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Isolation and Enumeration of *Vibrio vulnificus* and *Vibrio parahaemolyticus* from Coastal Virginia

An Honors Program Project Presented to

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James Madison University

by Zackary Alan Zayakosky

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Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors Program.

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Abstract

Vibrio vulnificus and *V. parahaemolyticus* are gram-negative, halophilic bacteria that are found throughout estuarial waters during the summer months, and are commonly associated with human infection. Gastroenteritis and other related symptoms can occur following infection from either organism, which most often occurs as a result of consumption of raw oysters or other seafood. *V. vulnificus* is particularly virulent, and can also produce wound infections that lead to severe septicemia and death. Due to the increasing rates of infection for these two organisms, recent research efforts have focused on potential environmental conditions and reservoirs that would be indicative of increased *Vibrio spp.* concentrations, and a higher potential for human exposure. This study was conducted in order to locate, isolate, and analyze reservoirs that potentially harbor both species of *Vibrio* near commercial and recreational water sources. Water, sediment, algae, fecal, and invertebrate samples were collected from the mud flats of the Eastern Shore of Virginia. These were processed and analyzed by means of dilution, vacuum filtration, and plating on selective media in order to accurately quantify the abundance of *Vibrio spp.* in various reservoirs on the coastal flats. Presumptive isolates will be confirmed with PCR, which will give an accurate estimate of the abundance of *Vibrio spp.* on the Virginia coastline. Future studies may include other qualitative analyses of the *Vibrio* isolates, such as Antibiotic Resistance Analysis (ARA).

Introduction

Vibrio vulnificus and *V. parahaemolyticus* are pathogenic, gram-negative bacteria that are naturally present throughout estuarial and coastal waters, during the summer months (Givens et al., 2014). The bacteria are found in, or on, nearly all seafoods, and occur in particularly high numbers amongst clams and oysters (Oliver, 2006). Oysters are more likely to contain *Vibrio* in the summer months, and temperatures of 12-17 °C are necessary in order to find any culturable *V. vulnificus*. The role of salinity is still being explored, but lower salinity levels are loosely associated with lower concentrations of *V. vulnificus* and *V. parahaemolyticus*. Approximately 95% of *V. vulnificus* cells associated with oysters are found within its tissue, rather than on the surface of the meat or the shell, posing an additional health risk for those that consume raw oysters (Froelich & Oliver, 2013).

Seafood makes up <1% of the typical US diet, but is responsible for approximately a quarter of all foodborne illness in the USA. Of these seafood related illnesses, 96% of cases are due to raw oyster consumption, and 95% of all seafood-related deaths are attributed to *V. vulnificus*, the most virulent of the *Vibrio* species (Oliver, 2013). *V. parahaemolyticus* is responsible for hundreds of hospitalizations each year, causing gastroenteritis and other related health complications. *V. vulnificus* infection rates remain relatively low, but have risen over time in concurrence with other *Vibrio* infections (Sims et al., 2011).

There appears to be some common characteristics among individuals that get infected by *V. vulnificus*, posing an additional risk for those that share specific traits. A review of data released by the FDA has shown that over 85% of *V. vulnificus* infections were reported in males. Additionally, over 95% of these patients had preexisting conditions, particularly liver diseases such as cirrhosis or hepatitis (Jones & Oliver, 2009). This epidemiological data helps explain why older males are often the victims of this pathogenic bacterium. Given the immunosuppression associated with many of the aforementioned conditions, it appears as though younger, healthier individuals are at less of a risk when exposed to *V. vulnificus*. Animal studies have shown that estrogen plays a role in the protection of women against infection, further explaining the disparity in infections between genders (Oliver, 2013). The growth of elderly and other at-risk populations could also partially explain the increased rates of infection that are being observed over time.

Data has shown that globally, average water and air temperatures have risen significantly since the beginning of the nineteenth century. A study performed in the Chesapeake Bay, for example, has shown a 0.3-0.4 °C increase in temperature every ten years for the last 30 years. Climate change is responsible for increasing the potential habitat of pathogenic vibrios, and lengthening the season in which they are most prevalent (Vezzuli et al., 2013). Over 75% of *Vibrio* infections currently occur between the months of May and October, suggesting that warmer temperatures are responsible for the increased rates of infection (CDC 2009, 2012). *V. parahaemolyticus*, in particular, has been shown to have a positive association with water temperature, but not salinity (Young et al., 2015). *V. vulnificus* grows optimally under halophilic conditions, but also seems to have the highest correlation with increased water temperature (Oliver, 2006).

V. vulnificus is a unique pathogen due to its multiple portals of entry. Despite its role as a foodborne agent, it is also capable of causing fatal wound infections. The incidence of wound infections in the US has also risen over time, with the average number of reported cases being

24/year between 1988 and1999, and 54/year from 2000 to 2010 (Baker-Austin et al., 2013). While mortality rates are lower than those from seafood consumption, wound infection cases have mortality rates of 24% (Oliver, 2005). Improved food processing and storage procedures reduce the risk of infection for seafood consumers, but increased concentrations of bacteria present in the environment will continue to pose a risk in recreational waters during the summer months.

Estuarial and marine waters during warmer seasons contain *Vibrio* in concentrations that are high enough for infection via wound exposure or oral ingestion. The U.S. Food and Drug Administration suggests that the ingestion infectious dose for *V. parahaemolyticus* with a 50% probability of illness is approximately 10^6 to 10^8 CFU g^{-1} (FDA, 2005). Risk of illness modeled by the World Health Organization determined an ingestion infectious dose of approximately 10^3 to 10^7 CFU g^{-1} for *V. vulnificus* (WHO, 2005). The non-ingestion infectious dose is currently unknown for *V. vulnificus* and *V. parahaemolyticus* (FDA, 2012). However, sub-cutaneous *V. vulnificus* inoculations in murine models have suggested that wound infection is possible with as little as 1000 CFU, making it conceivable that the concentration of *Vibrio* needed for human wound infection is a fraction of that needed for infection via oral ingestion (Thiaville et al., 2011).

