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Isolation, enumeration and antibiotic profiling of Vibrio vulnificus and V. parahaemolyticus from coastal Virginia

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Isolation, Enumeration and Antibiotic profiling of *Vibrio vulnificus* and *V. parahaemolyticus* from Coastal Virginia

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ABSTRACT

Vibrio vulnificus and *V. parahaemolyticus* are gram-negative, halophilic bacteria that reside in estuarine waters and are associated with infections in humans. These bacteria can cause gastroenteritis through their presence in raw fish and shellfish consumed by humans. *V. vulnificus* can also produce wound infections leading to severe septicemia, and in some cases, death if not treated promptly. With increasing incidence of infections due to these two organisms, research efforts have focused on potential reservoirs and environmental conditions that can increase human exposure to, and infection, with these species of bacteria. This study was conducted in order to examine the role of *Gracilaria*, a non-native invasive algal species, as a potential reservoir of *V. parahaemolyticus* and *V. vulnificus*. Water and sediment samples were collected weekly in triplicate from mud flats of coastal Virginia for a six week period from early July to mid-August. Plots were set up as two treatments — undisturbed and *Gracilaria* mats removed. Samples were taken for three weeks prior to algal removal to establish baseline data. Subsequently, algae were removed from half of the plots, and samples were collected for another three weeks. All samples were processed and analyzed by means of dilution, vacuum filtration, and plating on differential media in order to accurately determine the abundance of *Vibrio* spp. on the coastal flats. DNA was extracted, from "presumptive" *Vibrio* spp., and amplified by Polymerase Chain Reaction (PCR) using primers specific for the *vvhA* gene (*V. vulnificus*) and the *tdh* gene (*V. parahaemolyticus*) to confirm identification of isolates. A resistance profile was developed for confirmed isolates using a 12 antibiotic panel. The removal of *G. vermiculophylla* from the intertidal mudflats did not have a significant impact on the concentration of *V. parahaemolyticus* or *V. vulnificus* in either water or sediment. All *V. vulnificus* isolates (n=181) tested in this study were resistant in some aspect, intermediate or resistant, to all 12 antibiotics

tested. Additionally, 68.51% of *V. vulnificus* and 98.25% of *V. parahaemolyticus* tested were resistant in some aspect to multiple antibiotics. Future studies should aim at sampling over a longer period and including more sampling areas so that the association between the Vibrio species with the algae can be better understood and also give a more in depth picture of the development of antibiotic resistance among the same.

INTRODUCTION

Vibrio vulnificus and *V. parahaemolyticus* are halophilic organisms found in warm estuarine environments, including those along the eastern coast of the United States. First isolated from blood culture samples sent to the Centers for Disease Control and Prevention (CDC), *V. vulnificus* is differentiated from other *Vibrio* spp. by its ability to ferment lactose and by its higher tolerance for sodium chloride (Hollis *et al.* 1976). The first human isolate of *V. vulnificus* was recovered from an infected patient and called 'Lactose-positive *Vibrio* (Hollis *et al.* 1976). Lactose positive *Vibrio* was formally described as *Beneckea vulnifica* (Reichelt *et al.* 1976) and later transferred to *Vibrio* by Farmer *et al.* (1979) due to updated *Vibrio* spp classifications. *V. parahaemolyticus* was first discovered by Tsunesaburo Fujino after a shirasu food poisoning outbreak in 1950, but at that time the isolate was named *Pasteurella parahaemolytica* (Shinoda *et al*. 2011). Both *V. vulnificus* and *V. parahaemolyticus* are gram-negative bacteria that have a "curved rod" shape, prefer alkaline environments, and temperatures ranging from 8–31°C. Conditions such as these exist along the Gulf and eastern coasts of the United States near sites of high commercial and recreational use. Coastal Virginia provides the "perfect storm" as both of these species can exist in the estuarine environment. The usage of coastal waters in Virginia for recreation and swimming as well as commercial purposes, such as harvesting oysters, creates a high risk environment for *Vibrio* infections.

In the United States, *V. vulnificus* is responsible for 95 percent of all seafood-related deaths following ingestion of raw or undercooked seafood (Cantet *et al.* 2013). Moreover, *V. vulnificus* has often been associated with serious infections resulting from exposure of skin wounds to seawater. Different factors have been implicated in virulence of *V. vulnificus*,

including the *vvhA* gene that encodes hemolytic cytolysin (Cantet *et al.* 2013). The virulence of *V. vulnificus* and *V. parahaemolyticus* poses a risk of infection to anyone utilizing estuarine waters or ingesting raw or undercooked seafood. Recent research has sought to identify reservoirs harboring these harmful bacteria (Shaw *et al.* 2014) such as invertebrates, algae, and shellfish.

Gracilaria vermiculophylla is a non-native invasive macro alga from East Asia that has been introduced to environments around the world (Gulbransen *et al.* 2012). *G. vermiculophylla* accumulates on intertidal mudflats where it forms dense mats that remain for months to years due its attachment to *Diopatra cuprea*, a tube building polychaete (Gonzalez *et al.* 2014). The presence of *G. vermiculophylla* in Virginia was first confirmed in 2004 via genetic testing (Gonzalez *et al.* 2014). Gonzalez *et al.* (2014) examined the association between *V. vulnificus* and *V. parahaemolyticus* and *G. vermiculophylla*. This study seeks to further elucidate that association.

V. vulnificus Differentiation

V. vulnificus can be categorized into biotypes (I, II, and II) and ecotypes, representing conditions in which isolates are found and can remain viable (Bisharat *et al.* 1999; Chase and Harwood 2011). Biotypes I and III are primarily causative strains of human infection, whereas biotype II is specifically restricted to eel infections (Horseman and Surani 2010). The ability to ferment mannitol as well as growth temperature ranges separate biotype I and II strains. Biotype II strains will not grow at temperatures above 41°C, whereas biotype I can grow at this temperature (Oliver 2005). Biotype II strains typically lack the ability to ferment lactose and cellobiose and can further be divided into serovars, with only one serovar being linked to human infections (Bisharat *et al.* 1999). Biotype III of *V. vulnificus* has been geographically restricted to

Israel. In addition to biotype and strain characterization, *V. vulnificus* can also be differentiated based on ecotypes; clinical, *vcgC*, versus environmental, *vcgE*. Environmental isolates are favored under conditions which support rapid population growth whereas clinical isolates are better adapted to tolerating higher temperatures in order to remain viable (Rosche *et al.* 2010). Isolated *V. vulnificus* can be induced into a viable but non-culturable (VBNC) state by incubation at low temperatures (Oliver *et al.* 1995), which means the isolate is living but can no longer be cultured on media on which the isolate normally grows. This state presents an issue from a risk standpoint as the bacteria are not detected using growth media. *V. vulnificus* can be resuscitated from this state by increasing the temperature from below 10°C to favorable temperatures between 22–31°C.

V. parahaemolyticus Differentiation

V. parahaemolyticus is determined to either be pathogenic or non-pathogenic by the presence or absence of the virulence genes, thermostable direct hemolysin (*tdh)* or TDH-related hemolysin (*trh2)* (Cantet *et al.* 2013). Thermostable direct hemolysin (*tdh*), a pore-forming protein that contributes to the invasiveness of the bacterium in humans, and its homolog TDH-related hemolysin (*trh*), play a similar role in the disease pathogenesis. The strains isolated from environmental samples usually lack the pathogenic genes *tdh* and/or *trh*, which cause illnesses to humans and marine animals (Letchumanan *et al.* 2014). Most "environmental" *V. parahaemolyticus* strains are considered to be nonpathogenic due to low detection frequencies of *tdh* and *trh*, which is illustrated by detection of the *tdh* and *trh* genes in only 4.3% and 0.3% of environmental *V. parahaemolyticus* strains from highly populated areas of the South Carolina and Georgia coasts respectively (Gonzalez *et al.* 2014). A similar study by Letchumanan *et al.* (2015) found that although *V. parahaemolyticus* is harbored in seafood, not all strains are

pathogenic. The same study also reports that between 0–6% of the environmental samples may contain the hemolysin virulence genes (Letchumanan *et al.* (2015).

Infection and Disease Impact

In addition to humans, invertebrates are also subject to infection from *Vibrio spp*., including commercially farmed shrimp and oysters, and these infections can have a tremendously negative influence on the seafood industry (Molina-Aja *et al.* 2002). *Vibrio* illnesses and infections are becoming a major concern because of their increasing global distribution and their pathogenicity. Because *V. vulnificus* is indigenous in warm marine environments, water quality also has little impact on the risk of infection (Horseman and Surani, 2010). Isolates have been recovered from brackish water coastal areas along the Atlantic, Pacific and Gulf Coasts of the U. S. (Oliver 2005), Alaska (McLaughlin *et al.* 2005), Europe (Dalsgaard *et al.* 1999), Israel (Zaldenstein *et al.* 2008), and several East Asian countries (Oliver 2005). In Asia, *V. parahaemolyticus* is commonly isolated from seafood including shrimp. It has accounted for many food-borne infections throughout Japan, Hong Kong and Thailand, all of which are related to consuming raw seafood. Additionally, not only does consuming infected seafood cause disease, but recreational uses of estuarine waters that harbor *Vibrio* spp. also pose risk of bodily infection. In Virginia, prior research on *V. parahaemolyticus and V. vulnificus* includes studies on the antimicrobial susceptibility patterns of these microbes isolated from commercial and recreational areas located in the Chesapeake Bay and Maryland Coastal Bays (Shaw *et al.* 2014).

