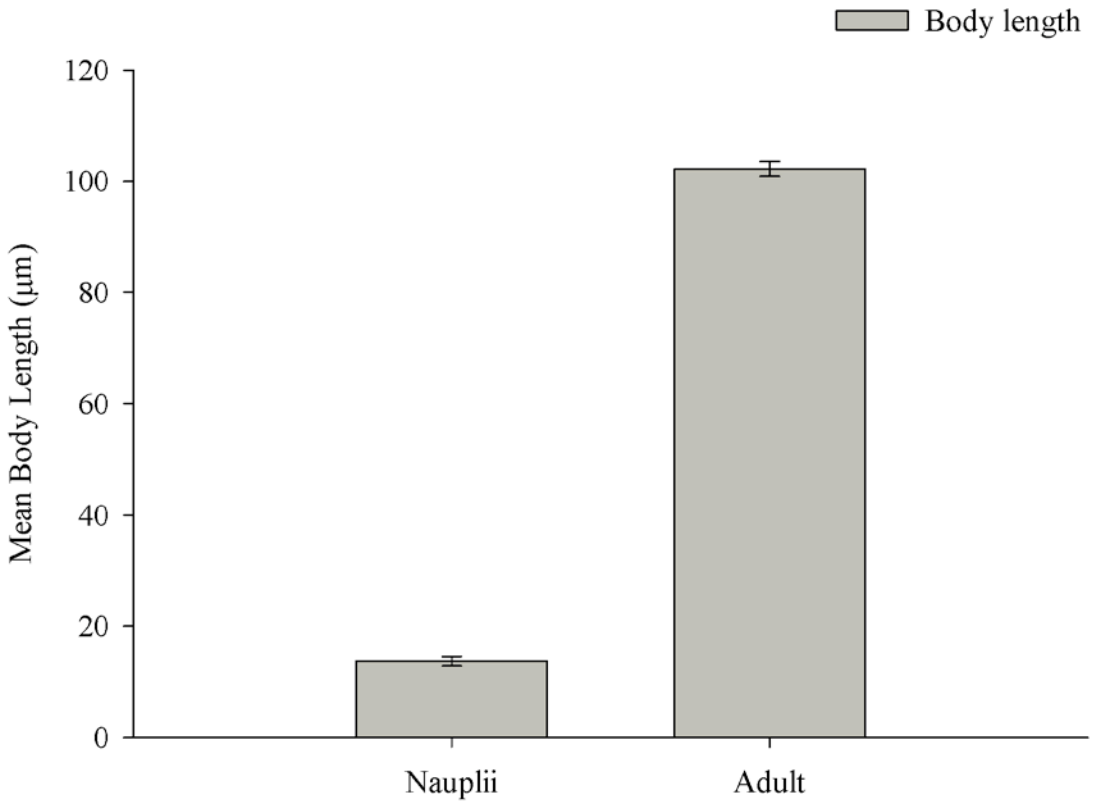

Figure 6.2. Mean body length (µm) of copepod nauplii and adult copepods (N = 10 for each).