Accurate ecological models of *V. vulnificus* and other less prevalent vibrios have been impossible to make due to lack of reliable data, but could be developed in the future as research continues (Urquhart et al., 2014). Modeling of these pathogens could help predict levels of vibrios after environmental changes, or during the summer months when recreational swimming, fishing, and boating are most common (Shaw et al, 2015). Estimates of oral ingestion rates of surface water during swimming have been used in conjunction with the average bacterial concentrations in surface water to predict the level of *V. vulnificus* ingestion that is possible throughout infected Virginia waters. Based on data collected from the Chesapeake Bay, a child (<18 years) consumes an average of 42,000 CFU of V. vulnificus per swimming event due to oral ingestion of surface water. Surface water concentrations of *Vibrio* have also been significantly associated with concentrations of *Vibrio* collected from hand wash samples, suggesting a method for health risk assessment. Recreational swimmers and individuals working in high-risk waters could reduce exposure with routine water testing (Shaw et al., 2015). Unfortunately, the impact of storm events on *V. vulnificus* and *V. parahaemolyticus* concentrations remain inconclusive, since enumeration data is highly variable between studies and sampling locations (Shaw et al, 2014).

One difficulty that researchers face when studying *V. vulnificus* and *V. parahaemolyticus* is their ability to enter a ‘viable but non-culturable state’ (VBNC). When environmental conditions are unfavorable, these microorganisms can alter their gene expression in order to lower their metabolic activity to a point of near dormancy. Bacteria are not culturable in this state, but are able to revert back to their more active and culturable forms once environmental conditions are favorable (Oliver, 2005). The primary environmental factor responsible for cells entering the VBNC is a drastic increase or decrease in temperature. Reducing temperatures to as low as 4 °C for a period of over two months has been shown to induce the VBNC state in certain strains of *V. vulnificus*. An increase of only two degrees is enough to resuscitate these cells, but maximum resuscitation is usually achieved by exposure to a temperature of 23-°C for 24 hours (Rao et al., 2014). Given the reliance on heat treatment and low temperature pasteurizing in the food industry, these findings are of particular importance. Quality assurance testing could result in false negative results if cells are in the VBNC state due to temporarily unfavorable conditions (Nowakowska & Oliver, 2013). This phenomenon also explains some of the apparent seasonal drop off in *Vibrio* concentrations (Nowakowska & Oliver, 2013).

Isolation and identification of different *Vibrio* species are frequently achieved by using one or more selective and differential media. Pathogenic *Vibrio* species are often isolated by first plating environmental samples on Thiosulfate-citrate-bile-salts-sucrose (TCBS) agar, followed by additional plating and/or molecular confirmation (Nigro & Steward, 2015). The pH indicator in TCBS agar differentiates between sucrose fermenting species of *Vibrio*, such as *V. cholerae* and *V. alginolyticus*, by reacting with fermentation products and turning yellow. Non-sucrose fermenting species, such as *V. parahaemolyticus* and *V. vulnificus*, do not produce these products, and colonies are green (Di Pinto, 2011).

Another commonly used differential and selective medium, CHROMagar Vibrio™ (CaV), allows for discrimination of *Vibrio* species based on the ability to metabolize chromogenic substrates. Colonies of *V. vulnificus* and *V. parahaemolyticus* appear blue and mauve respectively on CaV. Used in conjunction with TCBS or another type of medium, the number of false-positive isolates can be greatly reduced. However, this multi-plate method has its limitations, and presumptive identifications must be confirmed using molecular methods, such as polymerase chain reaction (PCR) (Hyun-Joong et al., 2015).

Phenotypic variability is high within *Vibrio* species, resulting in different ecotypes that have adapted to specific environmental conditions. Genomic analysis of *V. parahaemolyticus* suggests that there could be 100 or more ecotypes living stably throughout the Asian population alone (Cui et al., 2014). The taxonomy of *V. vulnificus* is also fairly complex, with genotypic variation resulting in at least three distinct biotypes. Biotype one is almost exclusively associated with human disease, and no two strains of this type have been found to have an identical genotypic sequence. Biotype two is similar to biotype one, but is primarily associated with infection of cultured eels. Negative indole and ornithine decarboxylase reactions and lack of mannitol fermentation or growth at 42°C differentiate biotype one strains from biotype two. Biotype three was first reported in 1999, and is most commonly associated with wound infections. Differentiation of biotype three from biotypes one and two include negative citrate and o-nitrophenyl-β-D-galactopyranoside tests, as well as the inability to ferment salicin, cellobiose, or lactose. Differentiation between clinical and environmental isolates has also been made, particularly in regards to virulence and differences in biochemistry (Oliver, 2006).

Two genotypes of *V. vulnificus* have been differentiated and are often referred to as the C-genotype and E-genotype, correlating with clinical and environmental sources respectively. Some studies have speculated that these two groups could even be considered separate ecotypes (Molles, 2005). Conditions that favor rapid population growth have been shown to favor the growth of E-genotype strains rather than C-genotype strains, while C-genotype strains may be able to protect themselves better from stressors such as osmotic shock (Rosche et al., 2010). While differences between these genotypes are still being explored, the versatility, complexity, and survivability of this microorganism is obvious. These differences are of particular importance as habitable regions for vibrios increase, and once separate gene pools begin to mix.