These bacteria can cause deleterious health effects such as; gastroenteritis, wound infections, and in some cases death, especially if they are resistant to clinical antibiotics. *V. vulnificus* is a particularly virulent organism that can cause severe wound infections in healthy persons, or causes primary septicemia in persons with preexisting chronic conditions,

particularly liver disease (Oliver 2015). Wound infections from *V. vulnificus* may range from relatively mild to severe, causing rapidly progressive cellulitis and myositis. Wound infections (e.g., injuries sustained while handling of live crabs) can result from exposure to seawater containing the bacterium which can progress to fatal necrotizing fasciitis (Oliver *et al.* 2005). Infections caused by ingestion commonly result in primary septicemia, and almost always require hospitalization. The most common vehicle for this infection in the United States is the consumption of raw or improperly cooked oysters (McLaughlin *et al.* 2005). Contamination with *V. vulnificus* can be difficult to detect in seafood, and especially raw oysters, because there is no noticeable effect on the contaminated food (appearance, taste, or odor). Mortality rates for *V. vulnificus* as a human pathogen are ~50% from food borne infections, and ~22% from cutaneous infections (Oliver 2013). Additionally *V. parahaemolyticus* has been found to be the primary source of vibriosis incidence and is becoming highly pathogenic with serotypes of *V. parahaemolyticus* emerging on a global scale (Newton *et al.* 2012). It is responsible for 20–30% of food poisoning cases in Japan and seafood borne diseases in many Asian countries and is the leading cause of human gastroenteritis associated with seafood consumption in the United States (Letchumanan *et al.* 2014; Newton *et al.* 2012).

Antibiotic Resistance

A study of Vibriosis conducted between 1996–2012 showed that infections due to *V. vulnificus* increased from 0.1 to 0.5 per 100,000 people (Newton *et al.* 2012). The Centers for Disease Control and Prevention (CDC) reported increased incidence of infection via consuming raw or under cooked oysters, and or seafood since 2000 (Han *et al.* 2007). With such an increase in incidence of infections and the severity of infections caused by *Vibrio* spp., the need for antibiotic resistance information is critical. The recommended course of treatment for *V.*

vulnificus infections, recommended by the CDC, is tetracycline for severe infection (Han *et al.* 2007). There are alternative treatments that involve a combination of antibiotics such as broad-spectrum cephalosporins and doxycycline or a fluoroquinolone alone (CDC 2017). The CDC recommends that pediatric infections be treated with a combination of trimethoprim-sulfamethoxazole and aminoglycoside. This regimen of treatment is solely for those for whom fluoroquinolones and doxycycline are not safe (CDC 2017). The CDC does not recommend any antibiotic treatment for *V. parahaemolyticus*, advising only to drink plenty of fluids to replace lost fluids from vomiting and diarrhea (CDC 2006).

This study aims to determine whether *V. vulnificus or V. parahaemolyticus* isolates from the east coast of Virginia are susceptible to a range of antibiotics used to treat infections. This study advances previous work by Conrad (2015) who utilized twelve antibiotics associated with treatment of *Vibrio* infections and a similar study using eight similar antibiotics also associated with treatment of *V. vulnificu*s and *V. parahaemolyticus* infections in coastal Louisiana (Han *et al.* 2007). Over the past few decades, several bacterial genera have become resistance to antibiotics. This evolutionary response is primarily due to excessive use of antimicrobials in humans, agriculture and aquaculture. With the increase in incidence of *Vibrio* infections, it becomes imperative that these organisms isolated from the environment are tested to confirm the efficacy of currently recommended antibiotics and for signs of antibiotic resistance (Han *et al.* 2007). This study will develop an antibiotic resistance profile for each isolate that contain virulence genes; *tdh* for *V. parahaemolyticus* and *vvh* for *V. vulnificus*.

Antibiotic resistance profiles can be generated using the standard Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute M02-A11). The cleared spaces, zones of inhibition, surrounding the disks after incubation, are measured via the BIOMIC™

(Giles Scientific Inc., Santa Barbara, CA) automated reading system. The diameters serve as the resistance "fingerprint" of the bacterium, and indicate its sensitivity to the antibiotic. This analysis will help determine whether increased antibiotic resistance is occurring in the coastal environment and if so, to what antibiotics is resistance being developed. This could also lead to the reduction in exposure to these harmful pathogens in commercial and recreational waterways through increased education of the public of the potential risk of infection (CDC, 2006).

Research Proposal Summary

V. vulnificus and *V. parahaemolyticus* are pathogenic bacteria that are widely spread in marine and estuarine environments. They pose direct harm to humans via wound infections and the consumption of undercooked or mishandled seafood. Even though infection rates have been on the rise since the 1990's with a reported 78% increase in *V. vulnificus* wound infections between 1996 and 2006 (Froelich and Daines 2020), *Vibrio* infections became nationally notifiable by the CDC in 2007 only. Tack *et al.* (2019) also reported that just between 2015 and 2018, the number of *Vibrio* infections in the United States increased by a staggering 311%. *V. vulnificus* wound infections increased by 272% between 2008 and 2018 and infections due to shellfish consumption are also on the rise (Froelich and Daines, 2020). *V. parahaemolyticus* infections have also been increasing, being implicated in outbreaks in the Pacific North West in 2012 and also along the Atlantic coast in 2014 and 2016 (Froelich and Daines, 2020). Infections can potentially be fatal, in particular if not treated quickly and properly. Warmer waters promote the growth and abundance of these two species of *Vibrio*; however other known ecological factors can also contribute to increased abundance. Crustaceans, mollusks, algae, sediment, and water-birds have all been implicated as potential reservoirs, hosts, and/or vectors for the bacteria. Gonzalez *et al.* (2014) demonstrated a relationship between the invasive macroalga, *Gracilaria*

vermiculophylla, and the abundance of the pathogenic *Vibrio* spp. *G. vermiculophylla* has become a dominant species of macroalga in many shallow intertidal and sub-tidal areas throughout Virginia's coast. Ocean side lagoons of the Eastern Shore have an active and growing hard clam (*Mercenaria mercenaria*) aquaculture as well as some oyster culture and natural oyster harvest. Aquaculture production is economically significant as 2013 sales of cultured clams and oysters were more than $36 million in adjacent Northampton County (Murray 2014).

Given the potential role of *G. vermiculophylla* in facilitating *Vibrio* abundance, this study examines the association of this macroalga with environmental *Vibrio* spp. The hypothesis is that *G. vermiculophylla* changes the concentration for *V. vulnificus* and *V. parahaemolyticus* in the surrounding water and sediment. The study will examine if removal of the algae changes the distribution of *Vibrio* in the surrounding water and sediment and potentially increase the risk of infection (Williams *et al.* 2014).

Research Questions:

1. Are *V. vulnificus* and *V. parahaemolyticus* species associated with the alga *G. vermicullophylla*?
    a. If so how do their concentrations on the alga compare with those in the surrounding water and sediment?
2. Would *Gracilaria* removal from a mudflat decrease the concentrations of *V. vulnificus* and *V. parahaemolyticus* associated with the mudflat?
3. Are any of the *Vibrio* spp., isolated from water and sediments resistant to antibiotics used to treat infection?

OBJECTIVES OF THIS STUDY

1. Investigate Virginia coastal waters for abundance of *Vibrio* spp. associated with *G. vermiculophylla*, water, and sediment and enumerate presumptive *Vibrio* spp. isolates from those sample sources.
2. Qualitatively analyze presumptive isolates, via selective media and PCR to confirm *V. vulnificus* and *V. parahaemolyticus* abundance in the environmental samples
3. Build antibiotic resistance profiles for confirmed isolates of both *V. vulnificus* and *V. parahaemolyticus*

METHODS

Site preparation and maintenance for algal removal

The 6-week field experiment was conducted at a large mudflat in Oyster Harbor (Figure 1), semi-enclosed by oyster reef and *Spartina alterniflora*. The mudflat is heavily vegetated with *Gracilaria*, *Ulva*, and other, but less abundant, species of seaweed.

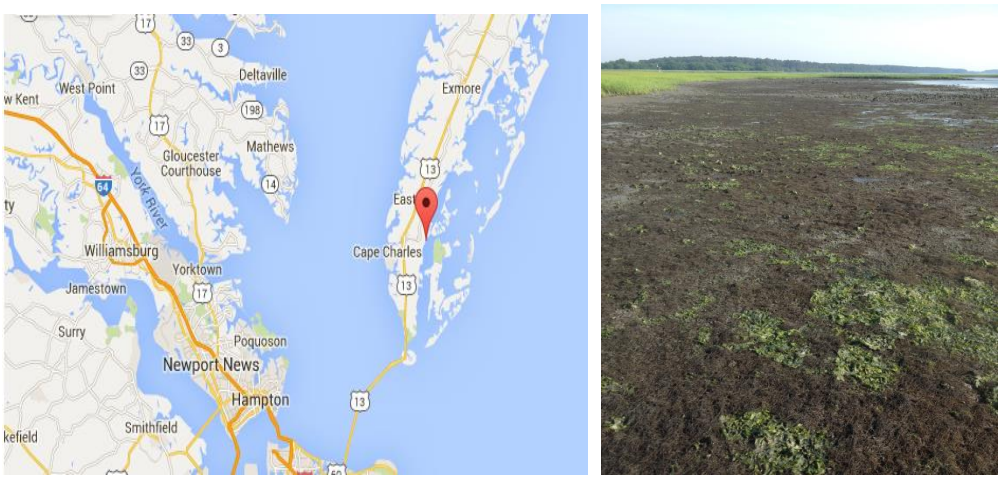

Figure 1. Geographic location of the University of Virginia Long Term Ecological Research Site (A). Dense *G. vermicullophylla* algal mat at the UVA LTER site (B).

