Populations of Vibrio vulnificus and Vibrio parahaemolyticus in Breton Sound and Barataria Bay

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POPULATIONS OF *VIBRIO VULNIFICUS* AND
*VIBRIO PARAHAEOMOLYTICUS*
IN BRETON SOUND AND BARATARIA BAY

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Environmental Sciences

by
Brian William Matherne
B.S., Louisiana State University, 2007
December 2009
DEDICATION

This master’s thesis is dedicated in loving memory of Rita Lorio Matherne and William Stanley Gorgas.
ACKNOWLEDGEMENTS

First I would like to thank Louisiana State University and the Northern Gulf Institute for helping make this project possible. Next I would like to thank my major professor Dr. Aixin Hou for her help, guidance, and patience in helping me achieve my academic goals.

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ABSTRACT

*Vibrio vulnificus* and *Vibrio parahaemolyticus* are halophilic gram-negative bacteria that are found in warm coastal waters. These two species are the leading cause of fatal shellfish poisoning. The objective of this study was to determine the population dynamics of *V. vulnificus* and *V. parahaemolyticus* as impacted by temperature and salinity in Breton Sound and Barataria Bay of Louisiana.

Water samples were collected from each water body along a salinity gradient transect monthly from September 2007 through July 2009. Furthermore, from March 2008 through July 2009 sediment and live oyster samples were collected exclusively from the Breton Sound. The population of *V. vulnificus* and *V. parahaemolyticus* was measured using the most probable number (MPN) method and plating method. The abundance of putative *V. parahaemolyticus* and *V. vulnificus* in Barataria Bay and Breton Sound waters was seasonally dominated by water temperature, but spatially controlled by salinity level. The plate counts indicated that *V. vulnificus* appeared less abundant in the Gulf waters during colder months than *V. parahaemolyticus*. Based on the laboratory testing, *V. parahaemolyticus* appeared to grow better overall at higher salinity levels. The average annual population of putative *V. parahaemolyticus* and *V. vulnificus* over all the sampling sites in Breton Sound was slightly higher than that in Barataria Bay. Like that in the waters of Breton Sound, the population of putative *V. parahaemolyticus* and *V. vulnificus* in Breton Sound sediments also followed a trend.

This research has shown a clear picture of the dynamics of putative *V. parahaemolyticus* and *V. vulnificus* populations in Breton Sound Estuary and Barataria Bay. However, further work is needed to (1) continue the PCR confirmation of *V. vulnificus* and *V. parahaemolyticus*.
parahaemolyticus and *V. vulnificus*, and (2) carry out statistical analysis for the relationships between environmental parameters and *V. vulnificus* and *V. parahaemolyticus* populations. The work would eventually lead to the establishment of a statistical relationship between the *Vibrio* concentrations and environmental parameters, in particular salinity and temperature, under a range of tidal, wind, and freshwater input conditions, which could be used to quantify the temporal and spatial variations of the *Vibrio* distributions in response to the environmental parameter.
1. INTRODUCTION

The Louisiana seafood industry generates approximately 2.3 billion dollars annually for the state. Louisiana is a national leader in producing many types of seafood such as shrimp, crawfish, and oysters. The FDA has stated that approximately 20 million Americans consume raw oysters each year. This has become a growing cause for concern because of the associated health risks with consuming raw oysters. Oysters harvested during warmer months often contain unsafe levels of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in their tissues. These two species are gram-negative bacteria that inhabit the warm coastal waters of the Gulf of Mexico. Ingestion of these bacteria often leads to gastroenteritis, but in severe cases septicemia and even death.

Accusations that raw oysters are dangerous have led to bans on oyster purchases. The state of California banned the acquisition of oysters harvested from the Gulf of Mexico between the months of April and October. This has been an estimated $20 million detriment to producers in the Gulf South. The purpose of this thesis is to work towards an understanding of the population dynamics of *V. vulnificus* and *V. parahaemolyticus* in Breton Sound and Barataria Bay. This is not simply limited to the monitoring of *Vibrio* in these waters, but also seeking to understand the possible impacts of water temperature and salinity on the population.
2. LITERATURE REVIEW

2.1 VIBRIOS AND PUBLIC HEALTH

*V. vulnificus* and *V. parahaemolyticus* are ubiquitous gram-negative rods that occur in estuaries and other coastal regions worldwide (DePaola *et al.*, 1990; Warner and Oliver, 2008). These are the two primary causes of food-borne illness resulting from the consumption of raw or undercooked oysters and other shellfish. In addition, these *Vibrio* populations have been known to cause wound infections, and are particularly dangerous to people with preexisting medical conditions (CDC, 2008).

In the United States, *V. vulnificus* accounts for 95% of all seafood related deaths, and has a mortality rate of 50% (Blackwell and Oliver, 2008). The highest incidence of infections typically occurs during the warmer months of the year (See figure 2.1). The most common infections from *V. vulnificus* and *V. parahaemolyticus* cause gastroenteritis, with symptoms such as nausea, vomiting, diarrhea, and abdominal pain. *V. vulnificus* infections in people with suppressed immune systems, especially from chronic liver disease, can cause a blood infection known as sepsis. Sepsis is a life-threatening disease characterized by fever, chills, low blood pressure, and skin lesions. Blood infections caused by *V. vulnificus* must receive aggressive treatment due to a 25% fatality rate (Blackwell and Oliver, 2008). Severe *V. parahaemolyticus* infections are rare, and are typically limited to gastroenteritis that lasts for about 3 days. While more common with *V. vulnificus*, both *V. vulnificus* and *V. parahaemolyticus* can also cause skin infections when open wounds are exposed to warm seawater (CDC, 2008).

*V. vulnificus* and *V. parahaemolyticus* infections are often treated much differently. While antibiotics can be used to treat both strains of *Vibrio, V. parahaemolyticus* infections are generally left alone and allowed to run their course.
However, in severe or prolonged cases of *V. parahaemolyticus* infection, antibiotics such as ciprofloxacin and tetracycline can be administered. *V. vulnificus* infections are considerably more serious, and require aggressive treatment. This treatment consists of antibiotics, such as doxycycline combined with a third-generation cephalosporin, or various types of fluoroquinolones, such as ciprofloxacin or levofloxacin. Removal of dead tissue is usually required and, in extreme cases, amputation of limbs is necessary (CDC, 2008).

While often underreported, cases of *V. parahaemolyticus* and *V. vulnificus* infections occur annually throughout the world. In the United States alone, it is believed that there are approximately 4,500 *V. parahaemolyticus* infections every year. *V. vulnificus* infections are considerably more rare, with less than 1,000 cases reported along the Gulf Coast between 1988 and 2006. In 2007, a new national system for monitoring and reporting both *Vibrio* populations was implemented. This system has led to increased awareness, particularly along the Gulf Coast, and improvements in monitoring and reporting infectious *Vibrio* cases (CDC, 2008).
2.2 POPULATIONS OF *V. VULNIFICUS* AND *V. PARAHAELOMYCITUS*

*V. vulnificus* populations are positively correlated with water temperature, proliferating at a temperature range between 19°C and 32°C (Blackwell and Oliver, 2008). However, *V. vulnificus* is typically present only in a viable, but nonculturable (VBNC), state when water temperatures drop below 13°C (Warner and Oliver, 2007). Because seasonal temperature variations only explain about 50-60% of *V. vulnificus* profusion, it is also believed that correlations with temperature may be salinity dependent as well.

Nevertheless, the general consensus for the true correlation for salinity and *Vibrios* is quite controversial. Some research has shown that temperature and salinity effects on *V. vulnificus* proliferation are interdependent (Randa et al., 2004; Kaspar and Tamplin, 2003). Conversely, other studies suggest a positive temperature correlation at salinities of 20-25 g/L, but not at salinities between 5-10 g/L (Randa et al., 2004; Blackwell and Oliver, 2008). Furthermore, Randa et al. concludes that optimal salinities for *V. vulnificus* may in fact be between 5 to 10 g/L.

![Fig. 2.2: Correlation between *V. vulnificus* water isolates (dark circle) and water temperature (open circle). Adapted from (Blackwell and Oliver, 2008)](image-url)
*V. parahaemolyticus* follows the same trends as *V. vulnificus* for the correlation between water temperature and population growth. *V. parahaemolyticus* is difficult to culture during the colder months of the year, and is also believed to enter the VBNC state. However, unlike *V. vulnificus*, *V. parahaemolyticus* studies have not typically shown correlations between population growth and salinity (Blackwell and Oliver, 2008).

Both *V. vulnificus* and *V. parahaemolyticus* were found to have higher concentrations in sediment than in water (see figure 2.2). Each *Vibrio* population remained fairly constant throughout the year, despite decreased numbers found in water during the winter months. Studies have suggested that sediment may allow *Vibrio* populations to survive the winter, and then be re-introduced to the water column when water temperatures reach optimal levels (Blackwell and Oliver, 2008).

![Graph](image)

**Fig. 2.3:** Levels of pathogenic *Vibrio* in water (*dark circle*) and sediment (*open circle*). (A) *V. vulnificus* and (B) *V. parahaemolyticus* (Blackwell and Oliver, 2008)

### 2.3 OYSTERS AND VIBRIO

The eastern oyster, *Crassostrea virginica*, is native to estuaries along the Gulf of Mexico (See figure 2.4). These sessile mollusks are filter feeders that thrive in coastal waters, with a salinity range of 10 to 30 g/L. The influence of salinity on oyster life cycles is not always constant throughout its various growth stages, due to the fact that
oysters tend to grow better in areas that fluctuate within their normal range of salinity requirements (Turner, 2006).