Environmental *Vibrio* spp. are associated with nearly all seafoods, including oysters, clams etc., but also utilize other environmental reservoirs such as water and sediment. In particular, macroalgae such as *Gracilaria vermiculophylla* and other seaweeds have been shown to harbor pathogenic vibrios such as *V. vulnificus* and *V. parahaemolyticus* (Gonzalez et al., 2014). The ability to attach to chitin within a host or throughout the environment facilitates additional reservoir options for pathogenic vibrios. Variability in pilin gene expression has been associated with this capability, which makes gammarids and other invertebrates that live in algal mats another possible form of shelter for certain biotypes of *Vibrio spp.* (Williams et al., 2014).

Studies have shown that aquatic bird feces contain culturable *V. vulnificus* and *V. parahaemolyticus*, even throughout the winter months (Miyasaka et al., 2006). This suggests that birds could be a significant vector for pathogenic bacteria during avian migrations. Consumption of invertebrates and algae that may be harboring *Vibrio* spp. could lead to subsequent infection of avian hosts and the feces they disperse during travel. Furthermore, these findings support the idea that *Vibrio spp.* in the VBNC state are present in the environment throughout the winter months, and can resuscitate within a host (Miyasaka et al., 2006).

As *Vibrio spp.* prevalence has risen over time, so have the research efforts that reveal the ways in which these microorganisms proliferate and spread throughout the environment. Several environmental reservoirs of *V. vulnificus* and *V. parahaemolyticus* have been studied for decades, but new findings have suggested additional areas of interest that could further increase our knowledge of these pathogenic bacteria. In particular, several algal and invertebrate species could also be acting as reservoirs for these organisms, which are common food sources for many coastal bird species. Since these birds are capable of flying in migratory patterns that are hundreds of miles long, and the feces they drop could contain pathogenic vibrios, this phenomenon is of particular interest. Additionally, the VBNC capabilities of these microorganisms could allow for dormancy until seasonal conditions are favorable for subsequent spread and growth.

Study Objectives

Data was collected to increase the understanding of the levels of *V. vulnificus* and *V. parahaemolyticus* in estuarial waters throughout the Eastern Shore of Virginia. Performing this study has also elucidated preferential environmental reservoirs for these bacteria, as well as how they may spread to new environments. The results from this study compliment those of previous years, providing a more complete picture as to how changing environmental conditions can affect the occurrence of these microorganisms. Additionally, the use of current isolation and enumeration methodologies have been evaluated for their appropriateness for use in future projects. The primary objectives of this study were to isolate *V. vulnificus* and *V. parahaemolyticus* from the environment, determine their prevalence at the sample sites, maintain pure cultures of all isolates, and compare their growth on several selective media. It was estimated that *Vibrio spp.* concentrations would approach peak levels in June, however, the highest concentrations were expected during the warmest months of July and August.

Methods and Materials

Initial processing of samples.

Two 500 ml environmental water samples were obtained from different locations within the same estuarial site off the Eastern Shore of Virginia (S1 and S2, Figure 1). Additionally, approximately 14 grams of sediment, 13-17 *Gammarus*, approximately 2.5 g of *Gracilaria vermiculophylla*, and six fecal swabs were collected for analysis. The samples were stored on ice for approximately 4 hours prior to processing. All environmental samples were collected by Alice Besterman from the University of Virginia.

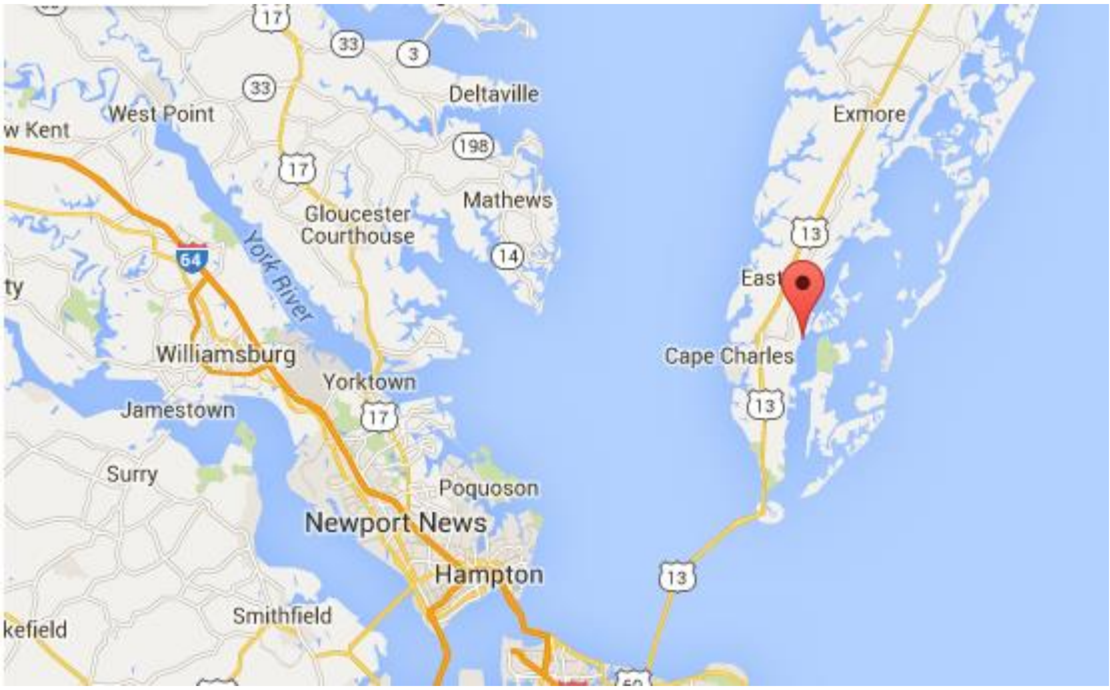

Figure 1. The estuarial site where all samples were collected, which were subsequently processed in Harrisonburg, VA. The DMS latitude was 37 °17’9.66”, and the DMS longitude was 75°54’57.08”. Each sample site was located within close proximity of the provided coordinates.