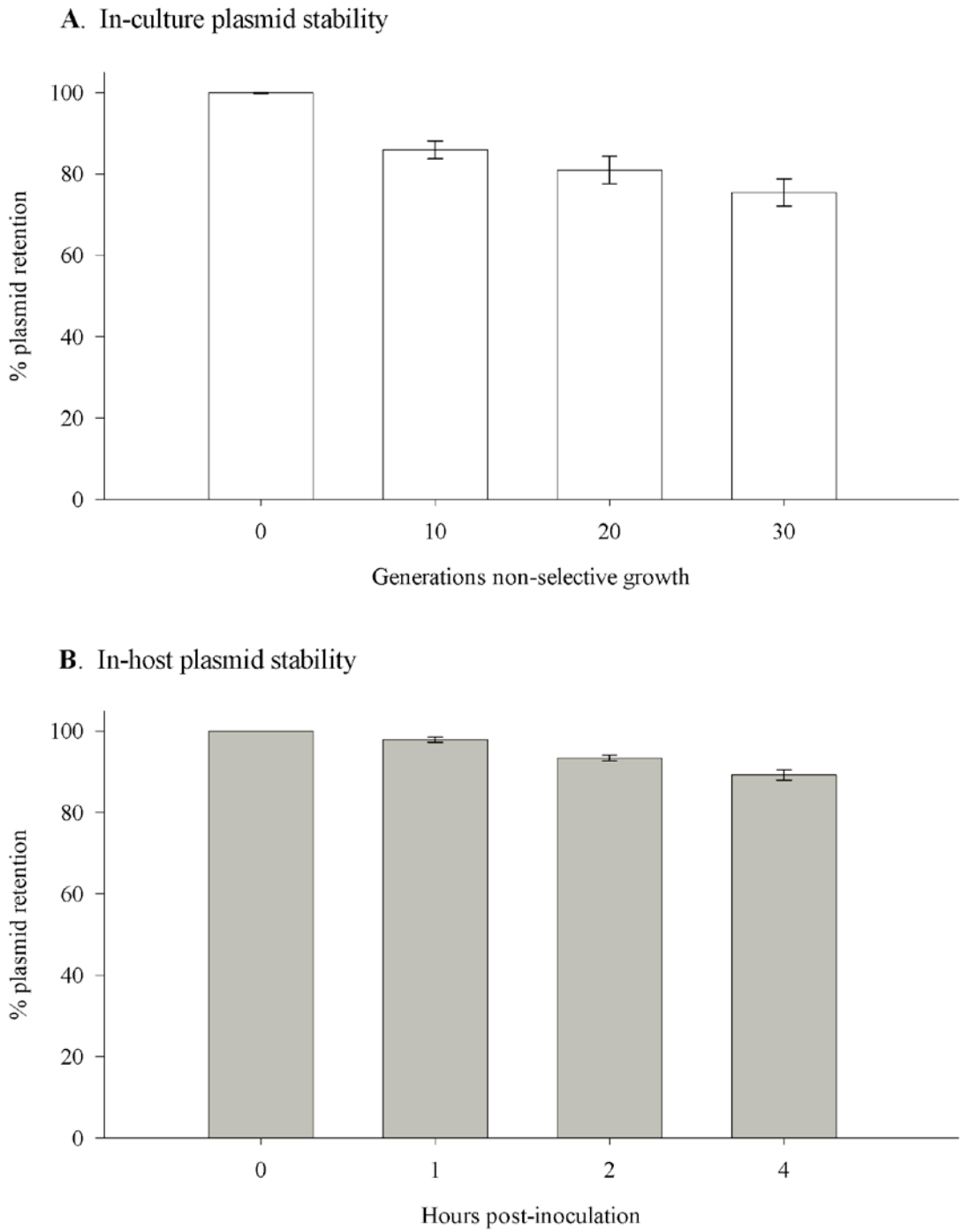

Figure 6.3. The mean percentage of cells retaining the *gfp*-pKV111 plasmid following 30 generations of non-selective growth (A. In-culture) (N = 10) and following 4-hours of association with the copepod host (B. In-host) (N = 3).

