EFFECTS OF TEMPERATURE AND SALINITY ON THE ABUNDANCE OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* IN *CRASSOSTREA VIRGINICA* IN WEST GALVESTON BAY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effects of Temperature and Salinity on the Abundance of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in *Crassostrea virginica* in West Galveston Bay

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Vibrio vulnificus and *Vibrio parahaemolyticus* are potentially harmful pathogens that are naturally found in the estuarine environment. These pathogens commonly cause illness from the consumption of raw or undercooked oysters, which are known to concentrate bacteria through filter feeding. It is suspected that these species become more abundant as the water temperature increases, resulting in an increased potential for human infection. It is important to understand the relationship between abundance of the pathogen and water temperature and salinity for a specific region in order to prevent infection. In this study, the abundance of *Vibrio* spp. was quantified from weekly oyster samples from West Galveston Bay using quantitative PCR. The correlation tests and multiple linear regressions did not reveal any strong correlations between bacterial levels and environmental conditions. However, it was found that the levels of *V. vulnificus* and *V. parahaemolyticus* were found in greater levels when the water temperature and salinity were in the ideal ranges as stated in Givens *et al.* (2014).

ACKNOWLEDGMENTS

This research was sponsored by the Seafood Safety Laboratory at Texas A&M University at Galveston. I would like to thank Dr. John Schwarz, Mona Hochman, and Claudia Schinnie for their assistance and guidance throughout this project. Additionally, I would like to thank Dr. Jessica Jones and her staff at the FDA Gulf Coast Research Laboratory for providing PCR training and additional guidance.

NOMENCLATURE

APW	Alkaline Peptone Water, standard growth medium for estuarine bacteria
MPN	Most Probable Number, the FDA-approved standard to quantify bacteria from a serial dilution by determining the best statistical estimate of the number of bacteria present.
PBS	Phosphate Buffered Saline solution, diluent for serial dilution
PCR	Polymerase Chain Reaction, method used to amplify DNA across several orders of magnitude
tdh	Thermostable direct hemolysin gene found in pathogenic *Vibrio parahaemolyticus*
tlh	Thermolabile hemolysin gene found in all *Vibrio parahaemolyticus*
trh	tdh-related hemolysin gene found in pathogenic *Vibrio parahaemolyticus*

CHAPTER I

INTRODUCTION

Disease and pathogenicity

Vibrio vulnificus and *Vibrio parahaemolyticus* are gram negative bacteria endemic to estuarine and marine waters (DePaola *et al.* 1990). These bacteria are of concern in the coastal environment as they have the potential to cause septicemia and acute gastroenteritis in humans, respectively (Ward and Bej 2005). The most common causes of infection by these bacteria are from the consumption of raw or undercooked seafood or through wound infections (Givens *et al.* 2014). Oysters are of utmost concern as they are often consumed raw and are known to concentrate these bacteria during the filter feeding process (Ward and Bej 2005).

Illness from *V. vulnificus* infection has the potential to be deadly to persons with preexisting health conditions (i.e. liver disorders, diabetes, or immunocompromising conditions). Consumption of infected oysters can result in a severe systemic infection known as primary septicemia, and in some severe cases, patients developed secondary lesions on their extremeties (Jones and Oliver 2009). Although *V. vulnificus* infections in humans are uncommon, they have one of the highest hospitalization rates (91.3%) and death rates (34.8%) of all foodborne pathogens (Oliver 2005). *Vibrio vulnificus* is responsible for approximately 90% of all seafood related deaths in the United States (Jones and Oliver 2009).

While *V. parahaemolyticus* infections have a much lower mortality rate, widespread outbreaks have been documented in the past because this bacteria can affect anyone who ingests it. The

largest outbreak in the United States occurred in 1998 from oysters harvested from Galveston Bay, resulting in 416 illnesses nationwide (Ward and Bej 2005). If ingested from raw or undercooked shellfish, *V. parahaemolyticus* can cause diarrhea, nausea, vomiting, fever, and chills, also known as gastroenteritis (Yeuna and Boor 2004). Although *V. parahaemolyticus* infections are typically less severe than those of *V. vulnificus*, these infections still have the potential to be fatal for patients with a preexisting medical condition.

Trends in abundance

As organisms of public health concern, it is important to study and understand the abundance and distribution of these harmful pathogens in the coastal environment. Previous studies have attempted to establish correlations between the abundance of *V. vulnificus* and *V. parahaemolyticus* and environmental factors such as temperature and salinity. It has been found that levels of *V. vulnificus* and *V. parahaemolyticus* increase when the water temperature rises above 20^{o}C and 15^{o}C respectively, with the highest densities occuring between 20-30^{o}C (Givens *et al.* 2014). Levels of these bacteria are therefore highest in the summer months, when the water temperature is at an optimum. Higher densities of *V. vulnificus* and *V. parahaemolyticus* have been observed when the salinity ranges from 10-20 ppt and 15-25 ppt respectively (Givens *et al.* 2014). These correlations, however, are affected by region and strain differences. Hence, it is important to examine the correlation between abundance and environmental factors for a specific region to determine the ideal conditions for these pathogens.

Objectives

The purpose of this study will be to determine any existing correlations between the abundance of *V. vulnificus* and two strains of *V. parahaemolyticus,* water temperature, and salinity for West Galveston Bay. Weekly samples of oysters will be collected from Sammy's Reef in West Galveston Bay and analyzed using quantitative polymerase chain reaction (qPCR) to determine the abundance of *Vibrio* spp. The abundance will be compared to water quality data from the time of collection in order to determine the presence, if any, of a correlation. It is expected that the abundance of both species will increase as the water temperature increases and decrease as the salinity increases.

CHAPTER II

METHODS

Collection of oysters

From December 2, 2014 to November 17, 2015, a team of scientists collected 15 live oysters randomly each week from Sammy's Reef in West Galveston Bay ($29^{o}15'$N, $094^{o}55'$W). The oysters were placed in a large bucket and submerged in the water from the sampling site. Additionally, air and water temperature were taken using a standard field thermometer. A water sample was gathered in a sterile plastic bottle, and taken back to the lab to measure the salinity in parts per thousand with a refractometer. Oysters were processed in the lab within an hour of being collected from Sammy's Reef.

Processing of oysters

Upon returning to the laboratory, three oysters were taken at random from the sample set. These three oysters were shucked and the temperature of the oyster meat was recorded. The remaining 12 oysters were scrubbed with a sterile wire brush to remove dirt and algae from the hinge and exterior of the oyster. Next, the oysters were shucked into a sterile, tared blender using a sterile glove and shucking knife. The weight of the twelve oysters was measured, and an equal weight of the growth medium alkaline peptone water, APW, was added to the blender. The oyster mixture was then blended for two minutes on high speed.

A serial dilution was created with the oyster mixture immediately after blending. The 10^{-1} dilution consisted of 20 grams of the 1:1 oyster:APW mixture into 80 grams of sterile phosphate

buffered saline solution, or PBS. After thoroughly mixing this dilution (shaking 25 times in a 1 foot arc within 7 seconds), 11 mL were transferred into the next dilution bottle containing 99 mL PBS to create the 10^{-2} dilution. Subsequent dilutions were created in the same manner to create a total of 6 dilutions. From each, 3 mL were taken to inoculate 1 mL into each of three test tubes containing 10 mL of APW.

Once all of the serial dilution test tubes were inoculated, the tubes were incubated overnight at 35^{o}C. After incubation, the test tubes were examined for turbidity, indicating the presence of bacterial growth. For each of the tubes that were positive for turbidity, one milliliter was pipetted aseptically into a sterile, conical microcentrifuge tube for DNA extraction.

Extraction of DNA

The microcentrifuge tubes containing the oyster samples were boiled for ten minutes at 100^{o}C in a dry bath. Immediately thereafter, the tubes were stored in a freezer at approximately -16^{o}C to -18^{o}C until ready to be run through real time PCR. Prior to PCR, the samples were thawed to room temperature and centrifuged for 30 seconds to form a pellet of cellular debris. The resulting supernatant was used as the template DNA during the PCR reactions.

In addition to the DNA extracts from the weekly oyster samples, overnight cultures of *Vibrio vulnificus* (K5057), *Vibrio parahaemolyticus* (tlh) (K4859), and *Vibrio parahaemolyticus* (tdh/trh)(DI-B9) were also extracted as control DNA. These control DNA extracts were used as positive and negative controls during all PCR runs.

Quantitative PCR

PCR runs were completed using the Cepheid Smart Cycler (Cepheid; Sunnyvale, CA). Each DNA sample was tested for three different strains of *Vibrio* using PCR: *Vibrio vulnificus*, *Vibrio parahaemolyticus* (tlh), and *Vibrio parahaemolyticus* (tdh/trh). Also included in each PCR reaction was an internal amplification control DNA template, which ensured that each run of PCR reactions was performed successfully. The PCR protocol for each strain of *Vibrio* required a specific master mix with species-specific primers and probes, which can be found in Table 1 below.

Table 1. Primer and probe sequences for target strains used during PCR, according to protocols established by Jessica Jones at the FDA, Dauphin Island, Alabama.

Target Strain	Primer or Probe	Sequence
V. vulnificus	Vvh_F Primer	5'-TGTTTATGGTGAGAACGGTGACA-3'
	Vvh_R Primer	5'-TTCTTTATCTAGGCCCCAAACTTG-3'
	Vvh_Probe	TEXAS RED-5'-CCGTTAACCGAACCACCCGCAA-BHQ2
V. parahaemolyticus (tlh)	tl_884F Primer	5'-ACTCAACACAAGAAGAGATCGACAA-3'
	tl_1091R Primer	5'-GATGAGCGGTTGATGTCCAAA-3'
	tlh_1043 Probe	TEXAS RED-5'-CGCTCGCGTTCACGAAACCGT-3'-BHQ2
V. parahaemolyticus (tdh/trh)	tdh_89F Primer	5'-TCCCTTTTCCTGCCCCC-3'
	tdh_321R Primer	5'-CGCTGCCATTGTATAGTCCTTTATC-3'
	tdh_269-20 Probe	6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ
	trh_20F Primer	5'-TTGCTTTCAGTTTGCTATTGGCT-3'
	trh_292R Primer	5'-TGTTTACCGTCATATAGGCGCTT-3'
	trh_133-23 Probe	TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ
Internal Control DNA	IAC_46F Primer	5'-GACATCGATATGGGTGCCG-3'
	IAC_186R Primer	5'-CGAGACGATGCAGCCATTC-3'
	IAC_109 Probe	CY5-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-BHQ2

A master mix containing all of the necessary reagents for real time PCR was created prior to each PCR run. The master mix consisted of the following components at these concentrations: 10X PCR reaction buffer, 50 mM magnesium chloride, 10 mM dNTPs (mixed equal concentration of each nucleotide), 10 μM of each forward and reverse primer, 10 μM of each probe, 5 units/μL of *Taq* polymerase, and the internal control template DNA. Sterile nuclease-free water was used to adjust the volume of each reaction to a total of 25 μL. All master mixes were created in a clean biosafety cabinet to prevent any source of contamination. Once complete, the master mix was transferred to a separate biosafety hood where it was allocated to smart cycler tubes. Each smart cycler tube was inoculated with 2 μL of a no template control (nuclease-free water), positive control DNA, negative control DNA, or sample DNA. These smart cycler tubes were then centrifuged and loaded into a Cepheid Smart Cycler to undergo PCR.