General filtration and plating of samples.

Membrane filtration was used to filter/process all sample types. Cellulose nitrate filter papers with 0.45 µm pores were used in conjunction with 50 mm plates. The filter papers were placed on CHROMagar Vibrio (CaV) and thiosulfate-citrate-bile salts-sucrose agar (TCBS) plates following filtration. The use of differential and selective media helped isolate the desired bacteria.

Throughout the filtering process, phosphate buffered saline (PBS) was used to rinse the walls of the filter apparati, both before filtration was performed and after samples were added. This rinsing was done to ensure that all of the bacteria from the sample volume were pulled through the filter. Pre-filter and post-filter rinses were also performed with at least 100 ml of sterile water. Pre-filter rinses were performed to confirm sterility of the filter apparati. Following filtration of the samples, each funnel was 'post-filter rinsed' to demonstrate bacteria had not been left on the funnel (i.e. rinsing between samples had been effective). These pre and post rinses were all plated onto TCBS plates, the less selective of the two media types.

For each sample filtered, approximately 4 ml of sterile PBS was used to wet each filter paper and lay it flat over the apparatus. The undiluted sample bottles were then shaken for 30 seconds to mix the bacteria prior to pipetting. The dilution tubes were also vortexed at high speed for ~30 seconds prior to filtration. Sample aliquots of less than 10 ml were suspended in approximately 10 ml PBS, and mixed to ensure even dispersal of bacteria. Between filtration sets, the previous plates were placed in an incubator to ensure that subsequent colony counting was performed as close to 24 hours as possible. In addition to filtration, spread plates were made using a sterile glass rod ('hockey stick') to spread 0.1 ml of the corresponding sample evenly across the media in all cases.

Filtration of water samples.

Three samples (A, B, and C), were filtered for each set of dilutions from each site (S1 and S2). Upon arrival, samples were diluted by method of serial dilution. A 10^{-1} dilution was created by adding 10 ml of the original sample to 90 ml of phosphate-buffered saline (PBS), and 2 ml of the 10^{-1} dilution was added to 18 ml of PBS to create a 10^{-2} dilution. The middle dilution was filtered in duplicate for both media types, which were thought to be the plates that would most likely yield countable numbers of bacterial colonies.

Each dilution was filtered from most dilute to least dilute. First, 10 ml of the 10^{-2} dilution was filtered for each sample and site, and the filter placed on TCBS plates. Next, 10 ml and 1 ml of the 10^{-1} dilution were filtered and placed on TCBS plates, then an additional 1 ml was filtered, and the filter placed onto CaV plates. Finally, 10 ml and 1 ml of the undiluted samples were filtered and placed onto CaV plates. All CaV and TCBS plates were incubated at 35° C and 37° C respectively for 24 hours. Since TCBS agar is more selective, lower dilutions were chosen to obtain countable numbers of colonies for all sample types. After incubation, pink and blue colonies (presumptive *V. parahaemolyticus* and *V. vulnificus* respectively) were counted on CaV plates, and yellow and green colonies were counted on TCBS plates to enumerate sucrose fermenting and non-sucrose fermenting *Vibrio spp.*

Filtration and plating of sediment samples.

Sediment samples were filtered onto filter paper and placed on 50 mm plates as described above, and also spread onto 100 mm CaV and TCBS plates. In order to make the dilution series for sediment, 10 grams of each sediment sample was weighed and suspended in 10 ml of sterile PBS. Diluted samples from 10^{-1} to 10^{-4} were prepared from the original sample. Samples were then filtered and placed on CaV from the 10^{-2} dilution, as well as from the 10^{-3} dilution. Similarly, aliquots from the 10^{-3} and 10^{-4} dilutions were also filtered and placed on TCBS plates. For the spread-plates, 0.1 ml of the 10^{0}, 10^{-1}, and 10^{-2} dilution were plated on CaV, while the 10^{-3} and 10^{-4} dilutions were plated on TCBS. A duplicate spread plate was performed on the 10^{-3} sample on TCBS to test for consistency in colony numbers. Spread plates were incubated at the same temperatures and time of incubation as the smaller CaV and TCBS plates. Total *Vibrio spp.* counts were obtained on both CaV and TCBS spread plates, while specific numbers of yellow and green colonies on TCBS, and pink and blue colonies on CaV were noted.

Filtration and plating of Gammarus samples.

Gammarus samples were filtered onto filter paper and placed on 50 mm plates as described above, and also spread onto 100 mm CaV and TCBS plates. In order to create the dilution series for *Gammarus*, live samples were suspended in 10 ml of sterile phosphate buffered saline (PBS), which were labeled as the stock solution. The total number of *Gammarus* added per sample varied, but were recorded, due to lower numbers obtained at certain samples sites. A 10^{-1} and 10^{-2} dilution were made with 18 ml and 9 ml of PBS, respectively. CaV spread plates were prepared using the original solution (10^{0}) and 10^{-1} dilution, whereas TCBS spread plates were prepared from the 10^{-1} dilution in duplicate, as well as the 10^{-2} dilution. For filtration, two 1 ml samples and a 5 ml sample of the 10^{0} sample were plated onto CaV, as well as 1 ml from the 10^{-1} dilution. Two 1 ml and 10 ml filtrations of the 10^{-1} dilution were plated onto TCBS, as well as 1 ml from the 10^{-2} dilution. All TCBS and CaV plates were incubated and counted in the manner described above.