*V. vulnificus* and *V. parahaemolyticus* naturally occur in the same coastal environments as oysters. As the oysters feed, they tend to concentrate the *Vibrios*, serving as a vector for disease. Seasonal temperature fluctuations usually play the greatest role in occurrences of infections. *V. vulnificus* is generally not found in oysters when the water temperatures dip below 13°C. The highest rates of *V. vulnificus* infection occur during the warmest months between May and October. The oysters harboring *V.*

![Fig. 2.4: (Top) Shows distributions of major oyster harvesting sites throughout the Gulf Coast. (Bottom) Shows the two major oyster harvesting sites for coastal Louisiana. Note that St. Bernard Parish is where Breton Sound is located, and Plaquemine Parish is where Barataria Bay is (Turner, 2006).](image-url)
*V. vulnificus* at this time of year typically contain between $10^3$ and $10^4$ CFU per gram (Warner and Oliver, 2007).

*V. parahaemolyticus* exhibits similar seasonal growth patterns to *V. vulnificus*, but they have a tissue concentration range of $10^2$ to $10^3$ CFU per gram (DePaola *et al.*, 2003). Unlike *V. vulnificus*, there has been debate about the significance of *V. parahaemolyticus* contamination in oysters (DePaola, 1990). Not all *V. parahaemolyticus* present in the environment are pathogenic and the occurrence of the pathogenic thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH) is not well understood. The presence of the *tdh* gene is known as the Kanagawa phenomenon, and it is found in over 90% of all clinical isolates. However, studies have shown only about 1-3% of all environmental isolates contain the *tdh* gene (Vongxay *et al.*, 2008).

<table>
<thead>
<tr>
<th>Sources</th>
<th>No. of isolates</th>
<th><em>tdh</em></th>
<th><em>trh</em></th>
<th>Hemolysin</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
<td>%</td>
<td>No. positive</td>
<td>%</td>
<td>No. positive</td>
</tr>
<tr>
<td>Clinical</td>
<td>25</td>
<td>21</td>
<td>84.0</td>
<td>3</td>
<td>12.0</td>
</tr>
<tr>
<td>Seafoods</td>
<td>191</td>
<td>3</td>
<td>1.57</td>
<td>7</td>
<td>3.66</td>
</tr>
</tbody>
</table>

2.4 CONTROL METHODS

With *Vibrio* infections on the rise in developing countries, it is important to understand the relationship between oyster consumption and disease. A variety of factors need to be taken into consideration, such as initial concentration of pathogens in the water and oysters, types of storage conditions, and implicated post-harvest treatment methods (Farame *et al.*, 2008). It is important to find reliable and cost effective methods
for post-harvest shellfish treatment. Techniques such as storing oysters on ice, freezing, heat-treatment, high-pressure processing, electrolyzed oxidizing water, and the use of diacetyl have all been studied as methods of reducing the levels of *Vibrio*.

Icing is the oldest and most basic storage technique used in oyster harvesting, in which oysters are put on ice immediately, or at the dock. When the oysters are put on ice during colder months, the reduced *Vibrio* levels in the shellfish are prevented from rapidly growing to a dangerous level (Gooch *et al.*, 2002). However, icing is not effective at reducing *Vibrio* populations that are already at unsafe consumption levels upon harvesting (Melody *et al.*, 2008).

Various forms of cold treatment have been studied to reduce levels of *Vibrio* in shellfish. Simply freezing oysters at temperatures < -20°C will reduce levels of *Vibrio* present over time, but *V. vulnificus* has been cultured from oysters stored at -20°C after a period of only 12 days. Therefore, basic freezing methods should not be relied upon to reduce the levels of *Vibrio* in shellfish. However, simple heating treatments in water greater than 45°C for ten minutes has been known to kill all *V. vulnificus* present in tissues. Since this process does not cook the oysters, it is a possible method for improving shellfish safety (Cook and Ruple, 1992).

Researchers have assessed several chemical methods of postharvest treatment. One such method is the use of electrolyzed oxidizing (EO) water. EO water contains chlorine, has a pH of 2.82, and an oxidation-reduction potential of 1131 mV. EO water reduces *Vibrio* to a non-detectable level within 15 seconds, but can damage the oysters with prolonged treatment (Ren and Su, 2006). Another method uses a component of butter known as diacetyl, which is very effective at reducing levels of *V. vulnificus* in oysters, but only after they have been shucked (Birkenhauer and Oliver, 2003).
Since 2000, the U.S. seafood industry has used high pressure to shuck and treat oysters. For instance, oysters may be put under a high pressurization at 300 MPa for 5 minutes at 25°C to reduce the levels of *Vibrio* below an infective dose. High pressure treatment is favored because it can be used effectively without causing any change in the quality of the raw meat. However, a major drawback to using high pressure is the expensive cost of the equipment used to run the pressurization (Kural and Chen, 2008; Kural *et al.*, 2008)

### 2.5 *Vibrio* Infections Following Hurricane Katrina

One of the major contributing factors leading to my research were the *Vibrio* caused illnesses and deaths following Hurricane Katrina (See figure 2.5). On August 29, 2005, Hurricane Katrina devastated several states along the Gulf Coast. There were twenty-two new cases of *Vibrio* caused illness, five of which resulted in death, recorded between August 29 and September 11. Eighteen were wound infections caused by *V. vulnificus* and *V. parahaemolyticus*, and four were gastroenteritis caused by nontoxicgenic *V. cholera*. Of the five deaths, two were caused by *V. parahaemolyticus* infections, and the remaining three resulted from *V. vulnificus* infections (CDC, 2005).

Eight of the wound-associated *Vibrio* cases originated in Mississippi, and the remaining ten originated in Louisiana. Seven of the eighteen reported cases were spread across Texas, Arkansas, Arizona, and Florida as a result of displaced hurricane victims. *Vibrio* speciation was achieved for seventeen of the wound associated cases; three were *V. parahaemolyticus*, and fourteen were *V. vulnificus*. Patients that received wound related illnesses ranged between 31 and 89 years of age. The majority of these patients were male (83%), and most required hospitalization. Thirteen patients had an existing history of another underlying health condition, such as heart disease, diabetes, or
liver disease (CDC, 2005).

The following is provided from a 2005 publication by the Centers for Disease Control and Prevention mentioning two cases of *V. vulnificus* and *V. parahaemolyticus* wound infections:

### 2.5.1 Patient A.

A 60 year old man with a medical history of alcohol abuse, hypertension, and stroke had spent three days wading through floodwaters in New Orleans, Louisiana. The man relocated to Texas on August 31, and went to the emergency room the following day. He was treated and released for bilateral ankle wounds and diarrhea. Later, *V. vulnificus* was found in his blood cultures, and the patient was contacted and admitted to the hospital on September 2. However, the man died the following day.

### 2.5.2 Patient B.

A 61 year old man with a medical history of coronary artery disease, hyperlipidemia, and HIV was examined in Mississippi on August 29th. He was initially treated for hypothermia and moderate lacerations to his trunk. Blood cultures revealed a
*V. parahaemolyticus* infection, and an antibiotic treatment was started. However, the man died the following day.

These cases were part of an increased incidence of *Vibrio* wound infections throughout the Gulf Coast, and are typical with post hurricane exposure. While each patient’s exposure histories are not available, it is believed that the *V. vulnificus* infections were the result of open wounds being exposed to flood water in individuals with already compromised immune systems. Interestingly, there were no *Vibrio* caused gastroenteritis cases reported in the days following Hurricane Katrina (CDC, 2005).

**2.5 DETECTION OF V. VULNIFICUS AND V. PARAHAELOMYTICUS**

Detection of these two species of *Vibrio* can be conducted in several manners. Simple methods, such as colony growth on selective agars, can be used for basic detections. Older confirmation assays, such as DNA probes, can be used as well. Recently, new and faster detection methods are being developed and perfected using various Polymerase Chain Reaction (PCR) techniques. Finally, methods utilizing remote detection are being developed to create real-time notices that can be made available via the internet.

Thiosulphate-citrate-bile salts-sucrose (TCBS) agar is commonly used in laboratories because it is highly selective for both *V. vulnificus* and *V. parahaemolyticus*. TCBS uses an alkaline pH, bile, and salt to repress the growth of other bacteria; it detects *Vibrio* based on the ability to ferment sucrose (Harwood *et al*., 2004). However, it is not possible to differentiate between *V. vulnificus* and *V. parahaemolyticus* on a TCBS plate (Pfeffer and Oliver, 2003). Therefore, a more selective media that contains an antibiotic known as colistin can be used to make cellobiose-colistin (CC) agar. CC agar takes
advantage of the resistance of *V. vulnificus* to the antibiotic, thus allowing only the growth of *V. vulnificus* (HØI *et al.*, 1998).

DNA probes are used in DNA colony hybridization. For example, the *vvhA* gene is used to detect *V. vulnificus* in environmental and clinical test strains. If a *V. vulnificus* gene is present, the *vvhA* gene will hybridize with its DNA. A list of DNA probes for *V. vulnificus* is in the following table (Harwood *et al.*, 2004).