Each target strain was assigned a specific PCR protocol. For *Vibrio vulnificus*, the PCR cycling conditions began with an initial denaturation at 95^{o}C for 60 seconds, followed by 45 cycles of amplification. An amplification cycle consisted of denaturation at 95^{o}C for 15 seconds, annealing at 57^{o}C for 15 seconds, and extension at 72^{o}C for 25 seconds. For all strains of *Vibrio parahaemolyticus*, the PCR cycling conditions began with an initial denaturation at 95^{o}C for 60 seconds, followed by 45 cycles of denaturation at 95^{o}C for 5 seconds, and a combined annealing and extension at 59^{o}C for 45 seconds.

Following the protocols described above, fluorescence readings were obtained for each sample, indicating a positive or negative result for the *Vibrio* spp. In order to determine the amount of

bacteria in each sample, a Most Probable Number (MPN) matrix was used to determine the best statistical estimate of the number of bacteria present. This method is the FDA-approvedstandard for quantifying bacteria from a serial dilution.

Statistical analysis

Upon completion of enumerating all *Vibrio* spp. from the weekly oyster samples, graphs were constructed to display the water temperature, salinity, and level of each species for the whole year. Microsoft Excel was used to run a correlation test between the level of each species and water temperature and salinity. Additionally, a multiple linear regression was performed to determine if a linear model could best explain the data.

Examination of these yearlong graphs revealed several noticeable peaks, or modes, within the four seasons (winter, spring, summer, fall). The data was split into seasons, with spring defined as March through May, summer defined as June through August, fall defined as September through November, and winter defined as December through February. These seasons were defined as stated based on mean water temperature in Galveston and noticeable trends or modes in the graphs. Again, graphs were constructed to show the level of each species, as well as the salinity and water temperature. Similar correlations and multiple linear regressions were run for each of the defined seasons to reveal any correlations between the variables.

CHAPTER III

RESULTS

Bacterial levels

The oyster samples were collected over the course of one year (December 2, 2014 to November 17, 2015), showing how the levels of *Vibrio* spp. vary over a long period of time. Figures 1 through 3 show the abundance of *Vibrio vulnificus* and *Vibrio parahaemolyticus* as well as the salinity and water temperature for each week.

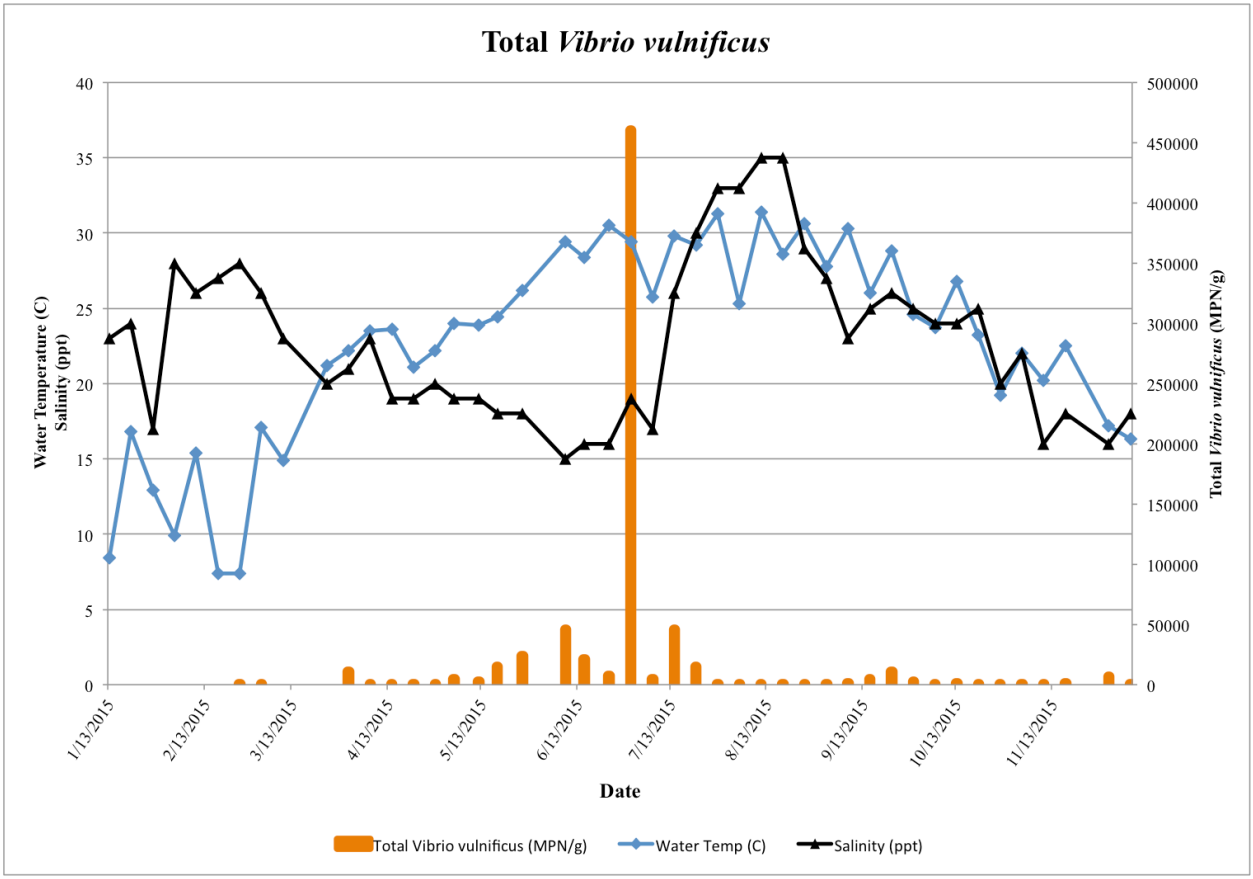

Figure 1. Levels of *Vibrio vulnificus* in relation to salinity and water temperature.

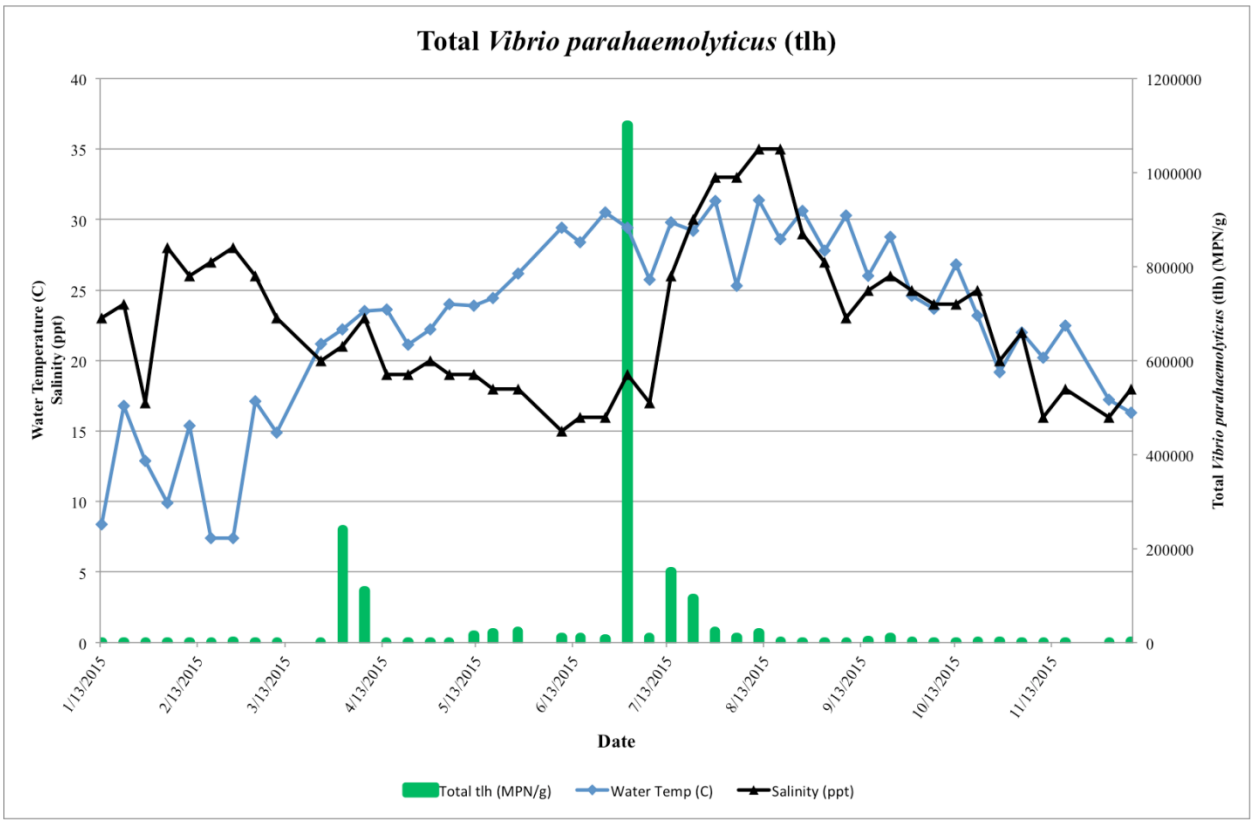

Figure 2. Levels of *Vibrio parahaemolyticus* (tlh) in relation to salinity and water temperature.