Filtration and plating of *Gracilaria* samples.

Gracilaria vermiculophylla samples were filtered onto filter paper and placed on 50 mm plates as described above, and spread onto 100 mm CaV and TCBS plates. In order to create a stock solution, 2.5 g of *G. vermiculophylla* was rinsed with 25ml of PBS to remove extra sediment, and then suspended in 25 ml of sterile PBS. Each of the stock tubes were vortexed for 5 minutes at maximum speed to separate the bacteria from the alga surface and suspend them within the solution. A dilution series was created from the stock, ranging from 10^{-1} to 10^{-4} by using tubes containing 9 ml sterile PBS. Spread plates for CaV were prepared from the 10^{-1} and 10^{-2} solutions, and a lab duplicate was created for the 10^{-1} dilution. Spread plates for TCBS were created from the 10^{-2} and 10^{-3} dilutions, and a duplicate was created for the 10^{-2} dilution. For filtration, 1 ml from the 10^{0}, 10^{-1}, and 10^{-2} dilutions were plated on CaV, and a lab duplicate was made for the 10^{-1} dilution. Additionally, 1 ml from 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on TCBS, and a lab duplicate was made for the 10^{-3} dilution. All TCBS and CaV plates were incubated and counted in the manner described above.

Filtration and plating of fecal samples.

Fecal samples were filtered on 50 mm plates as described above, and spread onto 100x15 mm CaV and TCBS plates. In order to create the dilution series, one inoculated swab was placed into a 5 ml solution of sterile PBS, and labelled as the stock solution (10^{0}). Next, 10^{-2} and 10^{-3} dilutions were created from the stock solution. Spread plates of the 10^{0} and 10^{-2} dilutions were plated on CaV, as well as a direct swab from the 10^{0} stock. Furthermore, spread plates of the 10^{-2} and 10^{-3} dilutions were plated onto TCBS. During filtration, 1 ml of the 10^{0}, 10^{-2}, and 10^{-3} dilutions were plated on CaV, and a duplicate was made for the middle dilution. Additionally, 1 ml of the 10^{-2} and 10^{-3} dilutions were plated on TCBS upon filtration, and a lab duplicate was created for the 10^{-3} dilution. Fecal swabs were also streaked directly onto CaV plates. All TCBS and CaV plates were incubated and counted in the manner described above.

Results

V. vulnificus and *V. parahaemolyticus* are naturally found in brackish waters throughout coastal regions, especially during the summer months when waters are warmest. Our results were consistent with this, and over 200 presumptive isolates were obtained from the water, sediment, *G. vermiculophylla*, and *Gammarus* samples collected in June from the Eastern Shore of Virginia. However, culturable *Vibrio spp.* were not present in any of the avian fecal samples collected during this study, either from the direct swabs or suspended fecal samples in PBS solution.

Table 1. Total number of presumptive sucrose fermenting vs. non-sucrose fermenting *Vibrio spp.* isolated from each sample type.

Sample Type	**Total Sucrose Fermenting *Vibrio spp.***	**Total Non-Sucrose Fermenting *Vibrio spp.***
Water	1.29×10^2 CFU/ml	1.10×10^2 CFU/ml
Sediment	1.35×10^5 CFU/g	7.55×10^3 CFU/g
Gracilaria	3.87 CFU/g	1.40 CFU/g
Gammarus	3.40×10^3 CFU/gammarus	4.00×10^3 CFU/gammarus

Table 2. Proportion of presumptive sucrose fermenting vs. non-sucrose fermenting *Vibrio spp.* isolated from each sample type.

Sample Type	**Percentage of Sucrose Fermenting *Vibrio spp.***	**Percentage of Non-Sucrose Fermenting *Vibrio spp.***
Water	53.9	46.1
Sediment	94.7	5.3
Gracilaria	45.9	54.1
Gammarus	73.5	26.5

Table 3. Proportion of presumptive *Vibrio spp.* isolates obtained from each sample site (S1 and S2) for all sample types.

Sample Type	**Percentage of Total *Vibrio spp.* from Site 1**	**Percentage of Total *Vibrio spp.* from Site 2**
Water	23.7	76.3
Sediment	60.0	40.0
Gracilaria	12.2	87.8
Gammarus	78.0	22.0

Water and *Gracilaria* samples had roughly equal proportions of sucrose fermenting and non-sucrose fermenting *Vibrio spp.* (Table 2). Sample site two also contained much higher *Vibrio spp.* concentrations in the water and *Gracilaria* samples compared to sample site one

(Table 3). However, Gammarus and sediment samples had over twice as many sucrose fermenting *Vibrio spp.* compared to non-sucrose fermenting *Vibrio spp.*, and the majority of these samples were obtained from sample site one (Tables 2 & 3).