<table>
<thead>
<tr>
<th>Probe/primer designation</th>
<th>Method</th>
<th>Target gene (size/type)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. vulnificus</em> probe</td>
<td>Colony hybridization</td>
<td><em>vvhA</em> (3.2 kb DNA probe)</td>
</tr>
<tr>
<td>VVAP</td>
<td>Colony hybridization</td>
<td><em>vvhA</em> (24 base probe)</td>
</tr>
<tr>
<td>V3VV</td>
<td>Colony hybridization</td>
<td>16S rRNA (24 base probe)</td>
</tr>
</tbody>
</table>

PCR protocols are now being developed as rapid detection methods by labs all over the world. Certain PCR techniques can be used to search for numerous genes at the same time through a method known as multiplex PCR. This utilizes special primers and probe sequences that look for specific target genes (See table 2.3). For example, PCR can determine the presence and type of *V. parahaemolyticus* by simultaneously searching for the total *V. parahaemolyticus* gene *tlh*, and the pathogenic gene *tdh*. Cells can be enriched with TCBS plates or in alkaline peptone water from an environmental or clinical sample, and a PCR protocol can be run to confirm the presence of *V. vulnificus* or *V. parahaemolyticus* (Panicker *et al.*, 2004).

Recently, the United States Food and Drug Administration set out to create warning systems for *V. parahaemolyticus*. They used remotely sensed sea surface temperature data to predict the levels of *V. parahaemolyticus* present in oyster reefs. This prediction system was designed to conduct risk assessment for the consumption of raw oysters. In the near future, researchers believe that a system could be posted online to
help people make educated decisions about the safety of oysters, without the need for constant laboratory testing (Phillips et al., 2007).

### 2.6 BRETON SOUND AND BARATARIA BAY

Breton Sound estuary and Barataria Bay are two separate bodies of water located along the Louisiana Gulf Coast. Both locations are important to the state of Louisiana due to the rich fisheries present in each. The Louisiana seafood industry generates approximately 3 billion dollars annually for the state. Louisiana is a national leader in the production of many types of seafood, such as shrimp, crawfish, and oysters. The state produces 35% of all the oysters harvested in North America alone (Scharnberg, 2005). According to the FDA, an estimated 20 million Americans consume raw oysters each year.

The Breton Sound estuary is a series of lakes, bayous, bays, canals, as well as fresh, brackish, and salt water marshes. In the upper estuary, it is geographically separated by a remnant of the Mississippi River distributary, Bayou Terre Aux Boeufs (Piazza and Peyre, 2007). The 1,100 km² of wetlands are also hydrologically restricted to

---

**Table 2.3: Primers used for multiplex PCR; (Panicker et al., 2004)**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Target gene</th>
<th>Primer and probe sequences</th>
<th>Oligonucleotide length</th>
<th>% GC content</th>
<th>Tm (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. vulnificus</em></td>
<td>vvh</td>
<td>F-VII: 5'-TTCACACTCCAAAAAGCATGTGAC-3'</td>
<td>25</td>
<td>40.0</td>
<td>61.3</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-VII: 5'-B-ATCCACCTTGAGATTGGAATAATGGTGTG-3'</td>
<td>24</td>
<td>45.8</td>
<td>62.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-VIII: 5'-GAAGGCGCCCGTGATCTGAAACGGGTCTACG-3'</td>
<td>30</td>
<td>60.0</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>viaB</td>
<td>F-VIIb: 5'-GGTGGGCGATCATAGTATA-3'</td>
<td>24</td>
<td>45.8</td>
<td>62.8</td>
<td>0.504</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-VIIb: 5'-B-GGCTGAACTGACTAAGCCGCTATCCG-3'</td>
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<td>59.9</td>
<td>66.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-VIIb: 5'-GAGAAAAGCGACCTCCTGCTATGCTTCTT-3'</td>
<td>30</td>
<td>50.0</td>
<td>68.7</td>
<td></td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>tol</td>
<td>F-TLH: 5'-AAAGGCTGTTATCGAAGAATGGACGTG-3'</td>
<td>24</td>
<td>45.8</td>
<td>62.8</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TLH: 5'-GCTGCTTTTACGTATTTCTCCTTCG-3'</td>
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<td>41.6</td>
<td>61.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-TLH: 5'-ACGGAGCGGCAAGCGAAGATCCTATGGTG-3'</td>
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<td>53.3</td>
<td>70.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TLH: 5'-TGGTGCCTGGTGGTGCCAGCGCTGTCTG-3'</td>
<td>30</td>
<td>60.0</td>
<td>72.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tdh</td>
<td>F-VDH: 5'-TGAAGGCGCTGACTGTTGAC-3'</td>
<td>23</td>
<td>43.5</td>
<td>57.7</td>
<td>0.260</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-VDH: 5'-CGCTGATCACTCCTTCTAACACCC-3'</td>
<td>24</td>
<td>41.7</td>
<td>61.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-TDH: 5'-ACGGAGCGGCAAGCGAAGGTGCTGCTGTCG-3'</td>
<td>30</td>
<td>53.3</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>thr</td>
<td>F-TRE: 5'-TGGGCTGATTTTATCGATATCC-3'</td>
<td>24</td>
<td>33.3</td>
<td>57.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TRE: 5'-CATTCAGCAATCATTCTAAGTTCTCCG-3'</td>
<td>24</td>
<td>40.0</td>
<td>61.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-TRE: 5'-AAACAACGCTAAGCAGACCGAAGATCCTG-3'</td>
<td>30</td>
<td>36.0</td>
<td>63.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TRE: 5'-CAGGCTAAGCAGACCGAAGATCCTGTTAG-3'</td>
<td>32</td>
<td>34.3</td>
<td>63.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-TRE: 5'-TGGAAGCTGCTACATTCATCCCTTCTCTCTA-3'</td>
<td>32</td>
<td>43.7</td>
<td>67.2</td>
<td></td>
</tr>
</tbody>
</table>
the north by natural levees of Bayou La Loutre, to the west by the Mississippi River levee, and to the east by the spoil banks of the Mississippi River Gulf Outlet (MRGO). Breton Sound estuary eventually connects to the Gulf of Mexico (Lane et al., 1999).

In 1991, the state of Louisiana implemented a freshwater diversion of the Mississippi River at Caernarvon, Louisiana, which leads into Breton Sound estuary (See figure 2.6). The state has also created several diversions, like Caernarvon, along the Mississippi River to mimic seasonal flooding events. When the Caernarvon diversion was first planned, it was intended to decrease salinity to boost oyster production in the surrounding areas. Now, the diversions are also being used to deliver sediment and nutrients to the now threatened wetlands (Lane et al., 1999).

Breton Sound has strong seasonal temperature changes, with winter lows less than 12°C, and summer highs in excess of 30°C. The water entering the estuary from the river
is typically cooler, and can be as low as 6°C, however, it normally balances out after a few kilometers. Naturally, the salinity of the upper estuary remains very fresh, and will increase up to 14 and 30 g/L along the Western and Eastern edges, respectively, as distance from the diversion increases. The salinity in the estuary is greatly affected by the level of river discharge. During large spring pulse events, the entire estuary can become fresh for a short period of time (<1 month). Also, there tends to be a delay of about two weeks between discharge and decreased salinity in the lower estuary (Lane et al., 2007).

Like Breton Sound, Barataria Bay is an estuarine wetland basin in the Mississippi Delta of coastal Louisiana. The bay encompasses approximately 6,000 km², and is made up of large lakes and tidally influenced marshes that connect to the bay. To the east are the natural levees of the Mississippi River, and to the west is Bayou Lafourche (See

![Fig. 2.7: Barataria Bay (Jones et al., 2002)](image-url)
The mouth of the bay is protected by a series of barrier islands known as the Grand Terre Islands (Emad et al., 2007).

Throughout the bay the salinity varies greatly with a range between 0.9 and 29.2 g/L. Salinity in Barataria Bay is lowest during the summer and winter, and highest during the fall and spring. Moving across a north-south gradient, salinity is greatest in the fall with a range of 17 g/L. Temperatures range between winter lows of 10.2˚C and summer highs of 32.0˚C. Thus, mean water temperatures vary considerably seasonally (Jones et al., 2002).

### 2.7 PURPOSE

The lack of agreement as well as deficiencies in understanding the true importance of salinity may be due to physical variations in sampling sites. Various research locations have unique salinity regimes that may be caused by any number of differing environmental factors. Thus, distinctive salinity patterns coupled with varying temperature ranges could create different growing conditions for various *Vibrio* populations. Closer analysis of seasonal and spatial patterns should be considered when attempting to fully understand optimal growing conditions for *Vibrios*.

The threat of infections caused by both *V. vulnificus* and *V. parahaemolyticus* is a serious issue along coastal Louisiana. Little is known about the actual *Vibrio sp.* populations in both Breton Sound and Barataria Bay. This study is a great opportunity to look at not only the seasonal variations in population growth, but spatial patterns as well. Coupling traditional culturing methods with cutting edge molecular techniques could provide a greater understanding of optimal *Vibrio* growing conditions.
3. MATERIALS AND METHODS

3.1 SAMPLING LOCATIONS

For this study two climatically similar, yet hydrologically different, locations were chosen for sampling. Each location is a vital part of the Louisiana Seafood industry, and also provides a plethora of recreational opportunities. For this study I sought to compare populations of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in both water bodies. Also, sediment and live oyster samples were collected exclusively from Breton Sound.