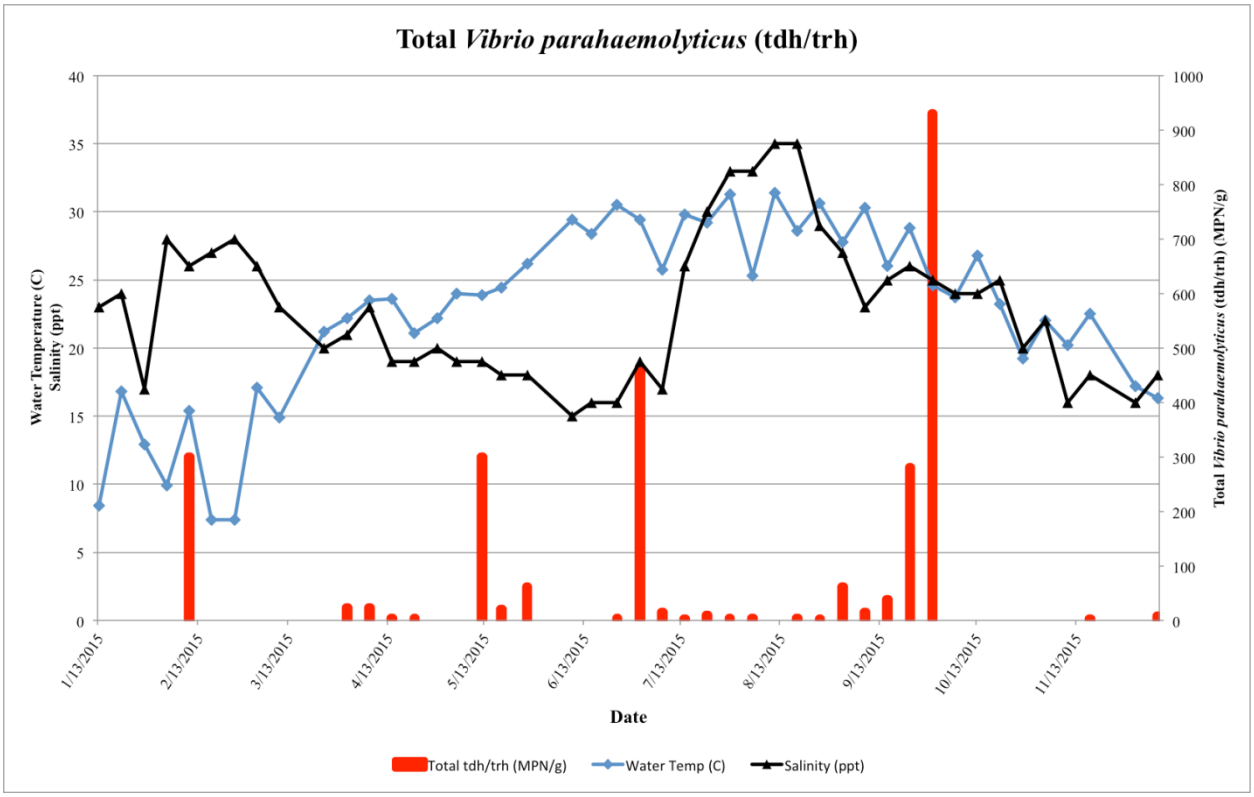

Figure 3. Levels of *Vibrio parahaemolyticus* (tdh/trh) in relation to salinity and water temperature.

The levels of each species experienced drastic fluctuations throughout the year, as seen by the large peaks within certain seasons. The highest levels of all three species occurred during the summer months, especially during June and July. *Vibrio parahaemolyticus* (tlh) was found consistently throughout the year in greater abundance, while *V. parahaemolyticus* (tdh/trh) and *V. vulnificus* were present in much lower levels or absent during several weeks of the year. There were four identifiable trends, or modes, found during the course of the year. Each of these modes occurred during the four seasons, allowing for smaller scale analyses of the bacterial levels, water temperature, and salinity.

Environmental correlations to bacterial levels

The two environmental variables, salinity and water temperature, varied at Sammy's Reef over the course of the year. Salinity ranged from 15-35 ppt, with the highest salinities occurring during the middle of the summer months and the lowest salinities occurring during the spring and winter months. The water temperature at Sammy's Reef ranged from 7.4-31.4^{o}C. Some weeks experienced large changes in salinity or water temperature, which resulted in a drastic change in the bacterial levels in the following weeks.

All of the correlations and multiple linear regressions for each of the species in relation to the water temperature and salinity over the entire year yielded weak correlations between variables. The r values from the correlation tests revealed weak correlations between levels of each *Vibrio* spp. and water temperature, as well as between each *Vibrio* spp. and salinity. The R^2 values from the multiple linear regressions were very close to zero, indicating that the variation in bacterial levels was not explained by the environmental conditions on the reef using a linear model. The r and R^2 values from each of the analyses are summarized below in Tables 2 and 3, respectively.

Table 2. R values from the correlation tests over the entire year for each species. An r value of 1 indicates a perfect correlation.

	R values – Correlation		
	Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
Water Temperature	0.2153	0.2087	0.1324
Salinity	-0.1531	-0.0994	-0.0032

Table 3. R^2 values from the multiple linear regression over the entire year for each species. An R^2 value of 1 indicates a perfect linear correlation.

R^2 Value – Multiple Linear Regression		
Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
0.0711	0.0542	0.0176

Spring bacterial levels and environmental correlation

The analyses for the spring season were performed for the months March, April, and May. Figures 4 through 6 below show the relationship between levels of *Vibrio* spp. and the water temperature and salinity.

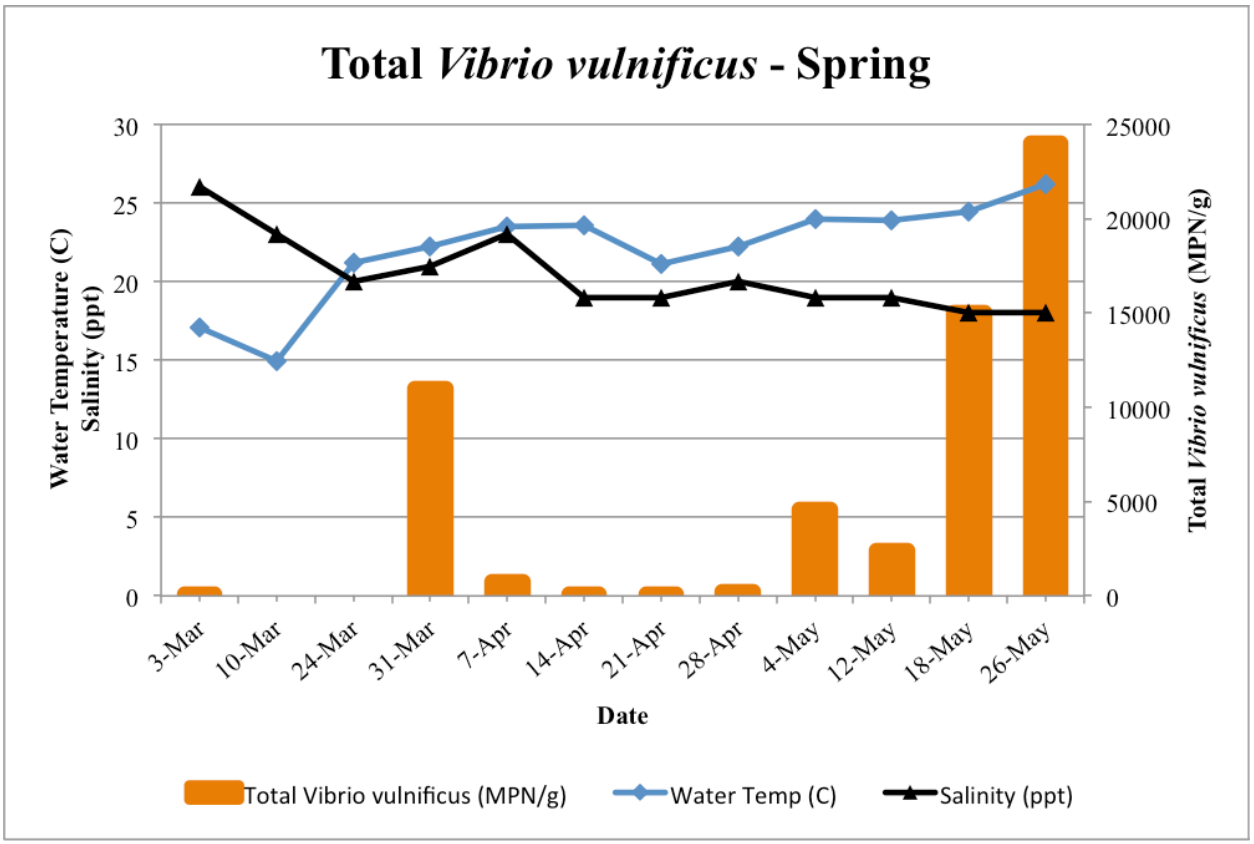

Figure 4. Levels of *Vibrio vulnificus* in relation to salinity and water temperature during the spring season.