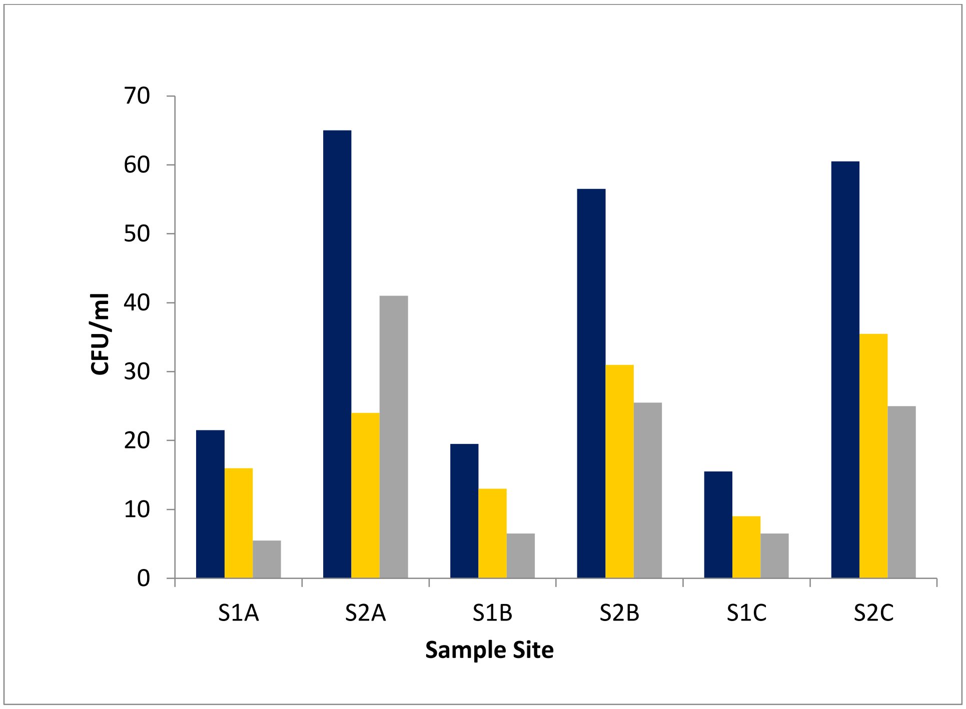

Figure 2. Water sample *Vibrio* concentrations. Total *Vibrio* colony forming units per ml of water are denoted by the navy blue bars, with sucrose fermenting *Vibrio spp.* and non-sucrose fermenting *Vibrio spp.* denoted by the yellow and gray bars respectively. Sample sites are denoted by S1 or S2. Locations within the sites are denoted by A, B, and C.

In general, concentrations of *Vibrio spp.* in water samples were moderate compared to numbers reported in previous studies. Additionally, considerable numbers of both sucrose fermenting and non-sucrose fermenting *Vibrio spp.* can be found at each of the sample locations during this time of year. With the lowest concentrations at 15.5 CFU/ml, there are significant numbers of *Vibrio spp.* suspended in the estuarial waters across all sample sites and locations. *Vibrio spp.* concentrations appear to vary greatly by sample site, but locations within each sample site are quite consistent (Fig. 2).

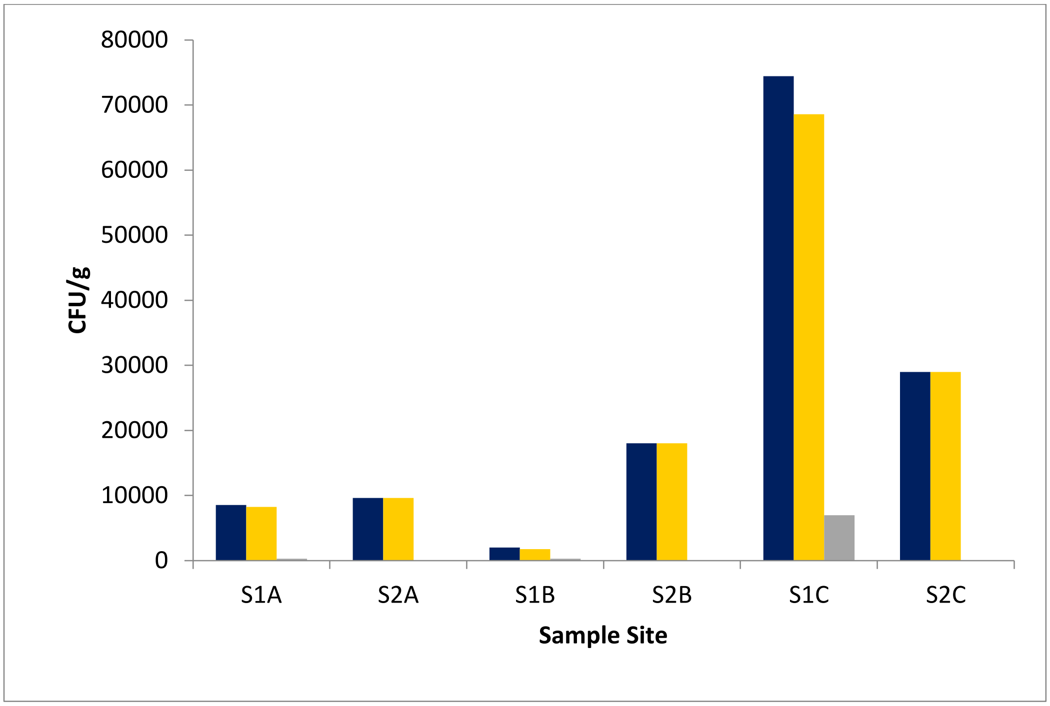

Figure 3. Sediment sample *Vibrio* concentrations. Total *Vibrio* colony forming units per gram of dry sediment are denoted by the navy blue bars, with sucrose fermenting *Vibrio spp.* and non-sucrose fermenting *Vibrio spp.* denoted by the yellow and gray bars respectively. Sample sites are denoted by S1 or S2. Locations within the sites are denoted by A, B, and C.