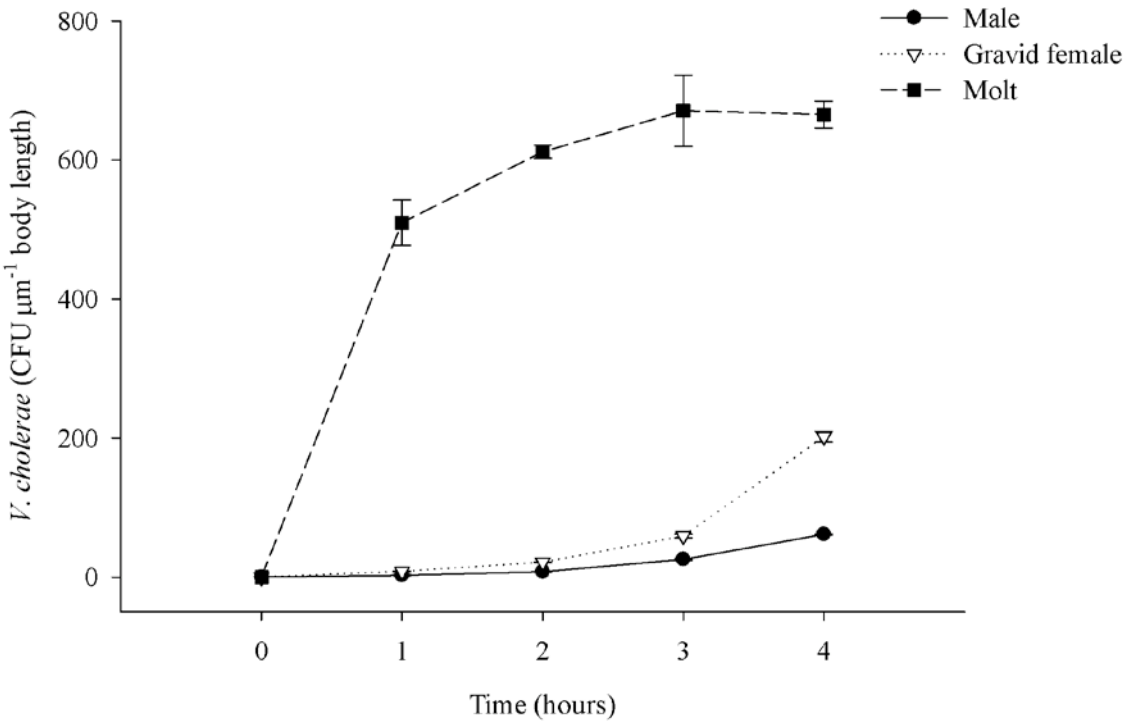

Figure 6.4. Differential colonization (CFU of *V. cholerae* µm body length^{-1}) of adult male copepods, adult gravid female copepods and copepod exoskeleton molts over time (hours).

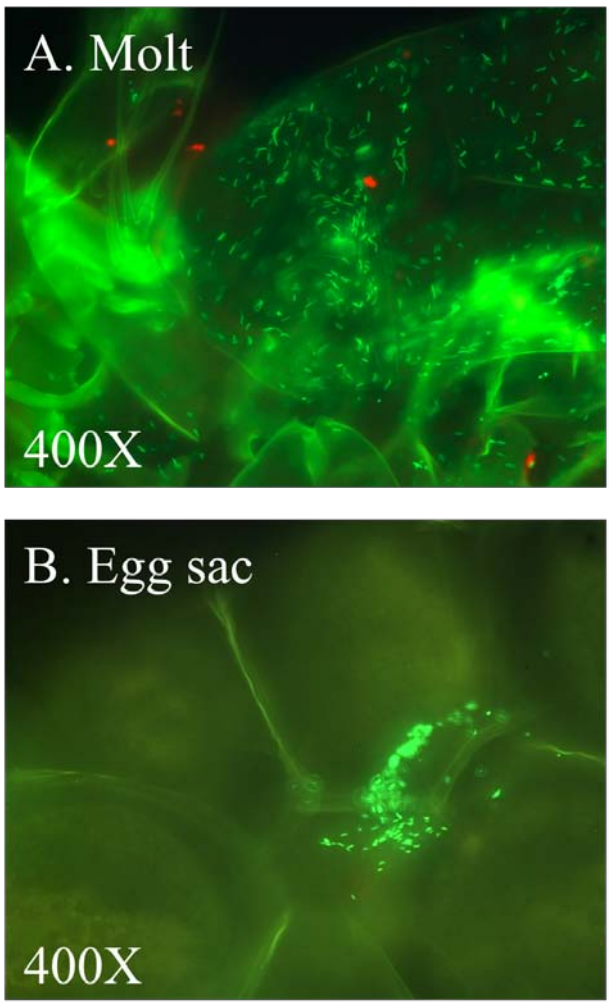

Figure 6.5. Fluorescent images of an exoskeleton molt from an adult copepod (A) and an egg sac from a gravid female copepod (B) colonized (green rods) by *gfp*-tagged *V. cholerae*.