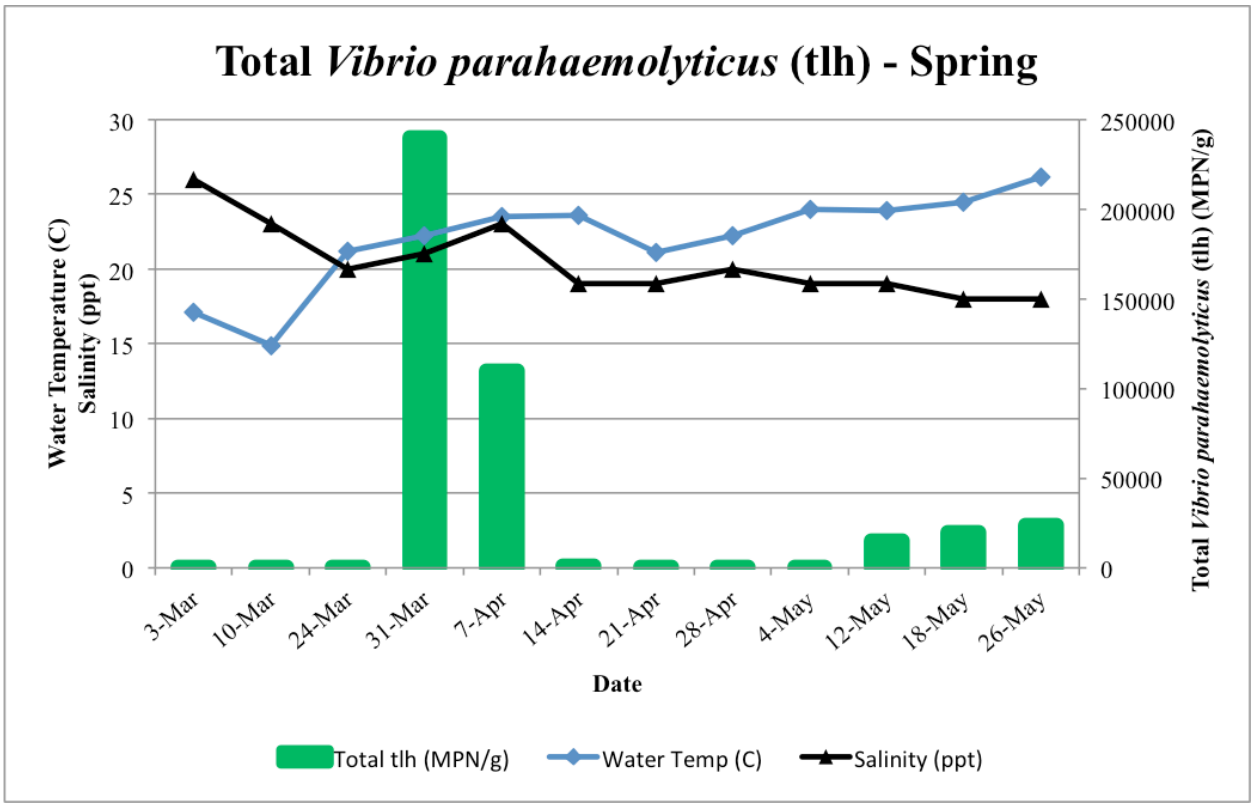

Figure 5. Levels of *Vibrio parahaemolyticus* (tlh) in relation to salinity and water temperature during the spring season.

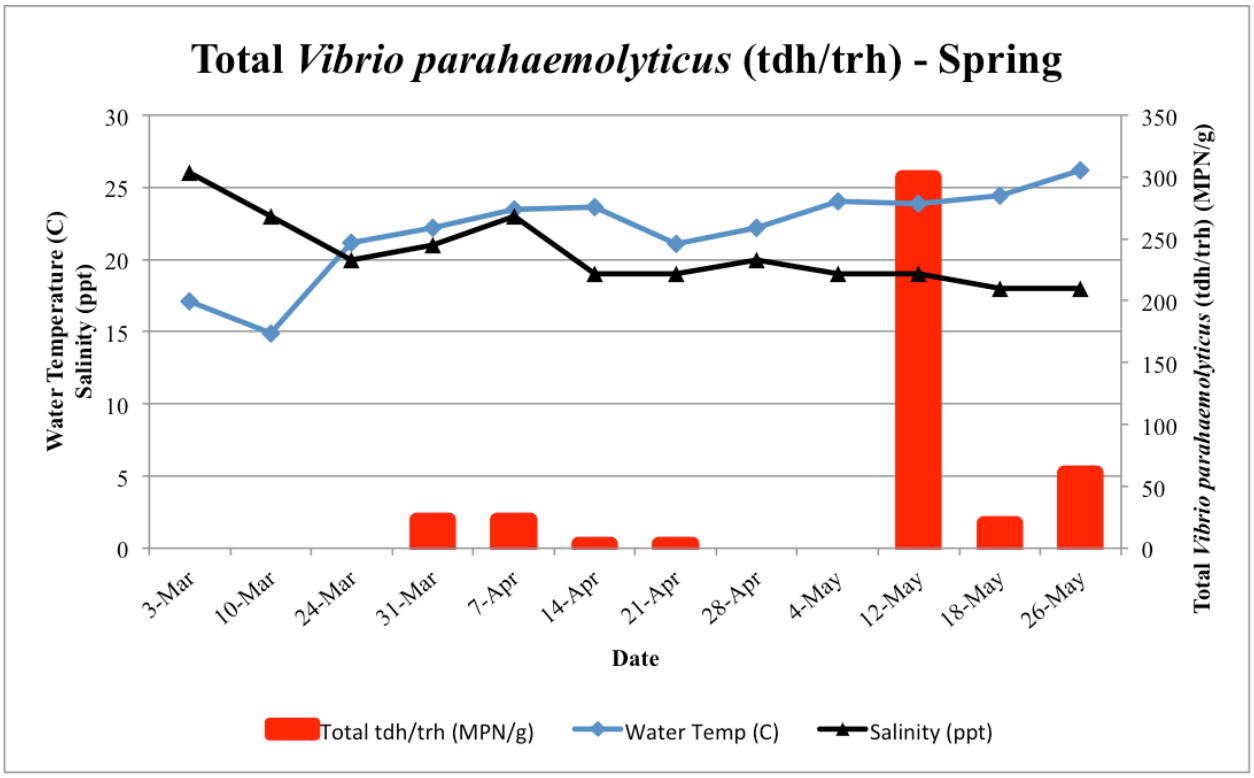

Figure 6. Levels of *Vibrio parahaemolyticus* (tdh/trh) in relation to salinity and water temperature during the spring season.

The first sharp increase in levels of all three species occurred during the week of March 31, as the water temperature rose above 20°C. The levels of all three species declined again during April as the salinity decreased and water temperature continued to increase. As the water temperature increased between 20-25°C and the salinity decreased below 20 ppt, the levels of *V. vulnificus* and *V. parahaemolyticus* (tdh/trh) spiked dramatically. During this time (May 12 to May 26), the levels of *V. parahaemolyticus* (tlh) also increased, but not as dramatically.

The correlation tests revealed a moderate positive correlation between *V. vulnificus* and water temperature, as well as a moderate negative correlation between *V. vulnificus* and salinity. Both species of *V. parahaemolyticus* yielded weak correlations with the environmental conditions. Table 4 below is a summary of the results from the correlation tests. The multiple linear

regressions for the three species still yielded weak linear correlations, indicating that the linear model did not fit the data from the spring season. The R^2 values from the regressions can be found in Table 5.

Table 4. R values from the correlation tests for each species during the spring season. An r value of 1 indicates a perfect correlation.

	R values – Correlation		
	Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
Water Temperature	0.5581	0.1513	0.3023
Salinity	-0.4619	0.1532	-0.2457

Table 5. R^2 values from the multiple linear regression for each species during the spring season. An R^2 value of 1 indicates a perfect linear correlation.

R^2 Value – Multiple Linear Regression		
Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
0.3157	0.1861	0.0922

Summer bacterial levels and environmental correlation

The analyses for the summer season included June, July, and August data. Figures 7 through 9 show the levels of the three species in relation to the water temperature and salinity.

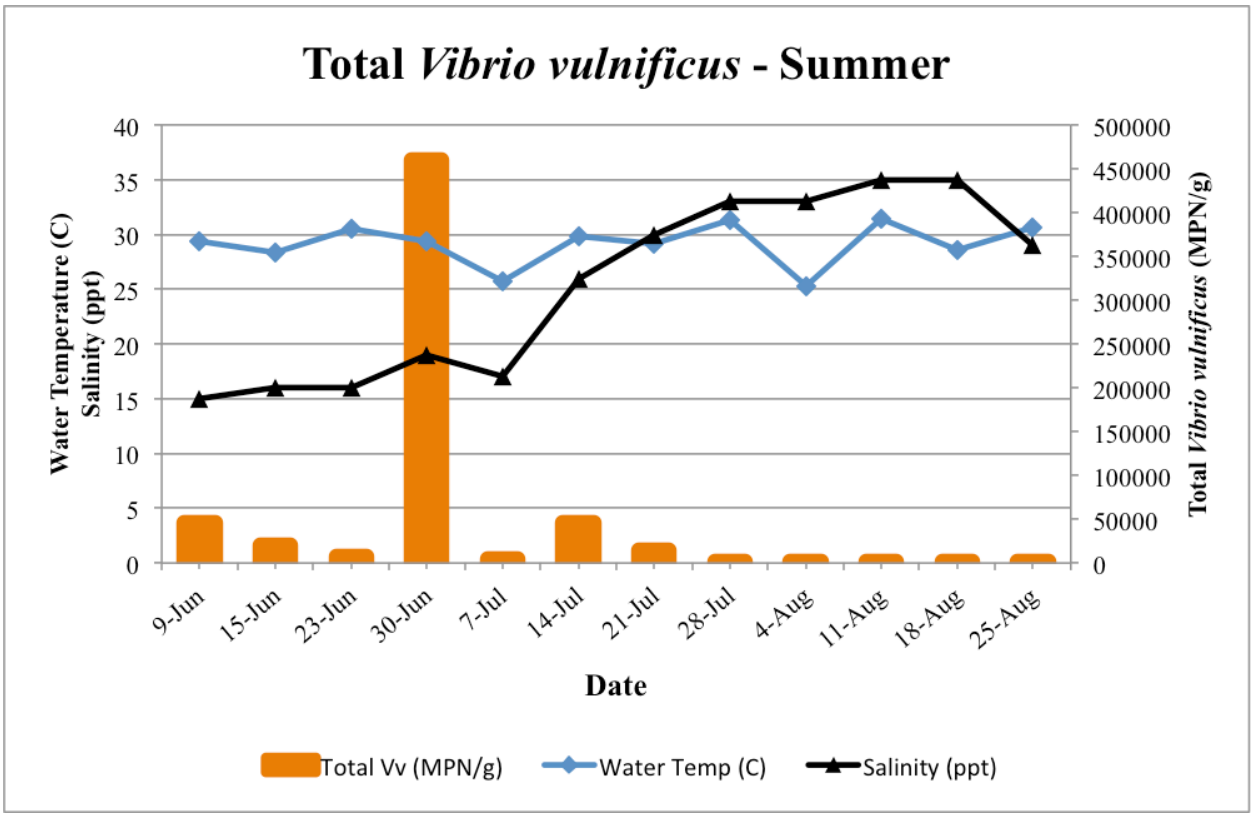

Figure 7. Levels of *Vibrio vulnificus* in relation to salinity and water temperature during the summer season.