The sample area was divided into two adjacent study plots, each measuring approximately 40 x 50m. During Week 3, one plot was cleared of algae using rakes to scrape the mudflat and by hand-picking remaining pieces. Diopatra worm tubes were also removed because they incorporate algae into their structure. Chicken wire fencing was put up around the border of the plot to prevent introduction of new algae onto the flat. Before and after algal removal, weekly collections of three replicate samples of seawater and three composite replicate samples of sediment were made from both plots at mid-tide for *Vibrio* enumeration for 6 weeks. Each week the flat was checked for algal growth and the fencing was cleared of any sediment or algae accumulation. The raking activities disturb the sediment, and likely the diatoms that help to bind the sediment and prevent erosion. Because there is evidence that sediment may be a significant reservoir for *Vibrio* (Gonzalez *et al.* 2014), and this disturbance may lead to temporary increases in suspended sediment, the plots were given a week to recover before another sample collection for *Vibrio wa*s taken.

Sample Collection:

All environmental samples were collected by Alice Besterman (Ph.D candidate, Department of Environmental Sciences at the University of Virginia) during the summers of 2015 and 2016 (6 July–10 August). Water and sediment sampling was conducted at the Virginia Coastal Reserve Long Term Ecological Research (LTER) site. Samples were placed in individual plastic bags, stored on ice for 4 hours while transported to James Madison University. Samples were processed at James Madison University. Serial dilutions of sediment and algae were made at concentrations of 10^{-4} and 10^{-3}, respectively. Each dilution was filtered via membrane filtration.

Membrane Filtration:

Samples were filtered via vacuum pump membrane filtration using 0.45 µm cellulose nitrate filter papers. For each sample, the filter was placed on the filtration apparatus, 10mL of sterile Phosphate Buffered Saline (PBS) was pipetted into the filter housing, the filter housing was opened and the PBS was pulled through the filter until all liquid was removed. The filter housing was then closed and the sample volume was pipetted into the same filter housing. The housing valve was opened again to allow the sample to flow through the filter, after which the housing was rinsed with PBS to ensure the entire sample passed through the filter. After the filter housing was rinsed and liquid removed and the housing was reclosed, the filter was removed and placed on one of various selective and differential media.

Isolation and Confirmation of Isolates:

Due to the natural presence of multiple *Vibrio* spp in estuarine environments, differentiation of species can be difficult. Many species have the ability to ferment the same sugars. *Vibrio* spp. are tolerant to alkaline pH; therefore, selective media used for this pathogen are often prepared at pH 8.6–pH 9.4, with the addition of 1–7% sodium chloride (NaCl) (Letchumanan *et al.* 2014).

Thiosulfate-citrate-bile-salts-sucrose (TCBS) agar is a medium used for isolating pathogenic *Vibrio* species. TCBS is a selective medium consisting of ox bile (0.8%), NaCl (1%) and alkaline pH 8.6, which suppresses growth of interfering gram-positive organisms. The main advantage of TCBS agar is its sucrose/bromothymol blue diagnostic system which differentiates sucrose-positive *Vibrio*s such as *V. cholerae* from other *Vibrio* species (Letchumanan *et al.* 2014). Sucrose fermenting colonies, such as *V. cholerae* and *V. alginolyticus* appear yellow, and

non-sucrose fermenting colonies, such as *V. parahaemolyticus* and *V. vulnificus* appear green on TCBS media (Fig. 2B and 2D); however some species of *V. vulnificus* can appear yellow.

Since both *V. vulnificus* and *V. parahaemolyticus* appear green, it is difficult to distinguish between the two species or enumerate them using TCBS alone. CHROMagar *Vibrio* (CaV), a similar medium, allows for the discrimination of *Vibrio* species based on the ability to metabolize chromogenic substrates (Nigro *et al.* 2015). *V. vulnificus* colonies appear turquoise/blue in color (Fig. 2C), and *V. parahaemolyticus* appear mauve/pink on CaV (Fig. 2E). Since the intensity of color as well as the size and shape varied considerably for the turquoise/blue colonies of *V. vulnificus,* all colonies were subjected to an additional confirmation step by streaking on *Vibrio vulnificus* Agar (VVA) on which *V. vulnificus* produce yellow colored colonies (Fig. 2A). Since *V. cholerae* colonies can also appear blue on CaV and some *V. vulnificus* colonies can appear green on TCBS, confirmation by a molecular method such as Polymerase Chain Reaction (PCR) becomes necessary. All colonies exhibiting flat, turquoise-colored morphology on CaV and yellow colony on VVA (for *V. vulnificus)* and mauve/pink colored colonies (for *V. parahaemolyticus)* were isolated and confirmed using PCR.

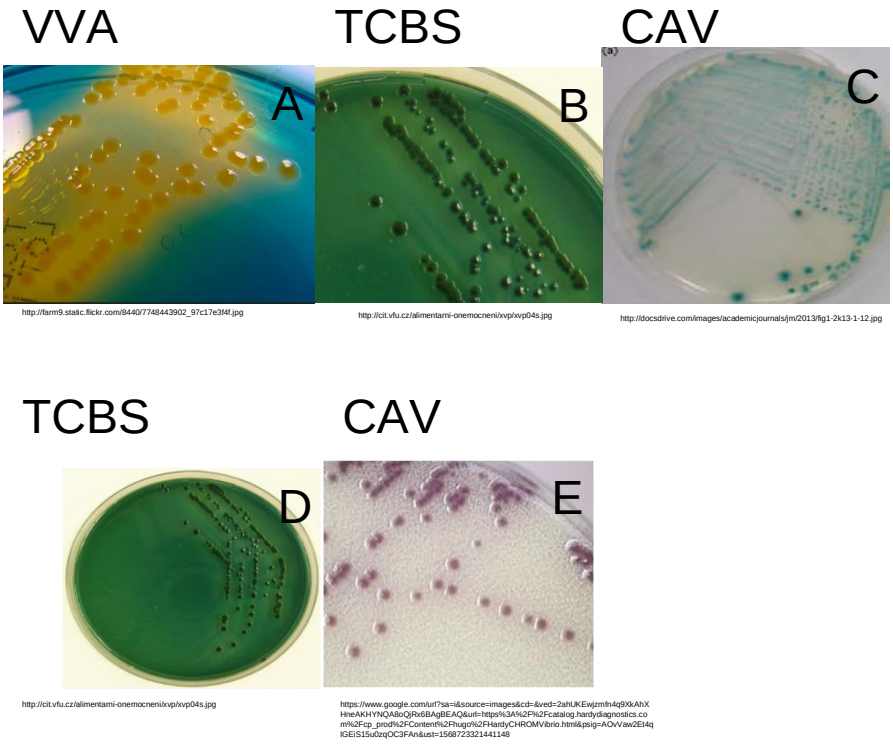

Figure 2. Typical appearance of *V. vulnificus* on *Vibrio vulnificus* agar (A), thiosulfate citrate bile salts sucrose agar (B), and CHROMagar™ Vibrio (C). Typical appearance of *V. parahaemolyticus* on thiosulfate citrate bile salts sucrose agar (D), and CHROMagar™ Vibrio (E)

Polymerase Chain Reaction (PCR) for confirmation of isolates:

The species specific gene, *vvhA* (205 bp) was targeted to detect *V. vulnificus*, and the *tdh* gene (265 bp) was targeted for *V. parahaemolyticus.* DNA was extracted from presumptive isolates after growing in 5ml of TSB containing 2% NaCl and incubating for 24 hours at 35^0C using the Ultraclean Microbial DNA isolation Kit. Template DNA, primers, and commercially available master mix were mixed in amounts consistent with the protocol followed by Conrad (2015). *V. vulnificus* PCR cycle parameters were as follows: 94°C for 120s and 30 cycles of: 94°C for 15s, 56°C for 15s, and 72°C for 25s followed by an infinite hold at 4°C. Settings for *V. parahaemolyticus* were identical to those for *V. vulnificus* with the exception of the annealing

temperature, which was set for 55°C (Panicker *et al.*, 2004). Gel Electrophoresis was performed to confirm amplification of each DNA sample, and agarose gels were stained with ethidium bromide to visualize results (Figure 3).