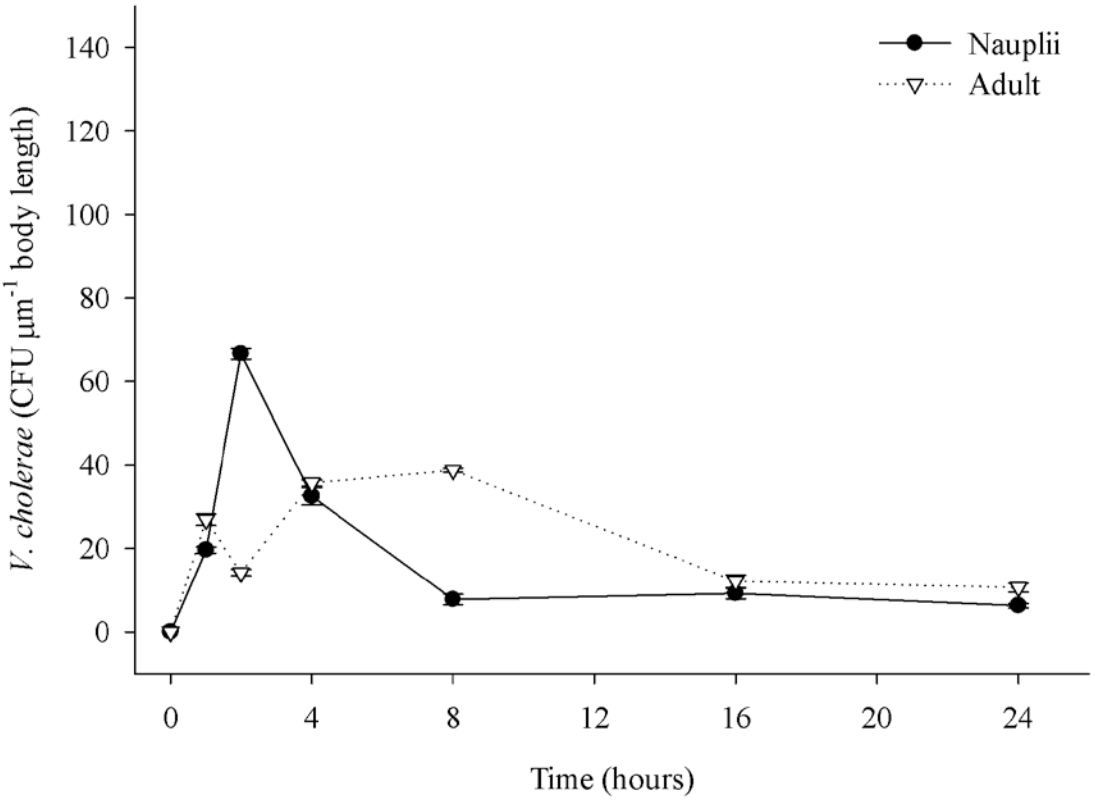

Figure 6.6. Differential colonization (CFU of *V. cholerae* µm body length^{-1}) of copepod nauplii and adult copepods over time (hours).

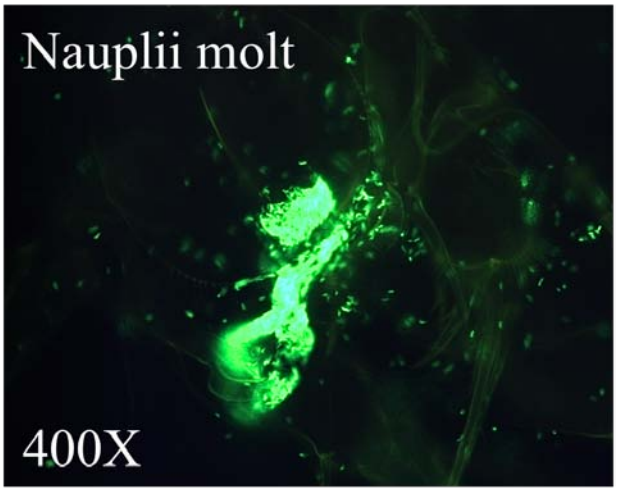

Figure 6.7. Fluorescent image of an exoskeleton molt from a copepod nauplii colonized (green rods) by *gfp*-tagged *V. cholerae*.