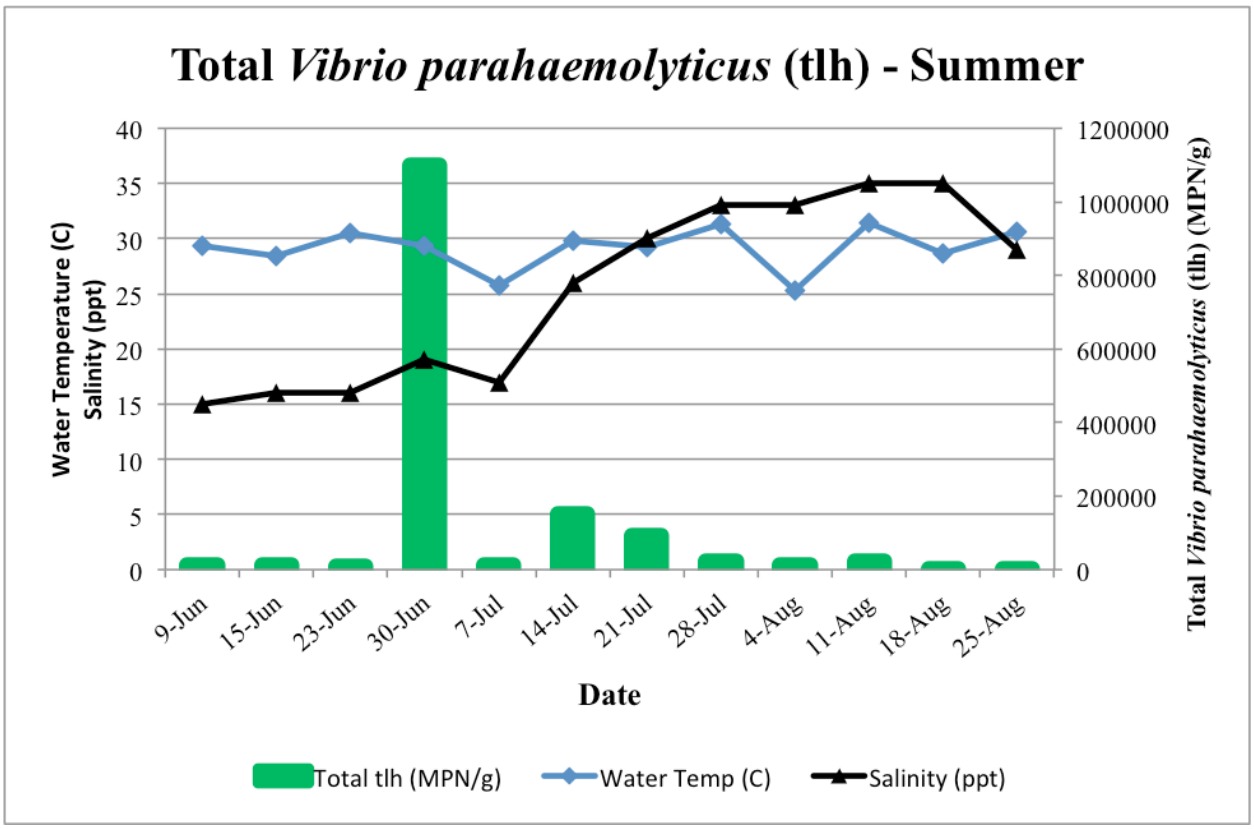

Figure 8. Levels of *Vibrio parahaemolyticus* (tlh) in relation to salinity and water temperature during the summer season.

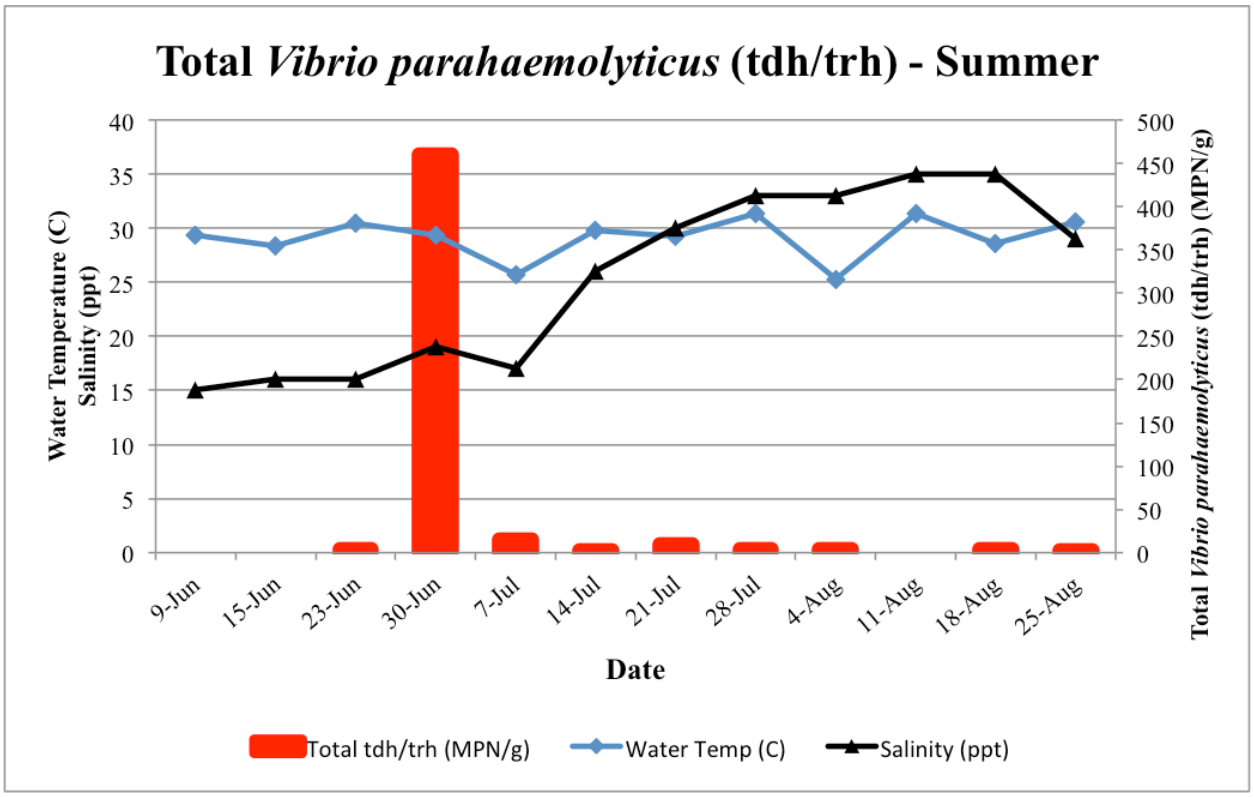

Figure 9. Levels of *Vibrio parahaemolyticus* (tdh/trh) in relation to salinity and water temperature during the summer season.

The levels of all three species remained very low during the first three weeks of June, while the water temperature remained roughly 29°C and the salinity 15 ppt. The week of June 30th resulted in a dramatic rise in the levels of all three species. Immediately following this rise in bacterial levels, the salinity rose to above 30 ppt. During this time of high salinity, the levels of *Vibrio* spp. all declined again to very low levels. The end of the summer season was characterized by temperatures around 30°C, salinities above 30 ppt, and relatively low levels of each species.

All three *Vibrio* spp. produced r values close to zero, resulting in weak correlations between bacterial levels, water temperature, and salinity. The correlation results are found in Table 6 below. The multiple linear regression produced similar results, as all of the R^2 values indicated

weak linear correlations between levels of each *Vibrio* spp. and environmental conditions. The R^2 values are summarized in Table 7.

Table 6. R values from the correlation tests for each species during the summer season. An r value of 1 indicates a perfect correlation.

	R values – Correlation		
	Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
Water Temperature	0.0526	0.0633	0.0264
Salinity	-0.3069	-0.2259	-0.2478

Table 7. R^2 values from the multiple linear regression for each species during the summer season. An R^2 value of 1 indicates a perfect linear correlation.

R^2 Value – Multiple Linear Regression		
Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
0.1049	0.0612	0.0659

Fall bacterial levels and environmental correlation

The fall season consisted of the months September, October, and November. Variations in the levels of *V. vulnificus* and *V. parahaemolyticus* as well as the water temperature and salinity can be found in Figures 10 through 12 below.

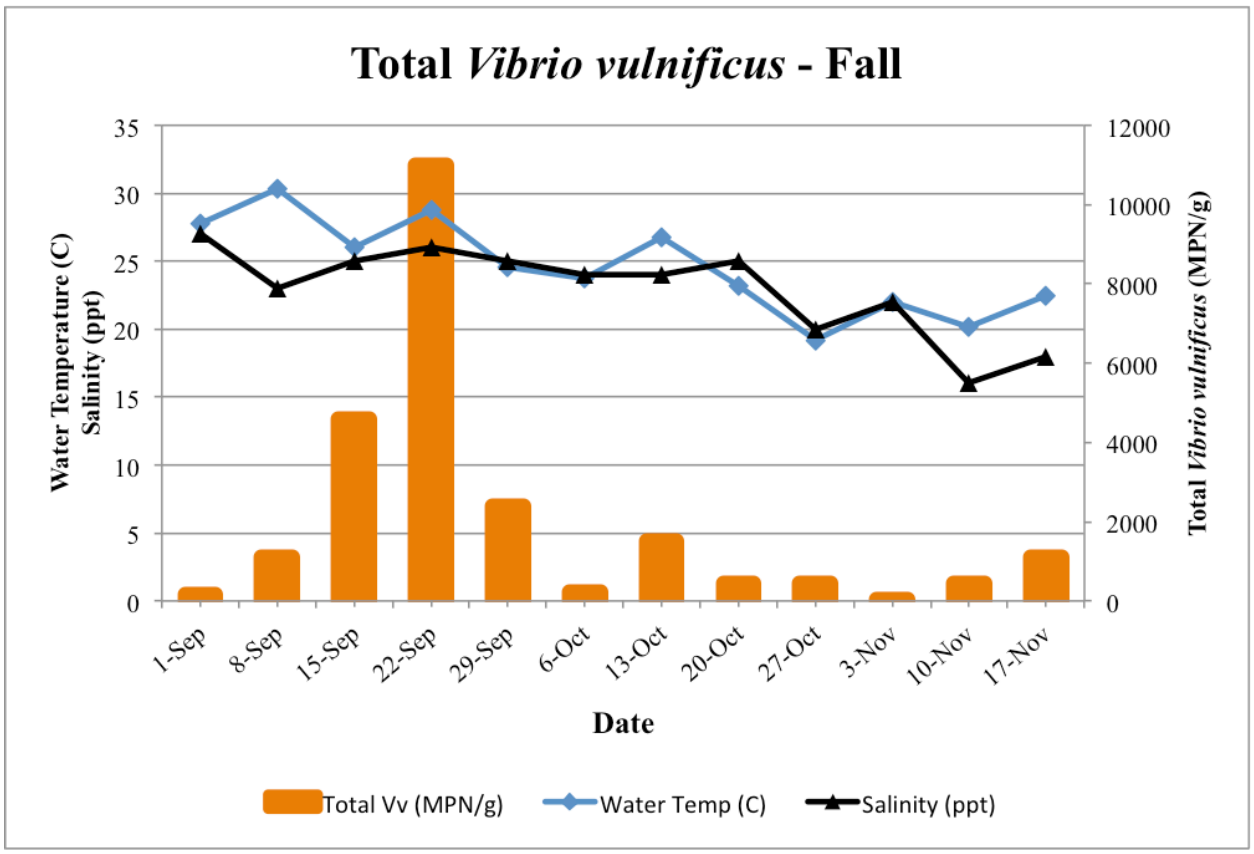

Figure 10. Levels of *Vibrio vulnificus* in relation to salinity and water temperature during the fall season.