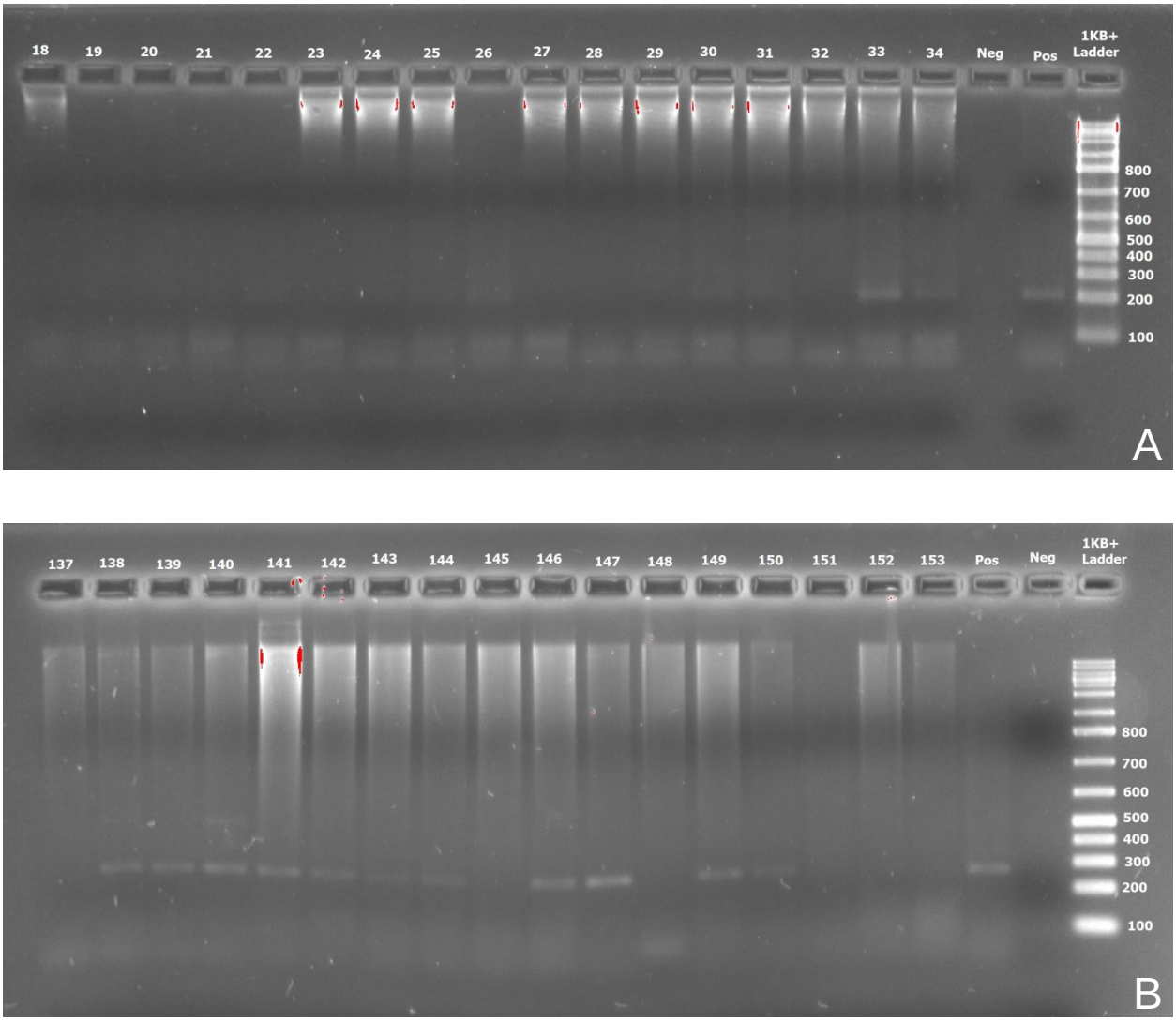

Figure 3. Agarose gel image of *V. vulnificus* isolates 18-34 and vvh primers (A) and *V. parahaemolyticus* isolates 137-153 with tdh primers (B), along with a 1 kb plus DNA ladder. Positive and negative controls are labeled on the wells.

Storage of Isolates

All confirmed *Vibrio spp.* isolates were transferred to cryogenic storage vials (2mL, 66008-284; VWR, West Chester, PA). The *Vibrio spp* isolates were first transferred from selective media to 5 ml of BD Difco™ Tryptic Soy Broth (TSB) (211825; Becton, Dickenson and Company, Sparks, MD). Cultures were placed in a rotary incubator and incubated at 35°C for 16–18 h. After incubation, a 500 µl culture sample was pipetted into a cryovial, followed by the addition of 500 µl of sterile glycerol, gently mixed, and placed in a vial box holder. The vial box was stored at -80°C. A duplicate vial of each *Vibrio spp. isolate* were made and placed in a separate -80°C freezer in case of reactivation failure.

Preliminary Sampling Study:

The 2015 sampling included collecting samples from two separate sites and collecting each type of sample listed above from each site. Sample processing, colony identification, DNA extraction and confirmation by PCR was done as described above.

Algal Removal Study:

Based on the association of *Vibrio* with algae (Gonzalez *et al.* 2014), a removal study was conducted to determine effect on *Vibrio* concentration. To test the hypothesis that removal of *Gracilaria* would change the concentration, six sites were chosen to measure the concentration of *V vulnificus* and *V. parahaemolyticus* for three weeks to provide a baseline concentration for each site. After the three weeks of sampling, the *Gracilaria* spp was removed from three sites while keeping the other three sites undisturbed. This allowed for a comparison of the *V. vulnificus* and *V. parahaemolyticus* levels before and after removal from the three sites to the three untouched sites. Water and sediment samples were collected from each site from 06

July– 10 August 2016. Sample sites are listed as AC1S–AC3S for control sediment sites and AR1S–AR3S for removal sediment sites. Water sites are listed as AC1W–AC3W for control water sites and AR1W–AR3W for removal water sites. Sample processing, colony identification, DNA extraction and confirmation by PCR was done as described above.

Antibiotic Resistance:

To develop an Antibiotic Resistance Profile for each sample, twelve antibiotics representing different classes and modes of action at specific concentrations were selected (Table 1). Antibiotic resistance analyses were conducted based on Clinical and Laboratory Standards Institute's guidelines in M02-A11 (2012). Isolation streaks of each confirmed isolate were performed on TSA plates incubated at 35°C for 18–24 hours. After incubation, isolated colonies were suspended in TSB broth media and the broth was adjusted to achieve a turbidity equivalent of 0.5 McFarland standard. This was confirmed by using an $O.D_{600}$ (optical density) of 0.08–0.1. A sterile cotton tipped applicator was used to inoculate a 150 mm Mueller Hinton agar (MHA) plate. The surface was swabbed three times, rotating the plate one third of a turn between each pass, and then allowed to dry with the lid ajar for 3–5min. Twelve antibiotic disks (Becton Dickinson) were applied with an antibiotic disk stamper. Plates were allowed to sit for 15min before being inverted and incubated 16–18h at 35°C. Control cultures were used for antibiotic quality control. Plates were read with a BIOMIC V3 imager and interpreted using CLSI standards for *V. vulnificus* and *V. parahaemolyticus*.

Table 1. Drug Panel of twelve antibiotics used to build antibiotic resistance profile for each isolate. The CDC recommended antibiotic to be used for treatment of infections is marked with,'*'.

Antibiotic	Abbreviation	Class	Concentration (µg)
Ampicillin	AM	Penicillin	10
Piperacillin-tazobactam	TZP	Beta-Lactam+ Beta-lactamase Inhibitor	100
Cefepime	FEP	Cephalosporin, 4th Gen	30
Ceftriaxone*	CRO	Cephalosporin 3rd gen	30
Imipenem	IPM	Carbapenem	10
Meropenem	MEM	Carbapenem	10
Amikacin*	AN	Aminoglycoside	30
Gentamicin*	GM	Aminoglycoside	10
Tetracycline*	Te	Tetracycline	30
Ciprofloxacin*	CIP	Fluoroquinolone	5
Chloramphenicol	C	Amphenicol	30
Sulfamethoxazole Trimethoprim*	SXT	Folate Pathway Inhibitor	23.75 /1.25

Statistical Analysis:

All statistical analyses to compare *V. vulnificus* (Vv) and *V. parahaemolyticus* (Vp) concentrations were done using ANOVA via interaction plots. Due to samples having skewed distributions, t-tests were used to provide more power in the comparison analysis of control and removal sites.

Results

Preliminary Sampling Study:

V. vulnificus and *V. parahaemolyticus* are found in estuarine waters of the coastal regions of the United States during the warmer summer months of the year. Selective, colorimetric media were utilized for isolation and enumeration of both *Vibrio* species. The concentration of both species of *Vibrio* in water, sediment and *Gracilaria* collected from two separate sites are presented in Tables 2 and 3, respectively.

Site 1 showed low association of *V. vulnificus* with sediment and *Gracilaria* and higher concentrations in the water (Table 2). *V. parahaemolyticus* was found in higher concentrations in sediment and *Gracilaria* samples than in water samples.

Table 2. Total number of *V. vulnificus* and *V. parahaemolyticus* isolated from each sample type from Sample Site 1 in 2015.

Sample Type	Total *V. vulnificus*	Total *V. parahaemolyticus*
Water	4.0 X 10^2/100ml	3.0 X 10^1/100 ml
Sediment	low #s none confirmed	1.39 X 10^4/gdw
Gracilaria	low #s no confirmed	6.67 X 10^3/g

Site 2 had higher concentrations of *V. vulnificus* overall than Site 1 with the highest concentration being in sediment than in water and *Gracilaria* samples (Table 3). *V. parahaemolyticus* was associated more with water and *Gracilaria* with only very low numbers in sediment samples.

Table 3. Total number of *V. vulnificus* and *V. parahaemolyticus* isolated from each sample type from Sample Site 2 in 2015.

Sample Type	Total *V. vulnificus*	Total *V. parahaemolyticus*
Water	4.2 X 10^2/100 ml	1.24 X 10^3/100 ml
Sediment	2.04 X 10^3/gdw	low #s none confirmed
Gracilaria	6.0 X 10^2/g	1.37 X10^3/g

These numbers indicate that there is indeed an association of *Vibrio* spp. with each sample type and the LTER site could be used for further sampling. The results were also consistent with finding presumptive *V. vulnificus* and *V. parahaemolyticus* isolates from water, sediment and *G. vermiculophylla* evidenced in Gonzalez *et al.* (2014).