Concentrations of *Vibrio spp.* were relatively the largest in sediment samples, with the lowest numbers being 2011.5 CFU/g at site S1B. However, extremely limited numbers of non-sucrose fermenting *Vibrio spp.* were present in the sediment samples (Fig. 3). An exception to this was site S1C, which had especially high concentrations of *Vibrio spp.* in sediment, but the lowest concentrations in the remaining sample types. This outlier skews the proportion of *Vibrio spp.* sampled from site 1, but appears more even otherwise.

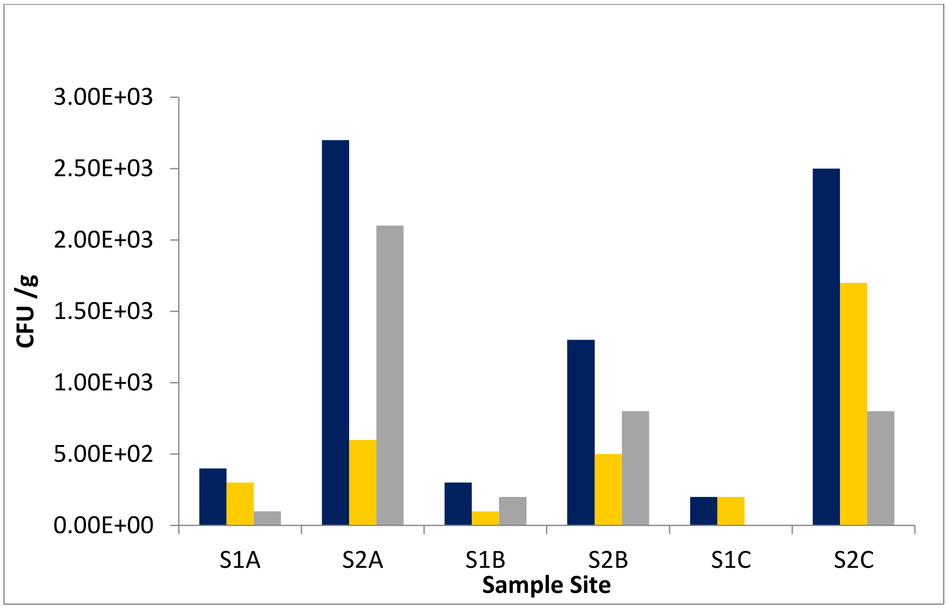

Figure 4. Algae sample *Vibrio* Concentrations. Total *Vibrio* colony forming units per gram of *G. vermiculophylla* are denoted by the navy blue bars, with sucrose fermenting *Vibrio spp.* and non-sucrose fermenting *Vibrio spp.* denoted by the yellow and gray bars respectively. Sample sites are denoted by S1 or S2. Locations within the sites are denoted by A, B, and C.

Vibrio spp. numbers were higher at site 2 compared to site 1 for *G. vermiculophylla* samples, which is also obvious for the water samples. In general, CFU/g concentrations are 1-2 log lower than those found in sediment. Variability in sucrose fermenting and non-sucrose fermenting *Vibrio spp.* are more pronounced between sampling sites and locations for *G. vermiculophylla* samples, but overall, roughly equal proportions of each type of *Vibrio spp.* are present (Fig. 4).

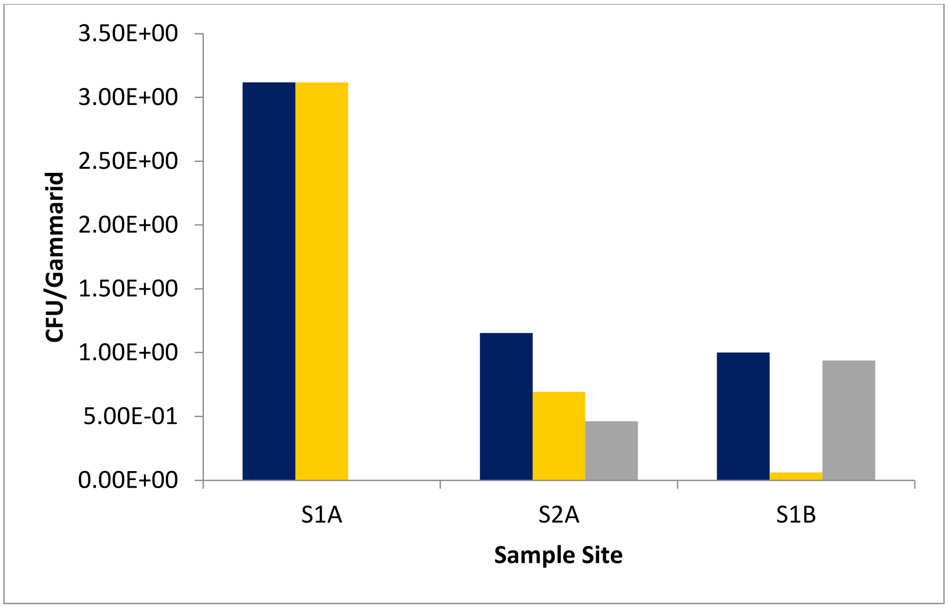

Figure 5. Invertebrate sample *Vibrio* concentrations. Total *Vibrio* colony forming units per Gammarid are denoted by the navy blue bars, with sucrose fermenting *Vibrio spp.* and non-sucrose fermenting *Vibrio spp.* denoted by the yellow and gray bars respectively. S1A contained 17 Gammarus from the S1A location, S1B contained 16 Gammarus from the S1B and S1C locations, and S2A contained 13 Gammarus from every location at site 2.