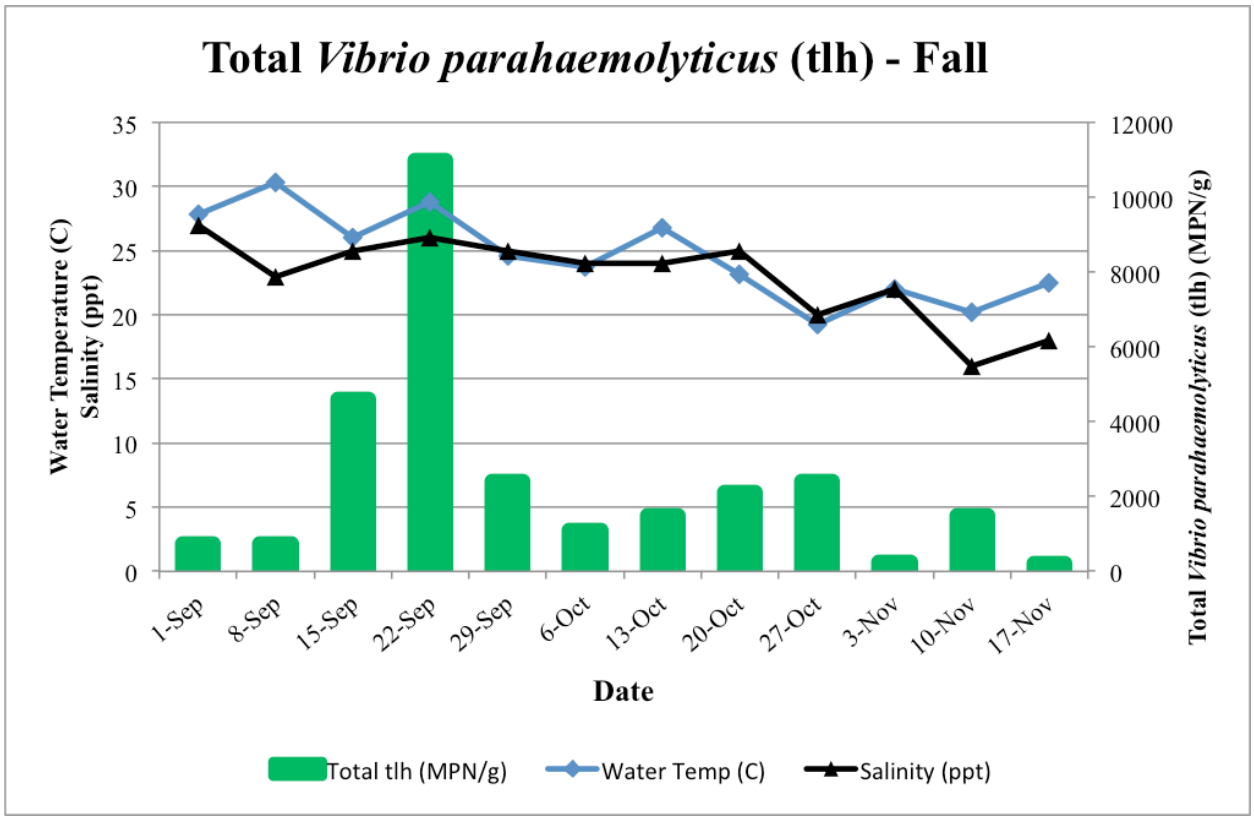

Figure 11. Levels of *Vibrio parahaemolyticus* (tlh) in relation to salinity and water temperature during the fall season.

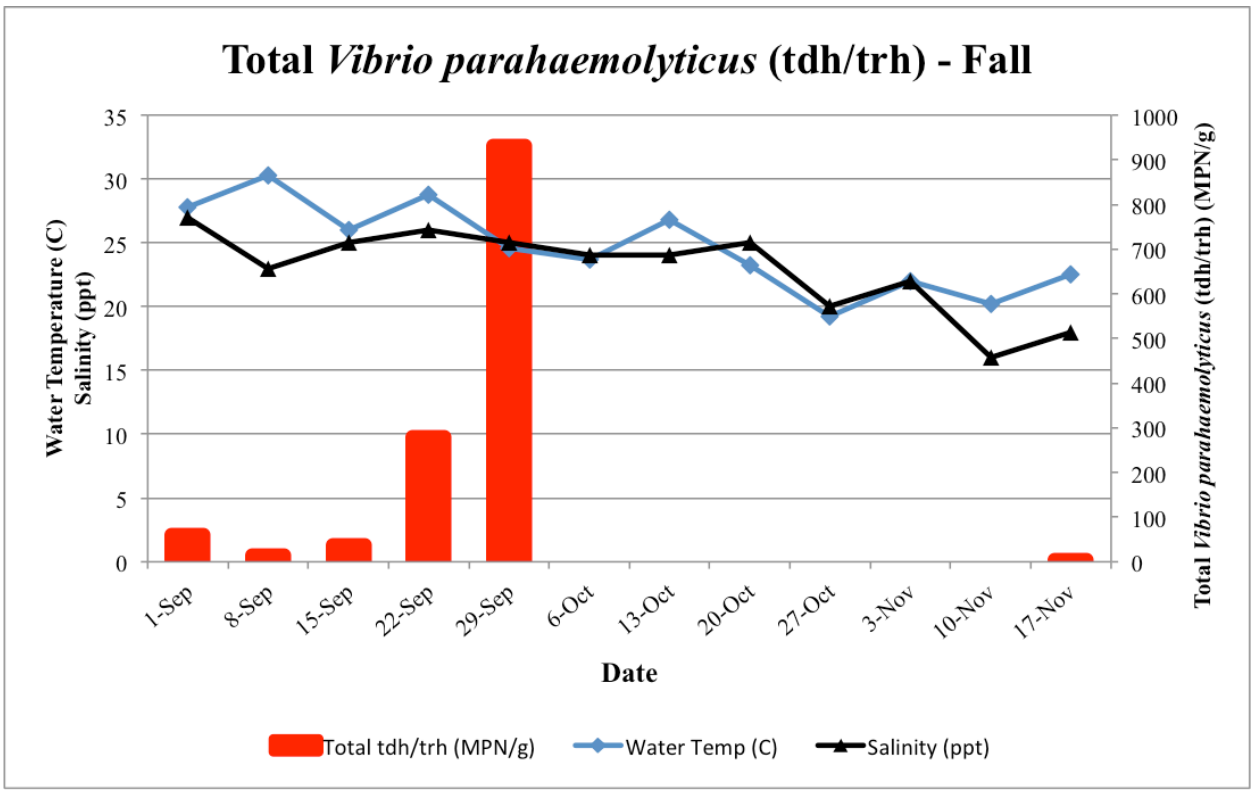

Figure 12. Levels of *Vibrio parahaemolyticus* (tdh/trh) in relation to salinity and water temperature during the fall season.

During the month of September, the water temperature fluctuated between 25-30°C and the salinity decreased from 23 to 21 ppt. The overall decrease in water temperature and salinity during the month of September were followed by another dramatic rise in the levels of *V. vulnificus* and *V. parahaemolyticus*. The water temperature and salinity remained fairly constant until the end of October, when both dropped to 20°C and 20 ppt respectively. The level of *V. vulnificus* decreased during this time, while the level of *V. parahaemolyticus* (tlh) decreased and then slowly rose again. There was no detectable *V. parahaemolyticus* (tdh/trh) from October to mid-November. The month of November was characterized by even lower salinities (16-22 ppt), resulting in slight increases in both *V. vulnificus* and *V. parahaemolyticus*.

Similar to the other seasons, the fall season did not produce any significant correlations between levels of *Vibrio* spp. and the water temperature or salinity. All of the r values from the correlation tests were still less than 0.5, indicating weak correlations. The multiple linear regression also proved that the data did not fit a linear model, as the R^2 values were all close to zero. Tables 8 and 9 contain the results from the correlations and multiple linear regressions, respectively.

Table 8. R values from the correlation tests for each species during the fall season. An r value of 1 indicates a perfect correlation.

	R values – Correlation		
	Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
Water Temperature	0.4673	0.3415	0.1486
Salinity	0.3665	0.3682	0.3138

Table 9. R^2 values from the multiple linear regression for each species during the fall season. An R^2 value of 1 indicates a perfect linear correlation.

R^2 Value – Multiple Linear Regression		
Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
0.2222	0.1504	0.1070

Winter bacterial levels and environmental correlation

The winter season consisted of the months December, January, and February. Figures 13 through 15 show the levels of *Vibrio* spp. and the water temperature and salinity from these months.