3.1.1 BRETON SOUND

Breton Sound is a 1,100 km$^2$ estuary in coastal Louisiana that consists of a series of lakes, bayous, bays, canals, and fresh, brackish, and saltwater marshes. Sampling locations along the transect were designed to follow a salinity gradient beginning at the Caernarvon freshwater diversion, and ending at the Gulf of Mexico.

Fig. 3.1: Map of Breton Sound showing sampling sites for LSU transects. Gold circles indicate sites specific to this study. White circles represent sampling sites for other researchers.
The numbered circles on the map indicate the various sampling sites along the Breton Sound transect. However, only samples from site 4 (Lake Lery), site 8 (Grand Lake), site 11 (Branville Bay), site 12 (Lake Cuatro Caballo), site 15 (Bayou Lery), and site 16 (Black Bay/Breton Sound) were used for this study. Water and sediment samples were collected from each of the 6 locations, but oyster samples were collected exclusively from site 16.

3.1.2 BARATARIA BAY

Barataria Bay is an estuarine wetland basin in the Mississippi Delta of coastal Louisiana. The bay encompasses approximately 6,000 km$^2$, and is made up of large lakes and tidally influenced marshes that connect to the bay. The sampling transect was designed to follow a salinity gradient beginning at Barataria Pass, and concluding in Lac Des Allemands.

![Fig. 3.2: Map of Barataria Bay with sampling locations marked.](image)
The following is a list of the six sampling sites chosen for this study: site 2 (Barataria Pass), site 8 (Upper Barataria Bay), site 16 (Little Lake), site 23 (Lake Salvador), site 30 (Bayou Des Allemands), and site 33 (Lac Des Allemands). Water samples were collected from each of the six sampling sites along the Barataria Bay transect.

3.2 SAMPLE COLLECTION AND STORAGE

Sample collection and storage methods were conducted in accordance with the U.S. Food and Drug Administration’s Bacteriological Analytical Manual. Water samples from each sampling site were collected in sterilized 2000 ml Nalgene® bottles. The bottles were kept in an ice chest, but were not allowed to come into direct contact with the ice packs. Exposure to the ice packs was prevented by a sheet of bubble wrap. This step was necessary because direct exposure to the ice packs could kill any Vibrio sp. present in the sampling bottles.

Sediment samples from all six Breton Sound sites were collected with a bottom grab fashioned from a cup attached to a long wooden handle. The collected sediments were then stored in sterilized 120 ml sampling cups. The sampling cups were kept in an ice chest, but were not allowed to come into direct contact with the ice packs. Exposure to the ice packs was prevented by placing a sheet of bubble wrap in between the cups and the ice.

Live oyster samples were collected from an oyster reef at site 16 from the Breton Sound transect. A cluster of oysters was removed from the reef using a shovel and a rake, and were stored in an ice chest. The oysters kept in the ice chest were prevented from coming into direct contact with the ice packs by placing a sheet of bubble wrap in between them.
3.3 SAMPLE PREPARATION

The various forms of buffer and growth media needed to process the *Vibrio sp.* samples are listed as follows: phosphate-buffered saline (PBS), alkaline peptone water (APW), thiosulfate-citrate-bile-sucrose (TCBS) agar, cellobiose-colistin (CC) agar, Luria-Bertani (LB) agar, and glycerol stock solution.

PBS was prepared by combining 7.65 g of NaCl, 0.724 g of Na$_2$HPO$_4$, and 0.21 g of KH$_2$PO$_4$ in 1 L of deionized (DI) water. The solution was thoroughly mixed, and the pH was adjusted to 7.4. Finally, PBS must be autoclaved before it can be used. PBS was used for serial dilutions and suspensions of water, sediment, and oyster slurry.

Three different types of APW were used for the most probable number (MPN) portion of our testing. The first formula (1X) used 10 g of peptone and 10 g of NaCl in 1 L of DI water. The second formula (4X) used 10 g of peptone and 2.5 g of NaCl in 250 ml of DI water. The third formula (10X) used 100 g of peptone and 10 g of NaCl in 1 L of DI water. Finally, each solution was mixed, had the pH adjusted to 8.5, and was autoclaved before use.

TCBS agar was one type of growth media used for my plating methods. It has been specially formulated for the selection of various forms of *Vibrio sp.*, and thus was used to detect the presence of both *V. vulnificus* and *V. parahaemolyticus*. TCBS media was made by mixing 89 g of TCBS powder in 1 L of DI water. The mixture was liquefied by boiling for one minute with frequent agitation. The media was then immediately cooled in a water bath until it reached a temperature of 45-50 °C. The cooled TCBS media was then poured into Petri dishes, and stored inverted at 2-8 °C until it was needed.
CC agar was the second type of growth media used for my plating methods. It contains a type of polymyxin antibiotic known as colistin. Colistin is effective at killing most Gram-negative bacilli, including *V. parahaemolyticus*. However, growth of *V. vulnificus* is not inhibited by this media, and thus CC agar was used to detect the presence of *V. vulnificus* exclusively. CC agar consist of 3 different solutions:

### Table 3.1: Formula for colistin cellobiose agar.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>20 g</td>
</tr>
<tr>
<td>1000X Dye stock solution*</td>
<td>1 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>DI water</td>
<td>900 ml</td>
</tr>
<tr>
<td>Bromthymol blue</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Cresol red</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Ethanol, 95%</td>
<td>100 ml</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>10 g</td>
</tr>
<tr>
<td>Colistin</td>
<td>400,000 units</td>
</tr>
<tr>
<td>DI water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Solution 1

The pH was adjusted to 7.6, and was the media was boiled with frequent agitation. It was then placed in a water bath until cooled to 48-55 °C.

*1000X Dye stock solution (Solution 3)

Solution 2

Cellobiose was dissolved in DI water by heating gently. The solution was cooled before colistin could be added.
Solution 1 was cooled to between 48-55 °C before solution 2 could be added. If solution 1 was too hot it would destroy the antibiotic. Once solutions 1 and 2 were thoroughly mixed they were poured into Petri dishes, and stored inverted at 2-8 °C.

LB agar was not used for the initial plating of the *Vibrio sp.* colonies. LB agar is not a selective media, and because it is not a harsh media it is useful when recovering cells from deep freeze. Also, LB media is not inhibitory of PCR, and colonies were grown on these plates before being extracted for molecular work. LB media was made by mixing 40 g of LB powder in 1 L of DI water. The mixture was liquefied by boiling for one minute with frequent agitation. Once boiled, the media must immediately be autoclaved because it is not very selective. The autoclaved LB media was then poured into Petri dishes, and stored inverted at 2-8 °C until it was needed.

Glycerol stock solution was used to store cultured colonies in a -80 °C freezer. This method kept the samples alive until needed. Glycerol stock was made from 2 g of peptone, 1 g of yeast extract, 2 g of NaCl, 180 ml of DI water, and 20 ml of glycerol. The pH of the solution was adjusted to 7.5, and was then autoclaved before storage.

**3.4 SAMPLE PROCESSING**

**3.4.1 MPN**

1) Preparation of culture tubes before sampling

   a) 2000 ml bottles for 1000 ml cultures: 2 [1 (replication) x 2 (sample#) x 1 (dilution)]

      i) Pipetted 100 ml of 10X APW into each bottle

   b) 150 ml bottles for 100 ml cultures: 18 [3 (replication) x 6 (sample#) x 1 (dilution)]

      i) Pipetted 10 ml of 10X APW into each bottle
c) 25 ml tubes for 10 ml cultures: 18 [3 (replication) x 6 (sample#) x 1 (dilution)]
   i) Pipetted 10 ml of 4X APW into each tube

d) 17 ml tubes for 1 ml culture: 18 [3 (replication) x 6 (sample#) x 1 (dilution)]
   i) Pipetted 9 ml of 1X APW into each tube

e) 17 ml tubes for dilutions: 24 [1 (replication) x 6 (sample#) x 4 (dilution)]
   i) Pipetted 10 ml of PBS into each tube
      ii) Labeled each tube in descending order (0.1, 0.01, 0.001, and 0.0001)

f) 17 ml tubes for dilution cultures: 72 [3 (replication) x 6 (sample#) x 4 (dilution)]
   i) Pipetted 9 ml of 1X APW into each tube

2) Inoculation of water samples (1000, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 ml)

   a) Poured 1000 ml of sample into each 2000 ml bottle
   b) Pipetted 100 ml of sample into each 150 ml bottle
   c) Pipetted 10 ml of sample into each 25 ml tube
   d) Pipetted 1 ml of sample into each 17 ml tube for 1 ml cultures
   e) Pipetted 1 ml of sample into a 17 ml tube labeled 0.1 and mixed
      i) Pipetted a 1 ml aliquot from the 0.1 ml tube, mixed, and injected into the 0.01 ml tube
      ii) Pipetted a 1 ml aliquot from the 0.01 ml tube, mixed, and injected into the 0.001 ml tube
      iii) Pipetted a 1 ml aliquot from the 0.001 ml tube, mixed, and injected into the 0.0001 ml tube
   f) Pipetted a 1 ml aliquot from each dilution tube, and transferred to the corresponding culture tubes

3) Incubated cultures at 37 °C overnight
4) Recorded positive growth, collected 1.5 ml of sample from each culture, and separated biomass by centrifuging.