Algal Removal Study:

***V. parahaemolyticus* concentration in Water and Sediment**

Water

V. parahaemolyticus concentration in water samples before and after removal of the algae are shown in Figure 4. The plots are presented by sample date comparison (Fig. 4A) and by sample site comparison (Fig. 4B). The concentration of *V. parahaemolyticus* in water when sorted by the date that samples were taken, and by the site that the sample where the sample was taken. Concentrations of *V. parahaemolyticus* for both sample site and sample date ranged from 1 cfu/mL to 12 cfu/mL. For comparison of *V. parahaemolyticus* concentration for time and treatment of each sample site, a two-way ANOVA was used for statistical analysis.

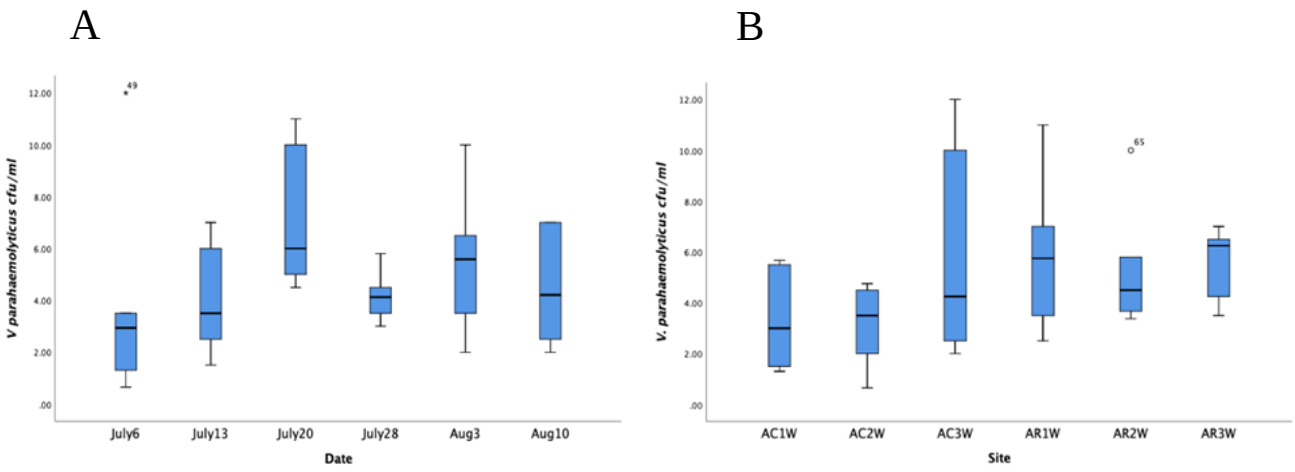

Figure 4. *V. parahaemolyticus* concentration in water samples by sample date (A) before removal (July 6th – July 20th) and after removal (July 28th – Aug 10th), and by sample site (B): AC1W-AC3W (control) and AR1W-AR3W (removal). The numbers represent a specific sample in the data set and the "*" and "°" represent outliers of the data set.

A two-way ANOVA analysis of the data shows that there is no statistical difference ($p=0.52$) between the concentrations of *V. parahaemolyticus* in water before or after removal of the algae. However, because of the assumptions of normal distribution and equal variances for

two-way ANOVA, which was not true in this study because of small sample size, further analysis with t-tests was done. For populations with non-equal variances, the Welch t-test which compares the concentrations of the "Before" groups with and without treatment, and the "After" groups with and without treatment for significant differences was employed. The analysis found no significant differences between treatments in the "Before" group ($p= 0.588$), however a significant difference ($p=0.02$) was noticeable between the treatments in the "After" group. This means that the removal of the algae from the mudflats resulted in a change in the concentration of the *V. parahaemolyticus* in water. To test the second assumption of normality violation, Wilcoxon ranked sum t-tests were utilized given the small sample size for each sampling. This analysis compared the "No treatment" and "Treatment" bars in the "Before" and "After" groups (Figure 5), which showed that there was no difference between the "Before" ($p= 0.678$) and "After" removal sites ($p=0.91$).

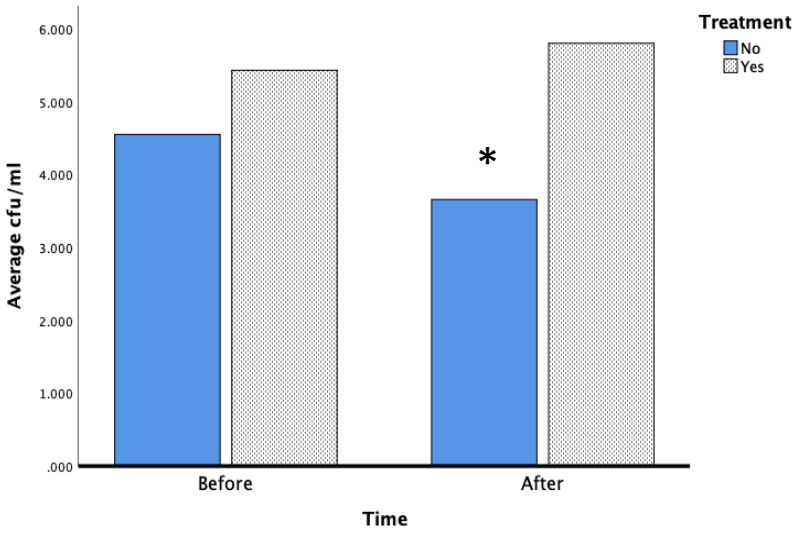

Figure 5. *V. parahaemolyticus* mean concentration in water samples before and after algae removal. Due to violations of assumptions of ANOVA analysis, Welch t-tests and Wilcoxon ranked sum t-tests were utilized for comparing the non-treatment groups (blue bars) and the treatment groups (gray bars) at the before and after time points. A "*" represents significant difference between treatments.

Sediment

V. parahaemolyticus concentrations in sediment ranged from $3.52x10^2$ cfu/ gram dry weight (gdw) to almost 5.11×10^3 for sample date comparison data (Figure 6A). The sample site data had concentrations ranging from about 4.06×10^2 to just under 3.58×10^3 (Figure 6B). A two- way ANOVA resulted in no significant difference in *V. parahaemolyticus* concentration (*p=0.53*) between time and treatment or due to removal of the algae from the mudflats. Further analysis using the Welch t-tests and Wilcoxon test, also resulted in no significant differences when comparing the non-treatment groups and treated groups in the before and after treatments (Figure 7).

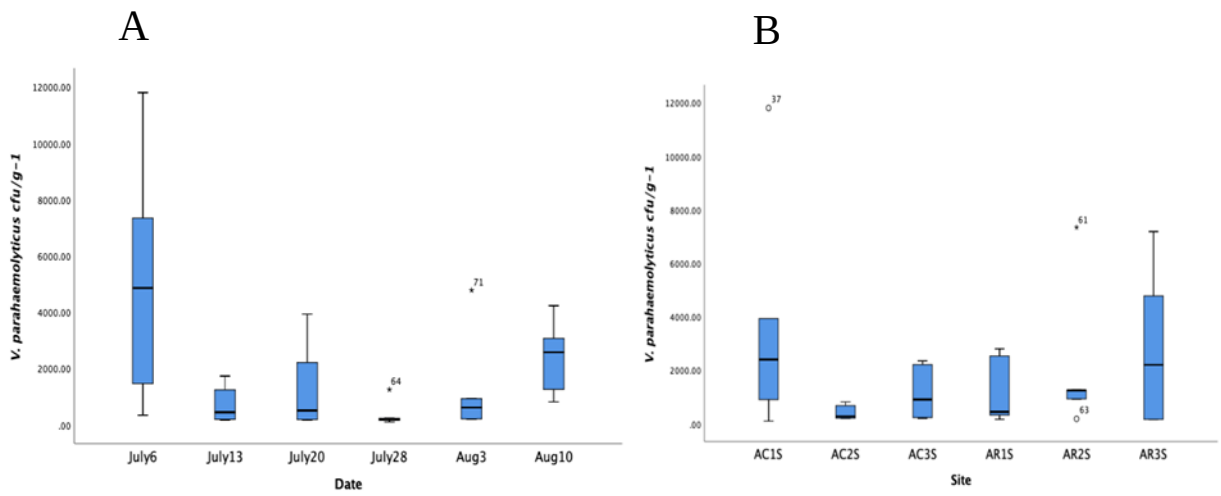

Figure 6. *V. parahaemolyticus* concentration in sediment samples by sample date (A) before removal (July 6th – July 20th) and after removal (July 28th – Aug 10th), and by sample site (B): AC1W-AC3W (control) and AR1W-AR3W (removal). The numbers represent a specific sample in the data set and the "*" and "°" represent outliers of the data set.