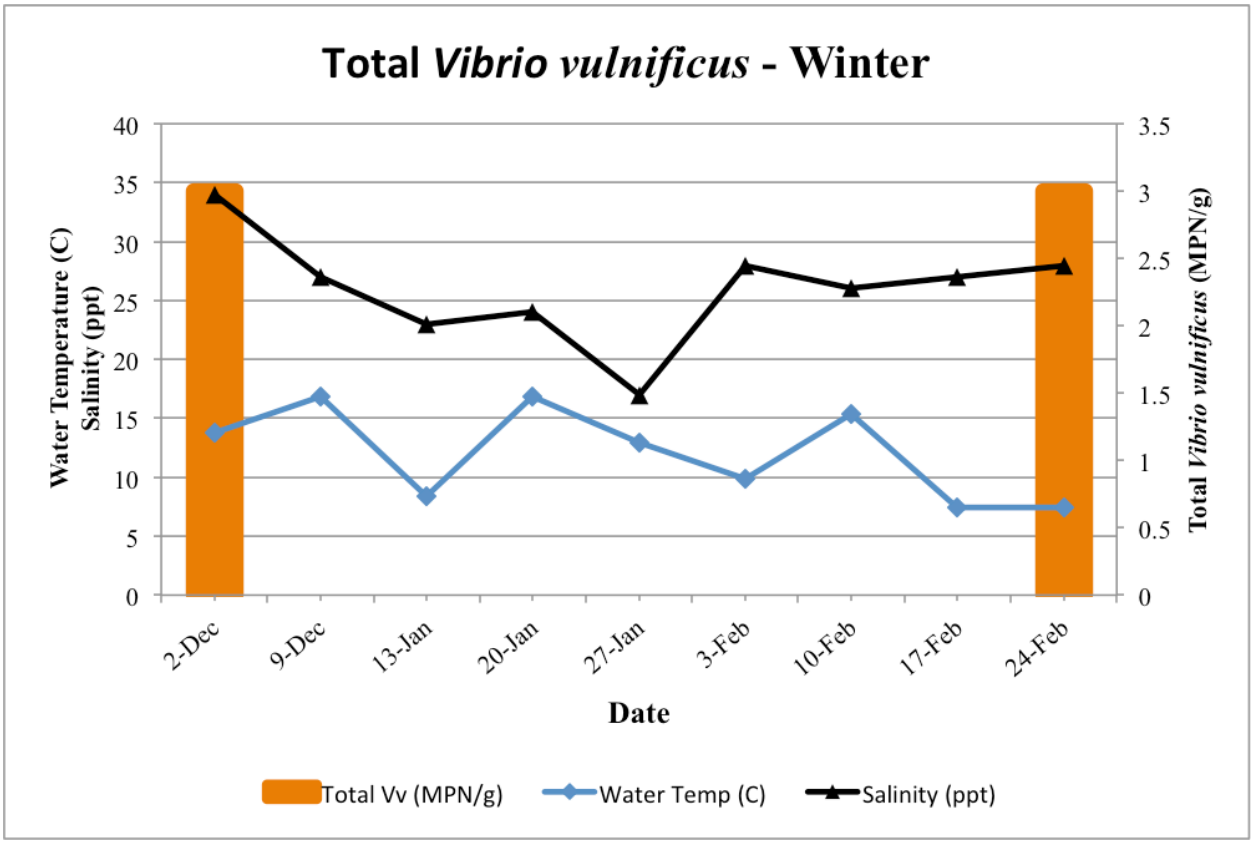

Figure 13. Levels of *Vibrio vulnificus* in relation to salinity and water temperature during the winter season.

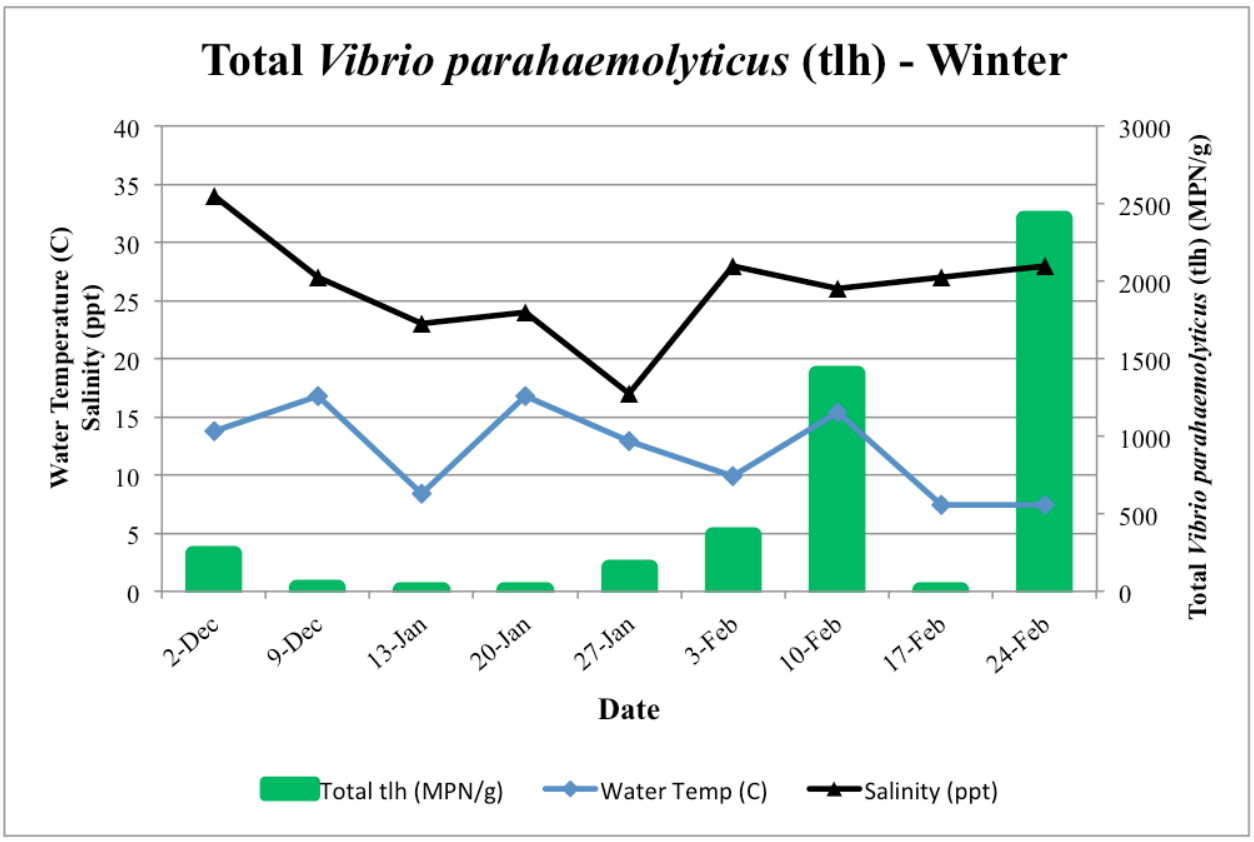

Figure 14. Levels of *Vibrio parahaemolyticus* (tlh) in relation to salinity and water temperature during the winter season.

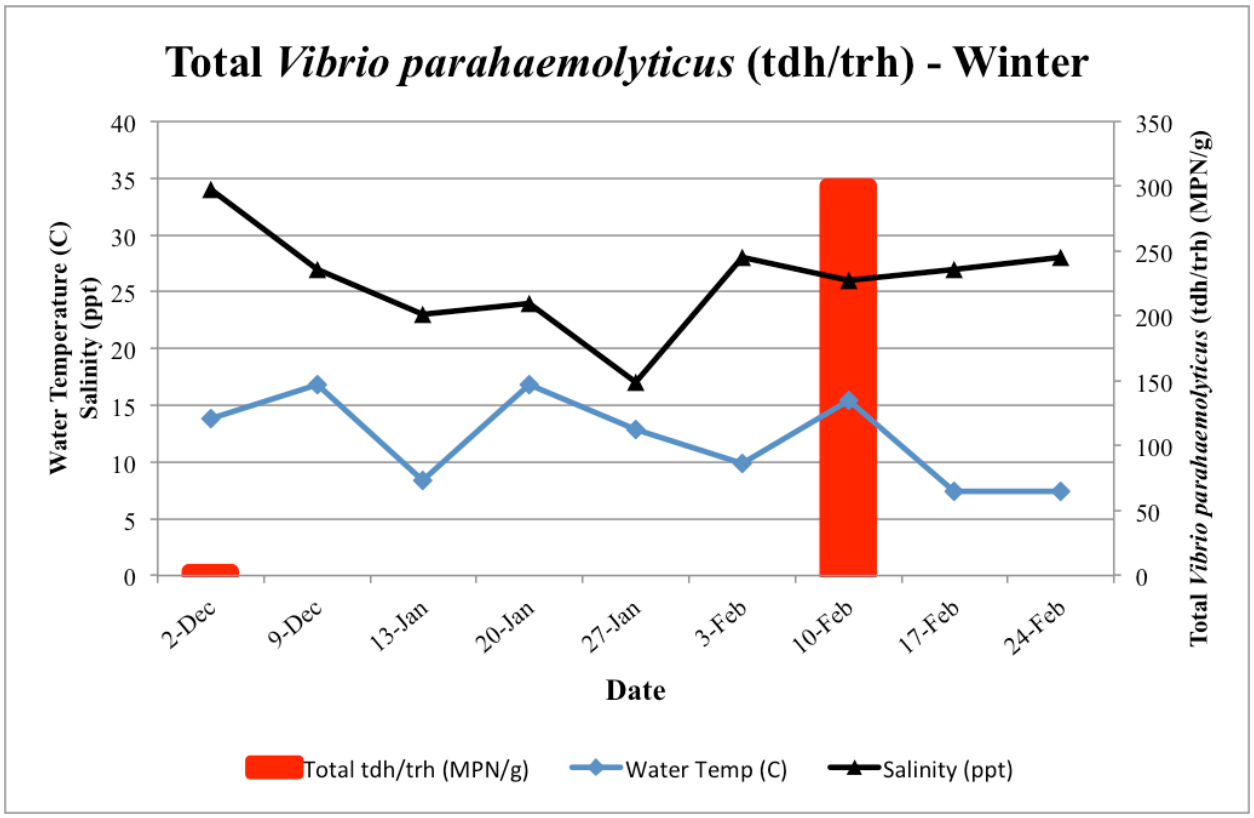

Figure 15. Levels of *Vibrio parahaemolyticus* (tdh/trh) in relation to salinity and water temperature during the winter season.

Environmental conditions during these winter months fluctuated greatly from week to week, with salinity ranging from 17-34 ppt and water temperature ranging between 8-16^{o}C. *Vibrio vulnificus* was detected at low levels at the beginning and end of this season, while the salinity was at its highest. *Vibrio parahaemolyticus* (tlh) was present during the entire season, with higher levels detected when the salinity rose above 25 ppt. *Vibrio parahaemolyticus* (tdh/trh) was absent during much of the winter season, except for a significant peak in mid-February when the water temperature warmed up to 15^{o}C.

The correlation tests for the three species and environmental conditions were all weak correlations, as seen by the values in Table 10. The multiple linear regressions also produced R^2

values that were close to zero. Therefore, the linear model was not a good fit for the levels of *Vibrio* spp. and environmental conditions during the winter season. The results from the multiple linear regressions can be found in Table 11.