5) Kept the collected pellet at -20 °C until needed for molecular work.

3.4.2 PLATING METHODS

The plating methods used followed the *Bacteriological Analytical Manual* procedures.

3.4.3 MEMBRANE FILTRATION

1) Water
   a) Filtered the desired amount of water (100, 50, or 10 ml) through Millipore Mixed Cellulose Ester Membranes (0.45 µl pore size) for 4 replicates for the 10 ml dilution, and 3 replicates for the 100 or 50 ml dilutions.
      i) 10 ml samples were suspended in ~100 ml of PBS before filtration.
   b) Placed duplicate filters from the 10 ml dilution onto both TCBS and CC plates.
   c) Placed duplicate filters from the 100 or 50 ml dilution on each TCBS plate, and the remaining filter on a CC plate.
   d) Incubated plates overnight at 37 °C.

2) Sediment
   a) 5 g of sediment was suspended in PBS to make a 50 ml solution, and was followed by a 1:10 dilution.
   b) Filtered the desired amount of sediment (10 or 1 ml) through Millipore Mixed Cellulose Ester Membranes (0.45 µl pore size).
      i) Sediment solution was suspended in ~100 ml of PBS before filtration.
   c) Placed duplicate filters onto both TCBS and CC plates.
   d) Incubated plates overnight at 37 °C.
3) Oyster

   a) Obtained 50 g of oyster meat and liquor, and blended for 90 seconds
   b) Added oyster meat to 450 mL of PBS to create a 1:10 dilution
   c) Blended for 90 seconds
   d) Created another 1:10 dilution with PBS
   e) Suspended 3 ml of sample in ~100 ml of PBS, and filtered through Millipore Mixed Cellulose Ester Membranes (0.45 µl pore size)
   f) Placed duplicate filters onto both TCBS and CC plates
   g) Incubated plates overnight at 37 °C

3.4.4 PLATE COUNTS

   Plates were collected after one night of incubation. Positive *V. vulnificus* and *V. parahaemolyticus* were shown by the presence of round green colonies on TCBS plates. Positive *V. vulnificus* was shown by the presence of round yellow colonies on CC plates. Positive colonies were then counted and recorded.

3.4.5 COLONY TRANSFERRING

   A grid was drawn on the back of a large TCBS plate for organization purposes. Positive colonies were then transferred using a sterile wire loop or toothpick and isolated within the grid of the large plate. Each grid location was labeled with the site number in which the colonies were taken from. Also, the front of the plate was used to record what type of sample the colony came from (i.e. CC oyster). The transferred colonies were then grown overnight in an incubator at 37 °C.

3.4.6 COLONY STORAGE
Each transferred colony was then moved to a tube containing 600 µl of glycerol stock solution. The tubes were then placed in a -80 °C freezer until needed for molecular work.

### 3.5 Multiplex PCR

For Multiplex PCR this study used Promega Hot Start Master Mix using the primers and procedure listed in the Panicker et. al., 2004 publication. The primers used for this study are shown in table 3.2

**Table 3.2: Primers for polymerase chain reaction**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Target gene</th>
<th>Primer and probe sequence</th>
<th>Oligo-nucleotide length</th>
<th>% GC content</th>
<th>Tm°C</th>
<th>Amplicon size (kbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. vulnificus</em></td>
<td>vvh</td>
<td>F-VVH: 5'-TTCCAACCTCAAACCGAACTATGAC-3'</td>
<td>25</td>
<td>40</td>
<td>61.3</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-VVH: 5'B-ATCCAGTCGATCGGAATACCTG-3'</td>
<td>24</td>
<td>45.8</td>
<td>62.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>viuB</td>
<td>F-VIU1728: 5'-GGITGGGCACTAAGCAGATATA-3'</td>
<td>24</td>
<td>45.8</td>
<td>62.8</td>
<td>0.504</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-VIU2231: 5'B-CGCGAGTGACTAAATACCGAC-3'</td>
<td>22</td>
<td>59.9</td>
<td>66.4</td>
<td></td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>tlh</td>
<td>F-TLH: 5'-AAAGCAGATTATGCGAAGCAGTCTG-3'</td>
<td>24</td>
<td>45.8</td>
<td>62.8</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TLH: 5'B-GCTACTTTCTGATCTTCTG-3'</td>
<td>24</td>
<td>41.6</td>
<td>61.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tdh</td>
<td>F-TDH: 5'-GAAGGACTTCTGACTTTTGAC-3'</td>
<td>23</td>
<td>43.5</td>
<td>57.7</td>
<td>0.269</td>
</tr>
<tr>
<td></td>
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<td>R-TDH: 5'B-TGGAATGAACCTTCTACTC-3'</td>
<td>24</td>
<td>41.7</td>
<td>61.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ith</td>
<td>F-TRH: 5'-TGTCCTTCTGATATTTCTC-3'</td>
<td>24</td>
<td>33.3</td>
<td>57.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TRH: 5'B-CATAACAAACATATGCCATTTCG-3'</td>
<td>25</td>
<td>40</td>
<td>61.3</td>
<td></td>
</tr>
</tbody>
</table>

Samples stored in glycerol stock were defrosted at room temperature, and rejuvenated on LB agar. Once the desired number of cells were transferred to LB plates, they were incubated overnight at 37 °C. After colonies were grown, a loop of cell mass was collected and placed in a PCR tube containing 100 µl of filtered DI water (DW). The PCR tubes were then placed in a BIO RAD MyiQ™ Single Color Real-Time PCR Detection System, and a boil sequence was run for 1 hour. When the boil sequence was complete, the samples could be used as template DNA for PCR testing.
The MPN samples were defrosted at room temperature. 1 ml of DW was added to each pellet, and the tube was mixed thoroughly. Small aliquots of each sample were pipetted into PCR tubes. Then the PCR tubes were placed in the PCR Detection System, and a boil sequence was run for 1 hour. Once the boil sequence was complete, the samples could be used as template DNA for PCR testing.

The following is a chart that describes the mixture used for PCR detection of:

Table 3.3: PCR reaction mixture for *V. vulnificus*

<table>
<thead>
<tr>
<th>PCR Mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Promega</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Primer</td>
<td></td>
</tr>
<tr>
<td>Fvvh</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>Rvvh</td>
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</tr>
<tr>
<td>FviuB</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>RviuB</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DW</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>Template DNA (1/10)</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>

Table 3.4: PCR reaction sequence for *V. vulnificus*

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
<th>Temperature</th>
<th>Time</th>
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</thead>
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<td>1</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Primer Annealing</td>
<td>65°C</td>
<td>1 min</td>
<td>X 35 cycles for steps 2-4</td>
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<tr>
<td>4</td>
<td>Primer Extension</td>
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<td>1 min</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final Extension</td>
<td>72°C</td>
<td>5 min</td>
<td></td>
</tr>
</tbody>
</table>

After PCR testing was complete, 3 µl of the sample were pipetted into a 1% agarose gel containing ethidium bromide dye. Gel electrophoresis was run for 20 minutes, and the gel was viewed under UV light using a gel doc camera. The presence or absence of *Vibrio* could then be determined.
Table 3.5 PCR reaction mixture for *V. parahaemolyticus*

<table>
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<tr>
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</thead>
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<td>Primer</td>
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<tr>
<td>F-tlh</td>
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</tr>
<tr>
<td>R-tlh</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>F-tdh</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>R-tdh</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>F-trh</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>R-trh</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>DW</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>Template DNA (1/10)</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>

Table 3.6: PCR reaction sequence for *V. parahaemolyticus*

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
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<tr>
<td>3</td>
<td>Primer Annealing</td>
<td>55°C</td>
<td>1 min</td>
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<td>Primer Extension</td>
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<td>1 min</td>
</tr>
<tr>
<td>5</td>
<td>Final Extension</td>
<td>72°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

3.6 Salinity Testing

A laboratory experiment was conducted to determine the salinity ranges that *V. vulnificus* and *V. parahaemolyticus* can live at. The test used 2 ppt increments beginning at 0ppt and ending at 36 ppt. A 1X APW solution was created, and the salinity was adjusted accordingly. The APW was pipetted into 100 ml bottles, autoclaved, and cooled overnight.
Confirmed strains of *V. vulnificus* and *V. parahaemolyticus* were used for the experiment. Cells of each species were transferred to tubes containing 60 ml of PBS. The suspensions were then vortexed until mixed, and 1 ml was pipetted in triplicate to each bottle. The bottles were placed in an incubator at 37 °C. The bottles were removed at various intervals over a 48 hour time period. 100 µl of samples were transferred from each bottle to separate centrifuge tubes, and the bottles were placed back into the incubator. The absorbencies of the transferred samples were then measured using a spectrophotometer.
4. RESULTS

4.1 SEASONAL VARIATIONS IN PUTATIVE *VIBRIO* POPULATIONS

4.1.1 BARATARIA BAY

Figure 4.1 shows seasonal abundance of putative *V. vulnificus* and *V. parahaemolyticus* at each sampling site in Barataria Bay. The data were plotted alongside the averages of water temperature and salinity for all the sampling sites in the bay. Seasonally, the TCBS plate counts appeared to be affected primarily by water temperature. However, there were several exceptions. For example, the June 24, 2008 samples had higher plate counts with an average of 284 CFU/100 mL; in the July 2008 sampling, the average plate count dropped below 100 CFU/100 mL although the water
temperature (29.7 °C) was the same as that of the June 24 2008 sampling. The August 2008 sampling received little growth on TCBS plates even though average temperature and salinity were at 29°C and 4.7 g/L, respectively. Also, while not quite as low as August 2008, May 2009 showed a decrease in *Vibrio* counts from April 2009, although average temperatures had risen by 7°C. The average salinity had dropped by nearly 3g/L in the May 2009 sampling in comparison to the April 2009 sampling. Therefore, the decrease in salinity may have been enough to offset the effect of increased temperature. The exceptions to the overall temperature-impacted seasonal pattern of putative *V. vulnificus* and *V. parahaemolyticus* might have been attributed to the fluctuating salinities in the bay.