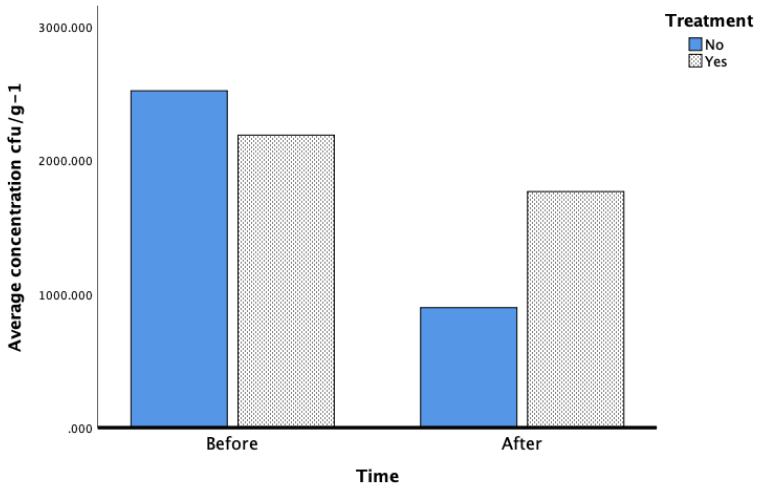

Figure 7. *V. parahaemolyticus* mean concentration in sediment samples before and after impact of algae removal. Due to violations of assumptions of ANOVA analysis, Welch t-tests and Wilcoxon ranked sum t-tests were utilized for comparing the non-treatment groups (blue bars) and the treatment groups (gray bars) at the before and after time points. A "*" represents significant difference between treatments.

V. vulnificus Concentration in Water and Sediment

Water

V. vulnificus concentration in water samples before and after removal of the algae are shown in Figure 8. Concentrations of *V. vulnificus* for both sample date (A) and sample site (B) ranged from 0.1 cfu/mL to 90 cfu/mL. The concentration of *V. vulnificus* in the water samples dropped to almost none in the last three sampling dates; July 28th, Aug. 3rd, and Aug. 10th (Fig. 8A).

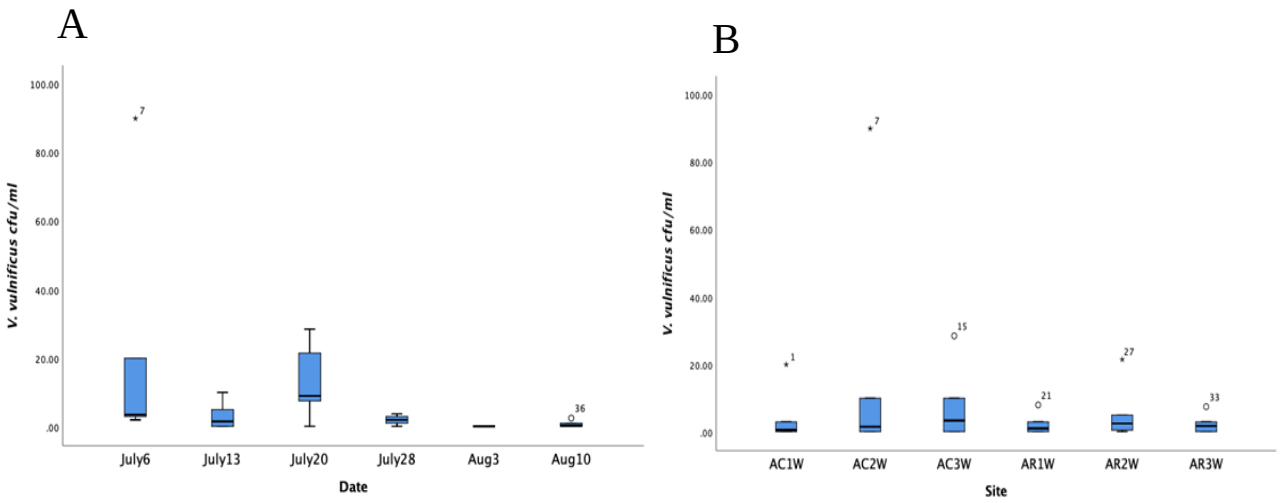

Figure 8. *V. vulnificus* concentration in water samples by sample date (A) before removal (July 6th – July 20th) and after removal (July 28th – Aug 10th), and by sample site (B): AC1W-AC3W (control) and AR1W-AR3W (removal). The numbers represent a specific sample in the data set and the "*" and "°" represent outliers of the data set.

Just as for *V. parahaemolyticus*, to do a comparison of the concentration for time and treatment of each sample site, a two-way ANOVA was performed which showed that the removal of the algae had no significant impact on the concentration of *V. vulnificus*. The Welch t-test, for populations with non-equal variances also resulted in no significant differences in the *V. vulnificus* concentration between both non-treated and treated before ($p=0.254$) and after ($p=0.292$) groups (Fig. 9). With the Wilcoxon test, there was no significant difference when comparing the concentration of *V. vulnificus* in the non-treated populations before and after algal removal. However, there was a significant difference when comparing treated ($p=0.012$) and non-treated groups over time indicated by the "*" in Figure 9.

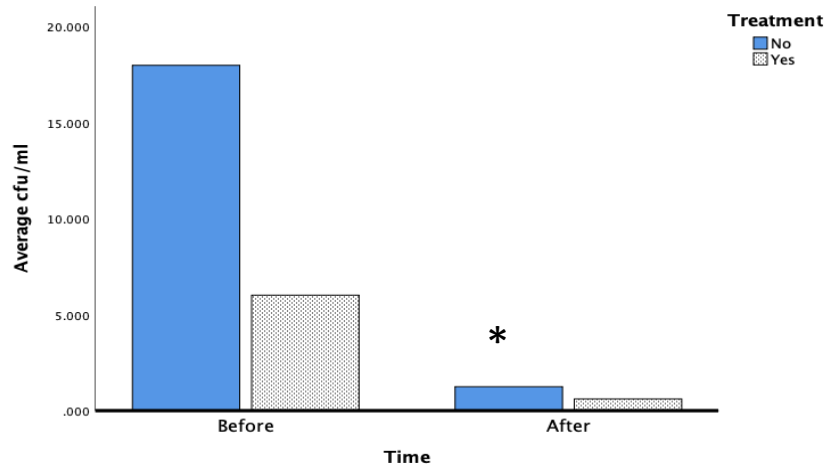

Figure 9. *V. vulnificus* mean concentration in water samples before and after algae removal. Due to violations of assumptions of ANOVA analysis, Welch t-tests and Wilcoxon ranked sum t-tests were utilized for comparing the non-treatment groups (blue bars) and the treatment groups (gray bars) at the before and after time points. The "*" represents significant difference between treatments.

Sediment

Sediment concentrations for *V. vulnificus* ranged from 6.84 $x10^2$ cfu/ gram dry weight (gdw) to 1.160 $x10^4$ cfu/gdw for sample date data (Fig. 10A). The sample site data showed concentrations ranging from about 2.329$x10^3$cfu/gdw to 6.99$x10^3$cfu/gdw (Fig. 10B). Two-way ANOVA analysis for *V. vulnificus* concentration in the sediment showed that there was no significant impact ($p=0.32$) of the removal on the algae. The Welch t-tests, for non-equal variances, comparing time and treatment resulted in both the before and after time point having no significant differences ($p=0.47$, and $p=0.403$), respectively. When looking at the Wilcoxon ranked sum t-tests for non-normal populations, for the non-treatment groups before and after removal of the algae, there was a significant difference, $p= 0.05$. When looking at the treated group before and after the impact of removal, there was no significant difference, $p= 0.37$ (Figure 11).

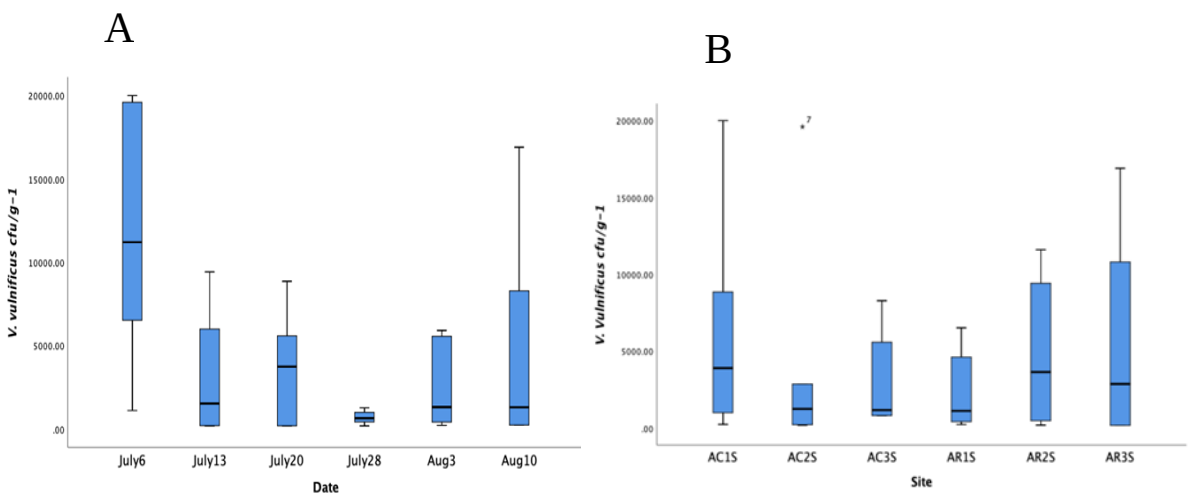

Figure 10. *V. vulnificus* concentration in sediment samples by sample date (A) before removal (July 6th – July 20th) and after removal (July 28th – Aug 10th), and by sample site (B): AC1W-AC3W (control) and AR1W-AR3W (removal). The numbers represent a specific sample in the data set and the "*" and "°" represent outliers of the data set.