Table 10. R values from the correlation tests for each species during the winter season. An r value of 1 indicates a perfect correlation.

	R values – Correlation		
	Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
Water Temperature	-0.2178	-0.2622	0.3212
Salinity	0.6186	0.1989	0.0066

Table 11. R^2 values from the multiple linear regression for each species during the winter season. An R^2 value of 1 indicates a perfect linear correlation.

R^2 Value – Multiple Linear Regression		
Vibrio vulnificus	*Vibrio parahaemolyticus* (tlh)	*Vibrio parahaemolyticus* (tdh/trh)
0.4197	0.1042	0.1036

CHAPTER IV

CONCLUSION

Annual trends in *Vibrio* spp. abundance

Throughout the course of the year, the three species reacted in different ways to the changes in water temperature and salinity. In Figure 1, it is easy to see that the levels of *V. vulnificus* were generally higher during warmer water temperatures (above 20^{o}C) and salinities between 15-22 ppt. Similarly, in Figure 2, the levels of *V. parahaemolyticus* (tlh) were generally higher when water temperatures exceeded 20^{o}C and the salinity was between 15-25 ppt. The levels of *V. parahaemolyticus* (tdh/trh) did not correlate to any trends in water temperature, as the significant blooms of this species occurred at water temperatures between 15-30^{o}C. However, *V. parahaemolyticus* (tdh/trh) did bloom when the salinity ranged between 19-26 ppt, as shown in Figure 3. Although the statistical analyses did not produce any strong correlations between the three species and the water temperature and salinity, the general trends observed over the year do correspond to the findings in Givens *et al.*(2014).

Spring season trends in *Vibrio* spp. abundance

The spring season was characterized by a salinity that decreased from 26 ppt to 18 ppt while the water temperature increased from 14^{o}C to 20^{o}C. These changes in the environmental conditions led to an increase in *V. vulnificus* (Figure 4) as the season progressed. Once the temperature rose above 20^{o}C, *V. vulnificus* experienced its first sharp rise in numbers and was present weekly thereafter in increasing quantities. Despite these trends illustrated in Figure 4, the analyses did not yield any strong correlations between bacterial levels and water temperature or salinity.

Throughout this season, *V. parahaemolyticus* (tlh) was present every week (Figure 5). Again, the first dramatic increase in the levels of this species occurred when the temperature rose above 20^{o}C and the salinity decreased to approximately 20 ppt. The rest of the season, however, did not prove to follow the same trend, as the levels of *V. parahaemolyticus* (tlh) declined again before slowly starting to increase. As a result, both tests showed weak correlations with environmental conditions.

The levels of *V. parahaemolyticus* (tdh/trh) followed the same pattern as *V. vulnificus*, as the bacterial levels first became present when the water temperature increased above 20^{o}C and the salinity decreased to 20 ppt (Figure 6). The bacterial levels decreased again until the temperature warmed to approximately 24^{o}C, resulting in another significant spike. Although these general trends can be seen for this species, the correlations and regression did not produce any strong correlations. Therefore, there was no significant correlation between *V. parahaemolyticus* (tdh/trh) and the environmental conditions.

Summer season trends in *Vibrio* spp. abundance

The summer months experienced a water temperature that oscillated between 25-31^{o}C, and a large spike in salinity from approximately 15 ppt to 35 ppt. The marked increase in salinity was a result of very little to no precipitation during the month of July, causing the salinity at the reef to double. As the salinity slowly increased to 19 ppt, the level of *V. vulnificus* experienced a dramatic increase in numbers at the end of June, as shown in Figure 7. Despite the optimal water temperatures (20-30^{o}C), the extremely high salinity caused the numbers of *V. vulnificus* to

drastically decline and stay at very low levels for this time of year. The r values and R^2 values from the statistical analyses were both close to zero, indicating no significant correlation between *V. vulnificus* levels and environmental conditions.

The levels of both strains of *V. parahaemolyticus* (tlh and tdh/trh) reacted in a similar way to *V. vulnificus* regarding the changes in water temperature and salinity (Figures 8-9). Both strains experienced a large increase in bacterial numbers during the last week of June before the large spike in salinity. As the salinity rose above 25 ppt, the levels of *V. parahaemolyticus* (tlh and tdh/trh) decreased to abnormally low levels for the summer season. These bacterial levels remained low as the salinity remained above 25 ppt through the end of August. No strong correlation was found for either species, however, as all of the r values and R^2 values were close to zero.

Fall season trends in *Vibrio* spp. abundance

The fall season experienced an overall decrease in both water temperature and salinity. As the salinity decreased to 25 ppt, the level of *V. vulnificus* began to rise, as seen in Figure 10. The highest number of this species was observed during the week of September 22. During October and November, the levels of *V. vulnificus* increased when the water temperature and salinity increased and decreased when the water temperature and salinity decreased. These parallels were not reflected in the correlations or regressions, however, as the r values and R^2 values still indicated weak correlations.

The levels of *V. parahaemolyticus* (tlh) responded to the changes in environmental conditions in a similar way to *V. vulnificus* (Figure 11). Once the salinity decreased to 25 ppt, the levels of this species experienced a drastic increase on September 22. Following this large spike in numbers, *V. parahaemolyticus* (tlh) increased and decreased with increasing temperature and salinity and decreasing temperature and salinity, respectively. These changes still produced weak correlations during the statistical analysis.

As shown in Figure 12, *V. parahaemolyticus* (tdh/trh) reacted differently to the changes in environmental conditions during the fall season. As the water temperature and salinity decreased during the month of September, the levels of this species increased and finally peaked on September 29. Immediately following September 29, the level of *V. parahaemolyticus* (tdh/trh) dropped to zero and remained there until November 17, when it was detected at a very low level. The correlations and multiple linear regressions again yielded weak correlations between levels of *V. parahaemolyticus* (tdh/trh) and environmental conditions during the fall season.

Winter season trends in *Vibrio* spp. abundance

The winter season experienced water temperatures below 16^{o}C and salinities between 15-35 ppt. The low temperatures were not tolerated by *V. vulnificus*, as this species was only present at the beginning and end of this season in very low amounts (Figure 13). These results were expected, as *V. vulnificus* prefers water temperatures above 20^{o}C (Givens *et al.* 2014). After performing the analyses, there were no strong correlations based on the r and R^2 values.

Unlike *V. vulnificus*, *V. parahaemolyticus* (tlh) was present throughout the entire winter season. During December and January, as the water temperature and salinity decreased, the levels of this species decreased as well. The month of February had a rise in salinity to above 25 ppt, followed by an increase in the levels of *V. parahaemolyticus* (tlh) despite the decline in water temperature. After running the correlations and regressions, all of the r and R^2 values were close to zero, resulting in weak correlations between this species and the water temperature and salinity.

During the winter, *V. parahaemolyticus* (tdh/trh) was only present when the water temperature was around 15^{o}C (Figure 15). This species was detected at a low level at the beginning of December, and again at a much higher level in February. Similar to *V. vulnificus*, this species did not tolerate the colder water temperatures as well. Both the correlation tests and the regressions produced r and R^2 values that indicated weak correlations between bacterial levels and environmental conditions.

Conclusion

The main objective of this study was to determine the abundance of *V. vulnificus* and *V. parahaemolyticus* (tlh and tdh/trh) in oysters from West Galveston Bay and determine any correlations between the bacterial levels and water temperature and salinity. The correlation tests run on the data resulted in no strong correlations between the three species and water temperature or salinity. Similarly, the multiple linear regressions indicated that the bacterial and environmental data for all three species could not be represented by a linear model. Although none of the statistical analyses indicated strong correlations, several substantial trends were identified visually from the figures for *V. vulnificus* and *V. parahaemolyticus* (tlh), giving

evidence for environmental conditions that encourage and inhibit the growth of these two species. Based on the data, *V. vulnificus* preferred water temperatures greater than 20^{o}C and salinity between 15-22 ppt. Similarly, *V. parahaemolyticus* (tlh) was more prevalent when the water temperature rose above 20^{o}C and the salinity ranged between 15-25 ppt. Although no significant trends were found from the figures for *V. parahaemolyticus* (tdh/trh), the data do suggest that the level of this species does correspond to previous findings (Givens *et al.* 2014). This species was most prevalent when the water temperature ranged from 15-30^{o}C, and the salinity ranged from 19-26 ppt.

Upon examining the graphs of the bacterial levels and the environmental conditions, it was found that there was a lag time from when the water temperature or salinity significantly changed to the reciprocating change in bacterial levels. For example, during the middle of July, when the salinity increased from 17 to 26 ppt, it took one to two weeks to observe the effects of the higher salinity on the levels of *Vibrio* spp. This lag time in bacterial response can be seen in several instances for the three species, and could be explained by the bacterial host. Oysters are filter feeders, but individual oysters do not filter the water at the same rate, especially during low tides and instances of low precipitation when the oyster reef is potentially exposed to air. The weather and changes in water level, in combination with unique filter feeding rates among oysters, could have produced this lag time in bacterial response.

The findings from this study are important when considering the possible consumption of raw oysters from reefs in Galveston Bay similar to Sammy's Reef. During the times of the year that experience water temperatures above 20^{o}C and salinities between 15-25 ppt, consumers should

be cautioned against eating fresh raw oysters from Galveston Bay. The levels of *V. vulnificus* and *V. parahaemolyticus* could be elevated under these conditions, and could cause human infection. Many consumers know the general rule of thumb for raw oysters which states that oysters can be consumed raw during months that end with the letter "r" (September, October, etc.). In Galveston Bay, however, the water temperature and salinity still remain within the optimum range for *Vibrio* spp., and levels of these species are still found through November. As a result, this rule of thumb is not applicable to Galveston Bay oysters due to the environmental conditions.

In order to encourage safe consumption of raw oysters, consumers should be informed about the extended period of increased risk of infection from *Vibrio* spp. Suggestions should be made to oyster consumers to avoid consuming raw oysters or to cook the oysters prior to eating during these times in order to prevent dangerous infections. Using this knowledge, the public can be informed of times during the year in which it is safe to consume these oysters raw.

Future research

In addition to the results summarized from this study, weekly samples should be collected from this reef to monitor levels of *Vibrio* spp. Increasing the number of samples from Sammy's Reef could influence the correlation and multiple linear regression results, and show different significant correlations between bacterial levels, water temperature, and salinity. A similar study could be performed on oyster reefs that are commonly harvested for human consumption, in order to determine the risk of *Vibrio* spp. infection during different times of the year, as well as during different environmental conditions.

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