Only 13 of the 46 Gammarus specimens were collected from site two, which led to the grouping of the Gammarus sample locations as they are reported in this study. No non-sucrose fermenting *Vibrio spp.* were found at sampling location S1A, and very few sucrose fermenting *Vibrio spp.* were found at sampling location S1B. In general, Gammarus specimens were scarcely found, and contained minimal concentrations of culturable *Vibrio spp.* (Fig. 5).

Discussion

All of the sucrose fermenting and non-sucrose fermenting *Vibrio spp.* in this study were isolated using the selective and differential media TCBS and CaV. On TCBS agar, yellow colonies are indicative of sucrose fermenting *Vibrio spp.*, and green colonies are considered non-sucrose fermenting *Vibrio spp.* Species of *Vibrio* such as *V. cholerae* and *V. alginolyticus* are sucrose fermenting, while *V. vulnificus* and *V. parahaemolyticus* are non-sucrose fermenting. On CHROMagar, blue and pink/mauve colonies are typically considered *V. vulnificus* and *V. parahaemolyticus* respectively. These presumptive identifications are quite reliable for total *Vibrio spp.* counts, but require additional confirmation at the species level. Slow fermentation of sucrose could have led to false negative results, and densely packed colonies could have been misinterpreted as sucrose fermenting if within close proximity of a neighboring colony. Molecular methods such as PCR or DNA sequencing will be performed in the future to confirm these presumptive identifications.

Gammarus samples were grouped in the manner that they were due to a lack of specimens collected at site 2. The highest concentrations were only 3.12 CFU/Gammarid, which is low compared to the concentrations in water and sediment. However, this remains consistent with the idea that sediment often contains some of the highest environmental *Vibrio spp.* concentrations. Perhaps a cascading effect is occurring, with *Vibrio spp.* preferentially choosing certain reservoirs such as *G. vermiculophylla*, and physically coming into contact with invertebrates such as *Gammarus*, but resulting in minimal attachment. Mechanisms have been proposed for chitin attachment within the *Vibrio* genus, which makes surface attachment much more likely for organisms of this size, rather than direct consumption of the bacteria (Williams et al., 2014). Since only the surface of the *Gammarus* samples were tested for the presence of *Vibrio spp.*, it is possible that *Gammarus* could correlate to higher numbers of *Vibrio spp.* internally, that were not observed in this study. Proportions of sucrose fermenting and non-sucrose fermenting *Vibrio spp.* from sample sites for *G. vermiculophylla* seem fairly consistent with the proportions found in the corresponding *Gammarus* samples, making this physical transfer appear a possible occurrence. It was expected that proportions in sediment samples would also correspond with those found in the other sample types; however, the relative lack of non-sucrose fermenting *Vibrio spp.* from sediment makes it difficult to support any direct association. The S1A *Gammarus* samples display a similar pattern to the sediment samples, but it is difficult to determine whether this is significant or coincidental.

With only 1-3 CFU of *Vibrio spp.* per *Gammarus* on average, it is likely that local birds eating a diet with relatively low *Vibrio spp.* concentrations would drop feces without culturable numbers of *Vibrio spp.* However, considering the varying freshness of the fecal samples, it is also possible that some *Vibrio spp.* present died due to unfavorable conditions, got diluted from periodically rising tides, or entered a VBNC state, making it difficult to obtain any culturable bacteria from the obtained fecal samples. While culturable *Vibrio spp.* have been obtained from birds in studies at different locations and times of year, it is possible that the birds in this region are eating prey with minimal or no *Vibrio spp.* concentrations (Miyasaka et al., 2006). *Gammarus* concentrations are typically highest during the summer months during their breeding season, but it is possible that there is a more abundant food source during this time that birds prefer to eat. Birds could act as a significant vector for spreading pathogenic *Vibrios* in other locations or times of year, but there was no evidence found in fecal samples taken from this location in June to support this theory. Catching and identifying birds while collecting fresh fecal samples could more accurately address this research question in the future.

There appeared to be a considerable contrast in the proportions of sucrose fermenting and non-sucrose fermenting *Vibrio spp.* isolated from the various sample types. The water and *Gracilaria* samples contained approximately equal proportions of sucrose fermenting and non-sucrose fermenting *Vibrio spp.* perhaps due to tidal fluctuations and contact with most other sample types (Figures 2 & 4). However, *Gammarus* and sediment samples contained over twice as many sucrose fermenting *Vibrio spp.* as non-sucrose fermenting *Vibrio spp.* In the case of sediment, 1.35×10^5 CFU/g of sucrose fermenting *Vibrio spp.* were present, compared to only 7.54×10^3 CFU/g of non-sucrose fermenting *Vibrio spp.* (Fig. 3). This suggests that there may be preferential environmental reservoirs for different species of *Vibrio* that could be characterized. In the case of *V. vulnificus* and *V. parahaemolyticus*, which are non-sucrose fermenters, enumeration studies could yield vastly different numbers depending on the sample type and location.

Overall, there were higher concentrations of *Vibrio spp.* at site two in the water and *Gracillaria* samples, compared to the higher *Vibrio spp.* concentrations at site one for the sediment and Gammarus samples. These similarities also appear to be consistent with the apparent trend in species composition. Since the *Gammarus* and *Gracilaria* samples were both washed before being processed, there is minimal possibility that the concentrations present in sediment and water would have skewed the numbers that were observed in the other sample types. These differences could suggest that different species of *Vibrio* spp. are clustered in environmental reservoirs by location, with certain microcosmic conditions being more favorable than others. Additionally, proliferation of any particular species could be isolated to a particular area by sporadic tidal levels or other natural barriers.

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