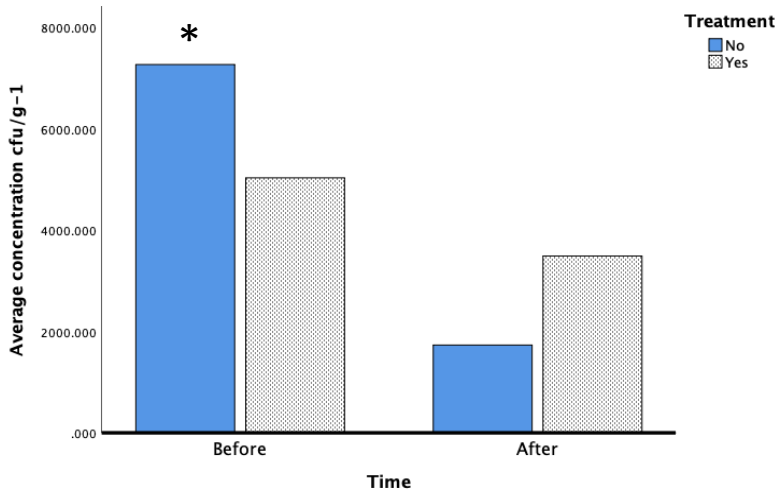

Figure 11. *V. vulnificus* concentration in sediment samples before and after impact of algae removal. Due to violations of assumptions of ANOVA analysis, Welch t-tests and Wilcoxon ranked sum t-tests were utilized for comparing the non-treatment groups (blue bars) and the treatment groups (gray bars) at the before and after time points. The "*" represents significant difference between treatments.

Antibiotic Resistance:

The objective of this part of the study was to determine if any of the *V. vulnificus or V. parahaemolyticus* isolated and confirmed by PCR were susceptible to antibiotics commonly used to treat infections and to develop an antibiotic resistance profile for each isolate that contains genes of pathogenicity; *tdh* for *V. parahaemolyticus* and *vvh* for *V. vulnificus*. Antibiotic resistance profiles were generated using the standard Kirby-Bauer disk diffusion method (Clinical and Laboratory Standards Institute M02-A11).

***V. vulnificus* antibiotic resistance**

All *V. vulnificus* isolates (n=188) taken and confirmed from the removal study were tested using a 12 antibiotic panel (Table 1) used by Conrad (2015). Several of the antibiotics used were selected based on CDC recommended treatment, including folate pathway inhibitors, quiniolones and tetracyclines for *Vibrio* infections. Of the 188 total *V. vulnificus* isolates, five were totally susceptible, 52 isolates exhibited single antibiotic resistance, 53 isolates showed resistance to two antibiotics, 56 isolates showed resistance to three antibiotics and 15 isolates showed resistance to four or more antibiotics. In total 97.24% of the isolates tested showed resistance to one or more antibiotics, with only 2.76% accounting for susceptible isolates.

Among the antibiotics recommended for treatment for *V. vulnificus* by the CDC, only 1.10% of the isolates exhibited resistance to amikacin and only 0.55% showed resistance to gentamicin (both belonging to the aminoglycosides group), 0.55% exhibited resistance towards tetracycline, while 2.76% had resistance against Ciprofloxacin (a fluoroquinolone). All *V. vulnificus* isolates were susceptible to the combination of trimethoprim-sulfamethoxazole (Table 5). However, almost 72% of the *V. vulnificus* isolates had resistance against ceftriaxone (a cell

wall inhibitor), which is concerning because it is also recommended for treatment. Resistance was also noticed against other cell wall inhibitors such as cefepime (58.01%), and ampicillin (28.18%). (Table 5).

V. parahaemolyticus antibiotic resistance

All *V. parahaemolyticus* isolates (n=400) taken from the removal study were also tested using the same 12 antibiotic panel used by Conrad (2015). Of the 400 total *V. parahaemolyticus* isolates seven were totally susceptible, 276 exhibited single antibiotic resistance, 51 showed resistance to two antibiotics, 41 showed resistance to three antibiotics and 25 showed resistance to 4 or more antibiotics. In total 98.25% of the isolates tested showed resistance to one or more antibiotics, with only 1.75% accounting for susceptible isolates.

V. parahaemolyticus isolates showed a similar pattern of resistance to the aminoglycosides, tetracycline, ciprofloxacin and trimethoprim+sulfamethoxazole as was exhibited by *V. vulnificus* (Table 4). *V. parahaemolyticus* also had most of its resistance against the cell wall inhibitors; however it was the highest for ampicillin (95.75%) and much lower for ceftriaxone (10.25%) and cefepime (10.5%) compared to *V. vulnificus*.

Table 4. Antibiotic resistance table for *V. vulnificus* and *V. parahaemolyticus* isolates.

	Antibiotic Resistance for *V. vulnificus* and *V. parahaemolyticus*											
	Penicillins and β-Lactam/β-Lactamase Inhibitors		Cephems		Carbapenems		Aminoglycosides		Tetracyclines	Quinolones	Metabolism Inhibitors	
	Ampicilli	Piperacillin-tazobactam	Cefepim	Ceftriaxone*	Imipenem	Meropenem	Amikacin	Gentamicin*	Tetracycline	Ciprofloxacin*	Chloramphenicol	Trimethoprim-sulfamethoxazole*
V. vulnificus (n=188)												
Susceptible	126	172	72	47	177	180	178	179	180	176	112	180
Intermediate	4	2	4	4	0	0	1	1	0	0	55	1
Resistant	51	7	105	130	4	1	2	1	1	5	14	0
% Susceptible	69.61%	95.03%	39.78%	25.97%	97.79%	99.45%	98.34%	98.90%	99.45%	97.24%	61.88%	99.45%
% Intermediate	2.21%	1.10%	2.21%	2.21%	0.00%	0.00%	0.55%	0.55%	0.00%	0.00%	30.39%	0.55%
% Resistant	28.18%	3.87%	58.01%	71.82%	2.21%	0.55%	1.10%	0.55%	0.55%	2.76%	7.73%	0.00%
V. parahaemolyticus (n=400)												
Susceptible	10	387	357	347	391	400	352	390	398	388	378	395
Intermediate	7	1	1	12	0	0	42	6	2	3	20	4
Resistant	383	12	42	41	9	0	6	4	0	9	2	1
% Susceptible	2.50%	96.75%	89.25%	86.75%	97.75%	100.00%	88.00%	97.50%	99.50%	97.00%	94.50%	98.75%
% Intermediate	1.75%	0.25%	0.25%	3.00%	0.00%	0.00%	10.50%	1.50%	0.50%	0.75%	5.00%	1.00%
% Resistant	95.75%	3.00%	10.50%	10.25%	2.25%	0.00%	1.50%	1.00%	0.00%	2.25%	0.50%	0.25%

Discussion

Preliminary Sampling Study

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 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 and therefore should appear green. This differentiation allowed the initial confirmation that identification of presumptive *Vibrio spp.* was correct according to colorimetric changes. The green colonies were further streaked for isolation on TSA media and then picked from TSA and streaked onto CaV media for the second stage confirmation. On CaV media, blue colonies are typically considered *V. vulnificus* and *V. parahaemolyticus* appear pink/mauve colored. These presumptive identifications are reliable for total *Vibrio spp.* counts, but require additional confirmation at the species level. Slow fermentation of sucrose could lead to false negative results, and densely packed colonies could have been misinterpreted as sucrose fermenting if within close proximity of a neighboring colony. Additionally the colors on CaV media weren't always uniform, potentially making them difficult to interpret. For *V. parahaemolyticus*, a higher confirmation rate was able to be achieved (90–95%), whereas *V. vulnificus* still only had a confirmation percentage of 50–60%. To further confirm these presumptive identifications, PCR targeting the *vvhA* gene for *V. vulnificus* and the *tdh* gene for *V. parahaemolyticus* were performed.

The data facilitated confidence in two areas: species confirmation ability and abundance. These two areas were used for informational purposes of determining the concentration of *Vibrio spp.* located in coastal waters of Virginia at the LTER site of UVA and that identification methods were correct. The hypothesis that *Vibrio spp.* concentration in the water and sediment created this cascading effect, where *Vibrio spp.* attached to invertebrates living in the sediment and water which are then eaten by birds and spread via fecal matter, allowed for further investigation of the area for association with such substrates. It was expected that *Vibrio spp.* were associated with water and sediment (Oliver, 2005, Baker 2008, Johnson 2012, Cantet 2013, however, this study confirmed results from Gonzalez *et al.* (2014) that *Vibrio spp.* were also associated with the non-native algal species *G. vermiculophylla*. Gonzalez *et al.* (2014) reported that *V. vulnificus* and *V. parahaemolyticus* concentration ranges fell within 2–6 log CFU $g^{-1}$for *G. vermiculophylla*. The preliminary study for Virginia LTER site also had *V. vulnificus* and *V. parahaemolyticus concentrations* within the reported range of the Gonzalez *et al.* (2014) study (Tables 2 and 3). The concentration of *Vibrio* spp from both sites in 2015 for *G. vermiculophylla,* were also consistent with their analysis comparison to Mahmud *et al.* (2008). Water and sediment numbers were also within the ranges that were reported by Gonzalez *et al*. (2014). This information was utilized in developing the 2016 algae removal study, with the hypothesis that removing the algae would cause a difference in *Vibrio spp*. concentration in the surrounding water and sediment.