CHAPTER 7

CONCLUSIONS

Bacteria belonging to the *Vibrio* genus are common marine heterotrophs and at least 12 *Vibrio* species are human pathogens. Plankton, a natural *Vibrio* reservoir, may play a role in the survival and persistence of these pathogens in the marine environment. In a series of environmental studies (Chapters 3, 4 and 5), we investigated how fluctuations in environmental factors (temperature, salinity and DO) and shifts in the plankton reservoir affect the prevalence and seasonality of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Testing hypotheses derived from these environmental studies, we applied a GFP-tagged *V. cholerae* to investigate the potential differential association of *V. cholerae* with copepod developmental stages (egg sacs, nauplii and adults) and exoskeleton molts (Chapter 6).

In the first environmental study (Chapters 3 and 4), temperature, salinity and DO were strongly associated with total *Vibrio* concentrations (CFU ml^{-1}, TCBS). Temperature was also a strong correlate with the prevalence of pathogenic species (i.e., *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*). However, fluctuations in physico-chemical environmental parameters alone were unable to account for the seasonal variation encountered in our field studies. Shifts in the abundance of plankton taxa were also associated with total *Vibrio* concentrations and the prevalence of pathogenic *Vibrio* species. In particular, the abundance of copepods was strongly associated with total *Vibrio* concentrations and the prevalence of *V. cholerae*. Similarly, the

abundance of diatoms and decapods were associated with the prevalence of *V. parahaemolyticus* and *V. vulnificus*, respectively.

In the second environmental study (Chapter 5), our sampling regimen reflected two major changes. First, the study period was limited to 6 months (April to November), when surface water temperatures did not limit *Vibrio* growth ($\geq 15^{o}$C). Secondly, in addition to quantifying plankton, we quantified detrital particulate organic matter (POM). Interestingly, shifts in the abundance of detrital POM played a particularly important role in the prevalence of total *Vibrio* and *V. cholerae*. The most apparent shifts in the abundance of detrital POM occurred in April and September and likely coincided with the phytoplankton bloom formation in this estuary and an increase in detection of *V. cholerae*.

Among the environmental studies, the analyses of different plankton size fractions resulted in a size-specific separation of copepod developmental stages. For example, copepod nauplii were more abundant in the smaller size fractions while adult copepods were more abundant in the larger fractions. We hypothesized that copepod developmental stages (nauplii and adult) could be differentially colonized by *V. cholerae*. For the purpose of visually documenting developmental stage specificity, we tested the utility of a *gfp*-tagged *V. cholerae* strain (Chapter 6). Although nauplii and adults were not differentially colonized by *V. cholerae*, egg sacs and exoskeleton molts were preferentially colonized. This finding confirms previous investigations and validates the utility of our GFP-tagged *V. cholerae* as a tool for investigating associations between *V. cholerae* and copepods or additional pelagic plankton.

Together, our results confirm the role of temperature and salinity in *Vibrio* seasonality and highlight an important and independent role for plankton composition in explaining seasonal changes in *Vibrio* prevalence. Further, this study shows that some plankton taxa, such as

copepods and diatoms, may be especially important reservoirs of pathogenic *Vibrio* species. Future*Vibrio* predictive models and *Vibrio* risk assessments could be improved by characterizing *Vibrio* prevalence in the context of seasonal changes in environmental parameters as well as shifts in the abundance of specific plankton taxa and detrital POM.