Figure 4.2: Seasonal putative *V. vulnificus* counts from Barataria Bay as grown on CC
Figure 4.2 shows seasonal CC plate counts at each sampling site in Barataria Bay. Like the TCBS plate count data, this chart also shows a trend that appeared to follow changes in temperature. However, there were several instances (April, May, July, and August 2008) where there was recorded little if any growth on CC plates even during times of warm water temperatures. Interestingly, both TCBS and CC plates had very little growth during the August 2008 transect. Conversely, February and March of 2009 appeared to have unseasonably high *Vibrio* growth. In fact, both months had the highest plate counts (1058 CFU/100 mL) that were observed throughout the two years of sampling.

**Figure 4.3:** Putative *Vibrio* seasonal variations for MPN counts in Barataria Bay
Figure 4.3 shows monthly MPN averages for Barataria Bay. With water temperatures averaging nearly 30 °C in August 2008, there was a high level of growth in the MPN tubes. Presumed average *Vibrio* growth then appeared to follow a normal seasonal pattern until May 2008. Thereafter, even with average water temperatures rising above 24°C, putative *Vibrio* levels began to drop. Furthermore, as water temperature averages pushed to an excess of 29°C for the following two months, the MPN numbers plummeted even further.

Average numbers in July 15, 2008 were shown to be lower than any other month of the year with an MPN of 2267 CFU/100 mL. Conversely, the following month’s MPN Vibrio levels were the highest for the two-year sampling period. In fact, August 2008 showed a putative MPN count of nearly 20000 CFU/100 mL higher than the next prolific month. MPN counts for the remainder of 2008 dropped off with the weather. As the weather began to warm in the spring of 2009 MPN counts began to rise and level off. MPN levels remained fairly even with numbers between 10000 and 5000 CFU/100 mL. However, June 2009 was unseasonably low with counts below even December of the previous year.

However, these MPN data have not been PCR-confirmed yet. The data would include *Vibrio* species other than *V. vulnificus* and *V. parahaemolyticus* and possibly some non-vibrio species.

4.1.2 BRETON SOUND

Sampling in Breton Sound began on September 9, 2007, and was carried out monthly until July 9, 2009. Seasonal variation in the *Vibrio sp.* throughout the waters of Breton Sound based on TCBS plate counts is shown in figure 4.4. Plate counts from each site were plotted alongside the average water temperature and salinity readings for all the
Due to broken equipment, temperature data was not collected for six of the first seven samplings, and salinity data was not collected for the first two samplings. Thus it is difficult to interpret two full years of sampling. Furthermore, plate counting was not carried out monthly during the first year of sampling. However, plate counts were conducted during March 2008 in which three samplings were carried out to monitor a freshwater pulsing event. In fact, salinity levels averaged less than 2 g/L across the sampling sites during this event.

During the March 2008 samplings, actual salinity levels for the first two trips remained below 1g/L in all sites except for 15 and 16. Site 15 remained just below 2g/L until the final March sampling of 2008. This sampling showed that all sites had actual salinities less than 1g/L with the exception of site 16. So, the average salinity remained less than 2g/L throughout March, dropped below 1g/L for April and May, and then rose to almost 3g/L in June. Finally, the average salinity dipped back below 2g/L during the August transect before climbing back up to 11g/L in September.
The first Breton Sound sampling (September 2007) had high counts for both TCBS and CC plates, with total site averages of $>1400$ CFU/100 mL and $>400$ CFU/100 mL, respectively. Putative *Vibrio* averages then dropped significantly to below 150 CFU/100 mL on TCBS plates, and to nearly 10 CFU/100 mL on CC plates for the month of October. Plate counts were then suspended until March 2008. Beginning in March, TCBS plate counts averaged below 100 CFU/100 mL until July. In July they reached an average of 165 CFU/100 mL, and in August they greatly increased to an average of 1287 CFU/100 mL.

Figure 4.5: Seasonal putative *V. vulnificus* counts from Breton Sound water as grown on CC.
CFU/100 mL. For CC plates, there was no visible growth for the first sampling in March, and then the average number stayed below 10 CFU/100 mL until April. For the remainder of the summer of 2008 the CC plate count averages steadily increased.

Plate count averages on TCBS were lower than expected in September 2008 while temperatures averaged above 25 °C. However, during the month of October, TCBS plate counts were at their highest for 2008 (>1400 CFU/100 mL). Most of the remaining samplings showed TCBS plate counts that followed seasonal temperature and salinity patterns. April 2009 showed the only deviation as plate count averages dipped to nearly 200 CFU/100 mL. While temperatures averaged above 21°C for April, there was a slight decrease in the average salinity. This change may have played a part in the drop of putative *Vibrio* numbers. CC plate count averages remained slightly more consistent as compared to TCBS plate counts from September 2008 to July 2009. Plate counts for September showed greater averages for the CC media with putative *Vibrio* numbers of ~800 CFU/100 mL. In fact, CC plate counts for November 2008 and May 2009 were greater than or equal to TCBS plate counts.

Because of the deficiencies of physical data shown in figure 4.6, it is hard to make assumptions about the first 6 samplings. However, based on the Barataria Bay data, one could deduce that water temperatures in Breton Sound from October through March could possibly also have averaged below 20°C. Now, if this is comparable to Breton Sound, then this could explain the reduced numbers in presumed *Vibrio* levels from November through March. Also, the freshwater pulse during March could be used to explain the reduced numbers through June. Water temperatures rose during May, but just like the plate counts there was no significant increase in growth until August 2008.

The most notable variance in MPN levels is from May 2009 to June 2009. May
temperatures were fairly cool (23.2°C), but quickly rose (29.8°C) in the following month. MPN counts suggesting increased Vibrio growth were shown at this time. However, a very significant drop in salinity (6.77g/L to 5.66g/L) appears to lower the Vibrio levels in July. While there was also a drop in salinity from May to June, the quickly rising temperatures seemed to play a larger factor in Vibrio proliferation.

While the Breton Sound samplings began on September 29, 2007, sediment samples were not collected before March 17, 2008. Yet, it was not until the June, 24 2008 sampling that the sediment protocol was perfected. Sediment was then processed monthly until July 2009.

Figure 4.7 shows that TCBS trends for sediment typically followed seasonal changes in water temperature and salinity. Still, there were a few exceptions such as June
2008 and May 2009. Sediments collected in June 2008 had similar results to the water plated on TCBS in that it showed a much higher count than the rest of the sampling dates. However, average plate counts were still significantly lower in sediment for TCBS. Figure 4.8 shows the yearly transect data for growth on CC plates. Unlike the water samples, these results appeared to follow a trend more closely related to average water temperatures than average salinities. A few exceptions are June 2008 in which there was no putative *Vibrio* growth on TCBS plates, and <2CFU/g were seen during May 2009. Both samplings had average water temperatures above 20°C, but only June had an average salinity below 10g/L. Live oysters were collected exclusively from site 16 in Breton Sound. The first oyster samplings began on March 28, 2008, and then oysters were collected upon

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Fig. 4.7: Seasonal putative *V. vulnificus* and *V. parahaemolyticus* populations from sediment samples as grown on TCBS plates
Fig. 4.8: Seasonal putative *V. vulnificus* populations from sediment samples as grown on CC plates

Fig. 4.9: Seasonal populations of putative *V. vulnificus* and *V. parahaemolyticus* from oyster samples as grown on TCBS plates
availability. Putative *Vibrio* numbers on TCBS plates appeared to follow seasonal temperature and salinity trends. CC plate counts were difficult to determine, and did not provide an adequate amount of data for analysis. However, the TCBS plate counts showed putative *Vibrio* levels as low as 33 CFU/g (February 2009), and as high as 4000 CFU/g (April 2009). During the winter months Vibrio levels were found to be uniformly low through all three phases of sampling. However, in warmer months, such as April

Fig. 4.10: Seasonal putative *V. vulnificus* populations from oyster samples as grown on CC plates
2009, *Vibrio* concentrations were 6X and 140X those found in sediment and water, respectively.