Gracilaria Removal:

V. vulnificus and *V. parahaemolyticus* are frequently found in estuaries of temperate waters with high salinity. The coastal waters of Virginia, specifically the UVA Long Term Ecological Research (LTER) site, provide such a habitat for abundance of these species of

bacteria. Concentrations of these species are elevated in the warmer summer months and reduced during winter months. The pathogenic characteristics of *Vibrio spp*. pose a threat to human health. *V. parahaemolyticus* is a causative bacteria for human acute gastroenteritis following the consumption of raw, undercooked, or mishandled marine products and it rare cases causes wound infection, ear infection or septicemia in individuals with pre-existing medical conditions. *V. parahaemolyticus* has two hemolysin genes, thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*), both of which play a role in pathogenesis. *V. vulnificus* is potentially more pathogenic than *V. parahaemolyticus*. It is a leading cause of seafood-associated fatality in the United States and there are approximately 50 cases of *V. vulnificus* infection with 45 hospitalizations and 16 deaths every year (CDC, 2013). *V. vulnificus* has been isolated from samples along the Gulf of Mexico, Pacific and Atlantic coasts in addition to the North Atlantic (Heng, 2017). The pathogenic nature of these two bacteria and their association with water and sediment led to further investigation through the removal study.

The removal study was based on the preliminary results of the 2015 study and Gonzalez *et al.* (2014) determining association of *V. parahaemolyticus* and *V. vulnificus* with the non-native invasive algal species, *G. vermicullophylla.* Six sites were sampled for six weeks of which, three sites had the algae removed. Control sites (AC1–AC3) had no removal activity for the duration of the study. Sites AR1–AR3 had the algae removed after the initial three weeks of sampling. Each sample was labeled further using the substrate where the sample was taken. For example water samples were labeled AC1W–AC3W and AR1W–AR3W for water and the sediment samples were labeled with AC1S–AC3S and AR1S– AR3S. The results of determining if the removal had any affect in changing the concentration of *V. parahaemolyticus* in water are inconclusive due to the mixed results from analysis. This is mainly due to the small sample size,

n=6, for the entire study and the large variances and non-normal distribution of *V. parahaemolyticus* concentration. The concentrations of *V. parahaemolyticus* in water were below what was found by Gonzalez *et al.* (2014) which had concentrations of 200 cfu/mL to 1300 cfu/mL for July, whereas concentrations for this study ranged from 1 cfu/mL to 12 cfu/mL. The concentration of *V. parahaemolyticus* in sediment in the current study aligns with the late August sampling of the Gonzalez *et al.* (2014) study, in which concentrations of *V. parahaemolyticus* ranged from 1.8×10^4 cfu/ gdw for vegetated sites to 4.1×10^3 cfu/ gdw on bare sites. *V. vulnificus* concentration in water for the Gonzalez *et al.* (2014) study, ranged from 1.1 cfu/mL – 8.8 cfu/mL for July and August sampling bare and vegetative sites. This is comparable to *V. vulnificus* concentration before and after removal of algae in this study. Concentrations ranged from 0.1 cfu/mL to 90 cfu/mL. The lower concentrations for this study were due to low or no confirmations for *V. vulnificus.* In comparison to the Gonzalez *et al.* (2014) study, the concentration of *V. vulnificus* ranged from 0 in July and beginning of August, to 30000 cfu/gdw. This shows that the removal study was potentially done too early. The analysis of *V. parahaemolyticus* and *V. vulnificus* concentrations in both water and sediment did change after the removal of the algae, however not on a significant level. Incorrect colony identification on selective media based on color led to not having confirmations for *V. vulnificus* and having to use 0.1 cfu/mL as a concentration causing results to seem significant. Large variances between concentrations of *Vibrio* spp, and sample size, presented challenges for the statistical analysis of the actual concentration of *Vibrio spp*., especially for *V. vulnificus* In addition sampling technique, sampling time, and/or the variable nature of *Vibrio spp*. could have caused incorrect concentration values for *V. parahaemolyticus* and *V. vulnificus. Vibrio spp*. have specific temperature and salinity ranges required for viability. When external conditions are

unfavorable they can enter a VBNC state in which they are alive, but cannot be enumerated on media (Oliver 2005). This state has the potential to affect the outcome of isolation and enumeration of the species. Additionally, colorimetric changes on media were found to be variable with colors ranging from light to dark turquoise on CHROMagar Vibrio (CaV). The challenge of identifying the difference between shades of turquoise and the VBNC state of the bacterium could potentially results in miscalculated concentrations of *V. vulnificus*.

Antibiotic Resistance Profiles:

Bacterial genera have evolved/adapted to become resistance to antibiotics primarily due to excessive use of antimicrobials in humans, agriculture and aquaculture (Han *et al.* 2007). Multi-antibiotic resistant bacteria are becoming an international health crisis in statements issued by the World Health Organization (WHO). WHO highlights antimicrobial resistance as a significant threat to human wellbeing (WHO, 2014). This crisis is ultimately the result of indiscriminant use of antibiotics in clinical cases, agricultural use as well as aquaculture industries (Heng, 2017). *Vibrio* infections, specifically *V. vulnificus* and *V. parahaemolyticus* infections are the leading causes of seafood related fatalities in the United States. Treatments of infections with antibiotics have become increasingly more challenging with the emergence of these multidrug resistant bacteria. Results from this study showed that 68.51% of *V. vulnificus* (n=181) samples were resistant (either intermediate or fully) to multiple antibiotics. Moreover, 98.25% of *V. parahaemolyticus* samples (n=400), showed at least intermediate resistance or full resistance to more than one antibiotic. Yang *et al.* (2017) found similar resistivity when *V. parahaemolyticus* was tested against 15 antibiotics, eight of which were used in this study. Both *V. vulnificus* (74.03%) and *V. parahaemolyticus* (13.25%) are developing resistance to

antibiotics used to treat infections specifically ceftriaxone. Similar findings were reported by Chen et al (2017), and Wang et al (2017) that above 80% of *Vibrio spp*. are resistant to ampicillin. *Vibrio* spp. were completely susceptible to meropenem, imipenem, trimethoprim/sulfamethoxazole, chloramphenicol, and tetracycline whereas there was resistance shown in this study. The results from the antibiotic resistance profile for *V. vulnificus* and *V. parahaemolyticus* isolates in this study show that antibiotic resistance is increasing in both these species of *Vibrio*. While the numbers couldn't be compared to a similar study due to the use of 2012 BIOMIC zone of inhibition definitions, there is enough evidence that resistance with these bacteria could pose a potentially larger threat to treating infections.

Summary

V. parahaemolyticus and *V. vulnificus* are pathogenic bacteria found in warm estuarine environments, such as those along the eastern coast of the United States. Both *V. parahaemolyticus* and *V. vulnificus* have the potential to cause gastroenteritis through ingestion of raw fish and shellfish consumed by humans. Both species of *Vibrio* can also produce wound infections leading to severe septicemia, and in some cases, death if not treated promptly. With increasing incidence of infection, my research focused on potential reservoirs and environmental conditions that can increase human exposure to, and infection, with these species of bacteria in addition to resistance to antibiotics recommended to treat infections.

Water and sediment samples were taken from the LTER site on the coast of Virginia. Presumptive isolates were identified using colorimetric changes on chromagar to differentiate between *Vibrio* species. Mauve colonies indicative of *V. parahaemolyticus* and turquoise colonies indicative of *V. vulnificus*, were streaked on to TCBS and VVA media for further

differentiation from *V. cholerae*. Virulence genes associated with pathogenicity of *V. vulnificus* (*vvh*) and *V. parahaemolyticus* (*tdh* and *trh*) were used for confirmation via PCR.

An impact analysis for the removal of *G. vermicullophyla* was conducted to assess the concentrations of *V. vulnificus* and *V. parahaemolyticus*, using three control sites and three removal sites. All sites were sampled for a total of six weeks; the first three weeks of sampling each site contained the alga, *G. vermicullophylla*. Following the initial three week sampling, the algae was removed from the removal sites. The removal *G. vermicullophylla* showed the potential impact of the algal species on concentrations of *V. vulnificus* and *V. parahaemolyticus*. ANOVA analysis showed that the removal while insignificant statistically, has potential to impact concentrations of both *Vibrio* spp. This study was only conducted over the course of six weeks and a more comprehensive study is necessary to fully understand if the impact of removal is truly significant.

Resistance profiles were developed for confirmed isolates using 12 antibiotics that were used in a study completed by Conrad (2015). Of those 12 antibiotics, six are recommended by the CDC for treatment of infection of *Vibrio* species. Testing of confirmed *V. vulnificus* isolates (n=181) revealed that they were resistant in some aspect to all antibiotics used in the panel specifically ceftriaxone (74.02%). *V. parahaemolyticus* was resistant to ceftriaxone (13.25%), and amikacin (12.00%). Additionally, 68.51% were resistant in some aspect to multiple antibiotics for *V. vulnificus* and 98.25% for *V. parahaemolyticus* respectively. Although there were confirmed isolates for both *V. vulnificus* and *V. parahaemolyticus* that were resistant to antibiotics in the panel, the recommended treatment by the CDC uses multiple antibiotics. This study shows that resistance is developing among these pathogenic species of *Vibrio*. Analysis of this site as well as other coastal parts of Virginia should be completed to provide a more

elaborate study of the association of higher concentrations of *Vibrio* species and the development of antibiotic resistance among them.

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