### 4.2 SPATIAL VARIATIONS IN PUTATIVE *VIBRIO* POPULATIONS

#### 4.2.1 BARATARIA BAY

![Graph](https://via.placeholder.com/150)

**Fig. 4.11** Top: Spatial patterns of putative *V. vulnificus* and *V. parahaemolyticus* in water column based on annual TCBS plate count averages from Barataria Bay

Bottom: Spatial patterns of *V. vulnificus* (putative) in water column based on annual CC plate count averages from Barataria Bay

Average annual water temperatures remained fairly consistent throughout Barataria Bay, but there was a clearly defined salinity gradient increasing from North to
South. Along the transect average temperatures ranged from 23.0°C to 24.2 °C. The average salinity in Barataria Bay was highest at site number 2 at nearly 19 g/L, and then gradually dropped as it moved towards site number 33 with a salinity of virtually 0 g/L.

Spatial distribution of *Vibrio* putatively based on plate counts appeared to roughly follow the salinity gradient. These numbers were highest in sites 2, 8, and 16, but then sharply decline in the remaining three sites. The first three sites averaged putative *Vibrio* counts of 276.8 CFU/100 mL on TCBS, and the remaining three sites on TCBS averaged 43.2 CFU/100 mL. Based on presumed CC media numbers, *V. vulnificus* levels followed a similar pattern with an average of 293.7 CFU/100 mL for the first 3 sites. These numbers dwarfed the final three sites that only averaged 5.7 CFU/100 mL on CC plates.

![Graph showing spatial variation of putative Vibrio counts in Barataria Bay](image)

**Fig. 4.12** Annual MPN averages showing spatial variation for putative Vibrio counts in Barataria Bay

Figure 4.12 shows spatial variations of putative *Vibrio* based on average annual MPN counts. The data were coupled with the average annual temperature and salinity for each site. Notice that the temperature data shown in figure 4.12 was slightly different
from those for the plate counts shown in Figure 4.11. This is because plate counts were not conducted monthly during the first year of sampling, and the temperature and salinity averages reflected only the months in which those tests were conducted. Annually, the temperatures averaged from 22.0°C to 23.1°C throughout the Bay.

The average salinity throughout the Barataria Bay clearly follows a gradient with salinities >19 g/L in site 2, and then decreased steadily to <1 g/L at site 33. Unlike the plate counting numbers, the MPN data does not show significantly higher projected numbers for sites 2, 8, and 16. Site 2 had a MPN of 24747 CFU/100 mL, which was higher than all other sites with the exception of site 30. Site 30 had an MPN of 27222 CFU/100 mL, which was the highest level along the entire transect. Following site 2, there was a sharp decline at site 8 which had the lowest MPN with an average count of 6693 CFU/100 mL. This was followed by an increase at site 16 to an MPN of 16094. Site 23 then dropped again to levels just above site 2, and then the number shot up to a larger average MPN count at site 30. The average MPN fell to 9080 CFU/100 mL at site 33.

Again, notice that these MPN data have not been PCR-confirmed yet.

4.2.2 Breton Sound

Figure 4.13 shows yearly averages of colony forming units (CFU) per 100 ml of sample as grown on TCBS and CC plates. These numbers were then compared to annual average water temperatures and salinity levels.

The counts of putative *V. vulnificus* and *V. parahaemolyticus* on TCBS for the first year of sampling show an increase beginning at site 4 and progressing towards site 16 with one exception. Site 8 showed a higher than expected amount of colonies with an average of 458 CFU/100 mL. There was a significantly lower level of colony growth for
site 4 with counts averaging 88.6 CFU/100 mL annually. While lower than site 8, site 11 averaged approximately 100 CFU/100 mL higher than site 12. Finally, sites 15 and 16 showed the highest means with respective levels of 522 and 865 CFU/100 mL.
Likewise, presumed CC averages were at their lowest at site 4 with counts of 75 CFU/100 mL, and at their highest at site 16 with numbers >600 CFU/100 mL. Temperatures remained relatively constant with averages ranging from 22.9°C to 24.2°C along the transect. However, the water was as fresh as 0.93 g/L of salinity at site 4, and progressed steadily towards 12.9 g/L at site 16.

Figure 4.14: Annual MPN averages showing spatial variation for putative Vibrio counts in Breton Sound

Figure 4.14 illustrates annual MPN, temperature, and salinity averages in Breton Sound. As previously mentioned, the temperature and salinity data were incomplete, and thus the numbers were not a fair representation of the yearly average for these parameters. Nonetheless the MPN data showed a slightly erratic oscillation that didn’t appear to follow the salinity gradient identified in the figure. Site 11 was the highest with a MPN of 52091 CFU/100 mL, while sites 15 and 16 were the lowest with MPNs of ~22000 CFU/100 mL each. It is interesting to note that the oscillation pattern has some
similarities to the one shown in the CC spatial patterns. The CC plate counts, like the
MPN data, showed higher averages at site 11. However, unlike the MPN spatial data, the
CC plate count averages at site 11 did not possess the highest numbers.

Average temperatures during the MPN collection months ranged from 21.1°C to
22.6°C, and salinity averages fell in a range between 0.95g/L and 13.4 g/L. While the
annual average of plate counts at each site positively correlated with salinity, the annual
average of MPN data did not show the same relationship. For sites 4, 8, 11, and 12 the
MPN positively correlated with the salinity, but sites 15 and 16 had negative correlations
between the MPN and salinity.

![Graph showing TCBS and CC plate count averages for Breton Sound sediment based on site number](image)

Fig. 4.15: Annual TCBS and CC plate count averages for Breton Sound sediment based on site number
Figure 4.15 shows annual averages of TCBS counts following the salinity gradient that started with site four’s average of 1.19 g/L, and then progressed steadily to site sixteen’s average of 15.99 g/L. This is comparable to the average TCBS plate counts shown in figure 4.4 from the second year of water sample collections. However, the sediment samples’ average only ranged from 28 CFU/g to 327 CFU/g while the waters’ ranged from 58 CFU/100 mL to 1301 CFU/100 mL. Unlike with the water samples, the average CC plate counts for sediment did not follow any specific trend. On the other hand, resembling the TCBS plate counts, the average CC plate counts for sediment were higher than in water with averages all <100CFU/g.

4.3 SALINITY TESTING

A laboratory study was used to find optimal salinity ranges for both *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Type strains of both species were obtained and grown and a temperature of 37°C.

![Vp Laboratory Salinity Testing](image)

Fig. 4.16: Laboratory growth test results of *Vibrio parahaemolyticus*
The laboratory test (Fig. 4.16 and 4.17) shows several things that are difficult to see from monthly sampling. First, the growth (measured as absorbencies) of tested *V. parahaemolyticus* type strain grew at a much quicker rate than *V. vulnificus* type strain. It also appeared that each species of *Vibrio* had an optimal salinity range. The *V. parahaemolyticus* grew best at 35g/L within a range of 0 to 35 g/L, while the *V. vulnificus* grew best at 17g/L. Based on the testing, *V. parahaemolyticus* appeared to grow better overall at higher salinity levels. In fact, it is evident that *V. parahaemolyticus* growth had a positive correlation to salinity within the tested range of 0 to 35 g/L. On the other hand, *V. vulnificus* showed an optimal salinity range between 5g/L and 17g/L.

**4.4 Polymerase Chain Reaction**

Successful execution of multiplex polymerase chain reaction (PCR) techniques were achieved in our lab. Several brands of polymerase were tried until finding one that
would work most effectively. The Applied Biosystems (AB) PCR kit was found to be least effective in quantification of gene products in our studies. Bands were very weak, and often hard to read. The Bio-Rad Laboratories Sybr Green was found to successfully amplify genes. However, it was difficult to repeat results using the Sybr Green mixture. Bands would sometimes be very clear, while other times be faded. Finally, Promega master mix was used, and could produce clear and consistent PCR products.

![Image](image.png)

**Fig. 4.18:** PCR results via gel electrophoresis

**Left:** Multiplex PCR detection of total and pathogenic genes of *V. vulnificus*. First column is negative strain, second column shows positive confirmation of total *V. vulnificus vvhA* gene, third column shows positive confirmation of *viuB*, and the fourth column shows confirmation of both vvhA and viuB genes.

**Right:** Multiplex PCR detection of total and pathogenic genes of *V. parahaemolyticus*. First column shows confirmation of pathogenic *tdh* gene, second column shows confirmation of total *V. parahaemolyticus tlh* gene, and the third column shows the confirmation of both the *tdh* and *tlh* genes.

For the PCR detection of *V. vulnificus*, putative *V. vulnificus* colonies that were cultured on CC plates were transferred to larger TCBS plates in order to increase cell mass. It was found that occasionally some of the transferred colonies would grow a negative yellow color as opposed to positive green. Using PCR, it was determined that
once grown on TCBS, only the green colonies were *V. vulnificus*. 43 of these colonies were tested, and a 100% success rate with PCR confirmation was achieved. Out of the 7 yellow transferred colonies tested, 100% were confirmed negative via PCR. These results showed a positive rate of 83.8% as true *V. vulnificus* among the tested putative colonies from CC plates. However, further testing on more isolates that were stored at -80°C should be conducted.

Preliminary PCR testing of MPN tubes has shown that out of the 331 positive growth tubes that were tested, 115 were *vvhA* positive and 9 were *tlh* positive. Of those 331 tubes tested, 122 of the tubes that were negative for both genes came from typically lower salinity sites. Predominately, these were sites 4 and 8 from Breton Sound, and sites 30 and 33 from Barataria Bay. Interestingly, if eliminating the tubes coming from lower salinity sites from the results, the *vvhA* positive rate increased from 35% to 55%. However, the positive rate only increased from 3% to 4.8% for *tlh*. Many samples still remain to be examined, and thus a better understanding could be gained after further testing is conducted.
Table 4.1: Examples of positively growing MPN tubes tested for presence of *V. vulnificus* and *V. parahaemolyticus*.

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<th>Location</th>
<th>Date</th>
<th>Type</th>
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<th>Site</th>
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<th>tlh</th>
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<td>-</td>
</tr>
<tr>
<td>Breton</td>
<td>July-09</td>
<td>MPN</td>
<td>10 ml</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Breton</td>
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<td>MPN</td>
<td>10 ml</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
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<td>10 ml</td>
<td>8</td>
<td>-</td>
<td>+</td>
</tr>
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<td>8</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Breton</td>
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<td>10 ml</td>
<td>8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Breton</td>
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<td>MPN</td>
<td>10 ml</td>
<td>11</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Breton</td>
<td>July-09</td>
<td>MPN</td>
<td>10 ml</td>
<td>11</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Breton</td>
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<td>-</td>
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<tr>
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<td>10 ml</td>
<td>12</td>
<td>+</td>
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</tr>
<tr>
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<td>10 ml</td>
<td>12</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Breton</td>
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<td>MPN</td>
<td>10 ml</td>
<td>12</td>
<td>+</td>
<td>-</td>
</tr>
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<td>16</td>
<td>+</td>
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5. DISCUSSION

Studies such as the one by Blackwell and Oliver (2008) have shown *Vibrio* populations to be strongly correlated with seasonal variations in temperature. It is important to determine if seasonality of a water body is the most important determinant of *Vibrio* populations. Coastal water bodies around the world are unique, and have various factors effecting microbial community structures in the systems. While this study confirmed that putative *Vibrio* numbers followed the natural ebb and flow of water temperature, it became clear that other factors, in particular salinity, were at hand.

Lane et. al (1999) and Jones et. al (2002) have shown that salinity is not constant throughout both Breton Sound and Barataria Bay. Little is known about the true effects of salinity on the population dynamics of *V. vulnificus* and *V. parahaemolyticus* in coastal waters. Randa et al. (2004) has suggested that the presence of *V. vulnificus* is interdependent to salinity, but has mixed results based on salinity ranges. Blackwell and Oliver (2008) believed that the presence of *V. parahaemolyticus* is completely unrelated to salinity. However, this research has indicated a strong correlation with regards to salinity and populations of putative *V. vulnificus* and *V. parahaemolyticus* for Breton Sound and Barataria Bay.

Based on plate counts, the populations of putative *V. vulnificus* and *V. parahaemolyticus* were shown to have a positive correlation to salt concentrations in both water bodies. Salinity typically increased with proximity to the Gulf of Mexico, and predominately the putative *Vibrio* populations did as well. While vibrios were found throughout each transect, they appeared to be in the greatest abundance at higher salinity sites. Furthermore, a freshwater pulsing event was shown to suppress *Vibrio* levels
throughout most of the Breton Sound. In addition, lab studies were conclusive in showing that each *Vibrio* species has its specific preferences for salt concentrations.

Previous research has shown that vibrios tend to be VBNC or nonexistent in the water column during cooler weather, but remain similar levels year round in sediments (Blackwell and Oliver, 2008). However, this study shows that the putative *V. vulnificus* and *V. parahaemolyticus* populations in sediments in Breton Sound typically followed the same seasonal pattern as the populations suspended in the water, but they were found at higher levels in general. The reasons for these differing findings remain uninvestigated. One possible explanation could be differences in water depth among different study systems.

Previous research has shown that *Vibrio* concentrations in oysters can be up to 100 times higher than in the water column. This may be due to an association of *Vibrio* and plankton. The *Vibrio* are thought to be concentrated in the oysters as they feed on the plankton (DePaola *et. al*, 1990). This study shows that putative *Vibrio* populations varied from month to month. On an annual average, putative *V. vulnificus* and *V. parahaemolyticus* populations were found to be about 5 times and 126 times those in sediment and water column, respectively, in Breton Sound.

Without PCR confirmation, the MPN results were misleading. This is because that the medium APW used for MPN tube cultures is not highly selective, and thus could allow other *Vibrio* species and aquatic microorganisms to proliferate. The preliminary PCR results with a limited number of tubes indicated that *V. vulnificus* might overwhelmingly dominate the sampling sites during warmer months. One explanation could be the lower salinity levels throughout all sampling sites in this study. However, one must bear in mind that only a small portion of samples collected have been tested
using PCR. Further work needs to be done for the PCR confirmation. In addition, statistical analysis needs to be carried out to analyze the relationships between environmental parameters and *V. vulnificus* and *V. parahaemolyticus* populations and to compare the differences between the two bays.
6. CONCLUSIONS

The putative data for *Vibrio* levels in Barataria Bay and Breton Sound waters generally followed a trend that was seasonally dominated by water temperatures, while spatially, salinity levels influenced the *Vibrio* populations. The plate count data indicate that *V. vulnificus* appeared less abundant in the environment during colder temperatures than *V. parahaemolyticus* as in most cases there was little if any discernable growth on CC plates versus low, yet consistent, levels of growth on TCBS plates during the winter.

Both field data and laboratory testing shows salinity as one of the major impacting factors in *Vibrio* proliferation. For example, site 2 of Barataria with an annually average salinity of 18.7 g/L had an annually average putative *Vibrio* population of 277 CFU/100 mL in contrast to an average *Vibrio* population of 1 CFU/100 mL at site 33 with an average salinity of 0.15 g/L.

Based on the laboratory testing, *V. parahaemolyticus* appeared to grow better overall at higher salinity levels. Within the salinity range of 0 – 35 g/L, *V. parahaemolyticus* growth had a clear positive correlation to salinity. On the other hand, *V. vulnificus* showed an optimal salinity range of 17g/L to 5g/L.

The plate counts showed that Breton Sound averaged 388 CFU/100 mL annually for all the sampling sites while Barataria Bay averaged 130 CFU/100 mL. Water temperatures throughout the sampling sites in Barataria Bay and Breton Sound remained relatively the same during the course of this study. The average annual salinity for sites in Barataria Bay ranged from 18.6g/L at site 2 to 0.15g/L at site 33. Likewise, the average annual salinity in Breton Sound ranged from 12.9g/L at site 16 down 0.93g/L at site 4. The differences in the salinity at the sampling sites in the two bays might have accounted for the differences in the *Vibrio* populations between the two bays.
Annual changes in *Vibrio* populations contained in Breton Sound sediment followed a trend that was heavily dominated by temperature change. During warmer months TCBS and CC plates produced similar growth suggesting higher levels of *V. vulnificus*. However, a near absent level of growth on CC plates during colder months suggests either the absence of *V. vulnificus* or the occurrence of the VBNC state. Spatial variation in sediment was relatively low throughout all sites. The only exception was a significantly higher average on TCBS plates at site 16. An average of 327 CFU/g was found at site 16, which all other sites were below 105 CFU/g.

As one would suspect, *Vibrio* found in oysters at site 16 in Breton Sound were found to be higher than what were typically shown in both water and sediment. Almost no *Vibrio* were identified on CC plates with the exception of the last month of our survey. However, TCBS plates showed a clearly defined seasonal variation in *Vibrio* levels. Water temperature at site 16 appeared to play a bigger role than salinity in *Vibrio* abundance.

The MPN method proved to be rather ineffective without the PCR confirmation of *Vibrios* as the media used for the MPN is not very selective. The protocols for PCR confirmation of *V. vulnificus* and *V. parahaemolyticus* have been successfully established in our laboratory during this research. Also a number of the MPN positive-growth tubes and selected colonies from the plates have been tested with the established protocols. Future work will continue the PCR confirmation work and carry out statistical analysis for the relationships between environmental parameters and *V. vulnificus* and *V. parahaemolyticus* populations and to compare the differences between the two bays.
7. REFERENCES


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Brian Matherne was born in June 1985 in Gulfport, Mississippi. Growing up overlooking the Mississippi Sound, Brian became fascinated with the coast at an early age. As a boy he spent several summers attending “Sea Camp” at the J.L. Scott Marine Education Center, and was also very active in scouting. In June of 2001 Brian became an Eagle Scout, the highest rank bestowed by the Boy Scouts of America.

He graduated from Mercy Cross High School in May of 2003, and enrolled at Louisiana State University that following August. While Brian chose to major in biological sciences, his childhood passion for the sea led him to concentrate his studies in marine biology. Realizing his passion he spent the summer of 2006 at the Gulf Coast Research Laboratory, and took two field courses in marine science.

After completing his bachelor’s degree in May of 2007, Brian pursued various interests before returning to Louisiana State University in January of 2008. Here he began his master’s degree in environmental science and toxicology under the guidance of Dr. Aixin Hou. While currently a master’s candidate at Louisiana State University, Brian has been accepted into a doctoral program at the University of North Texas. He will continue his studies in environmental science, and hopes to focus his efforts on solving water quality problems.