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The ecological impact of the Deepwater Horizon oil spill on Vibrio parahaemolyticus type III secretion system and the vibrio community

Erica L. Stephens
Louisiana State University and Agricultural and Mechanical College

THE ECOLOGICAL IMPACT OF THE *DEEPWATER HORIZON* OIL SPILL ON *VIBRIO PARAHAEMOLYTICUS* TYPE III SECRETION SYSTEM AND THE VIBRIO COMMUNITY

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ABSTRACT

Vibrio spp are gram-negative, halophilic bacteria that naturally occur in both shallow coastal waters and parts of the deepest oceans and are a known human pathogen. There have not been extensive studies that analyze an oil spill as an environmental stressor to vibrios. If there were to be an increase in the pathogenicity of vibrios it would be a cause for human health concern. The purpose of the two studies presented was to determine changes in the pathogenicity of *V. parahaemolyticus* pathogenicity and the vibrio community, with respect to *V.* cholera, V. *parahaemolyticus*, and *V. vulnificus*. The results showed that there was an insignificant change in *V. parahaemolyticus* pathogenicity, and there was not a significant trend in the species composition of the vibrio community when interacting with oil. Even though the results did not show statistically significant or consistent trends of both studies, the study was an innovative evaluation of the vibrio community and various oil concentration effects thereon.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Vibrio Background

Vibrio spp are gram-negative, halophilic bacteria that naturally occur in both shallow coastal waters and parts of the deepest oceans (Su & Liu 2007; Okada et al., 2005). This bacterium is relevant to humans because it is a known human pathogen that can cause gastrointestinal problems such as diarrhea, headache, vomiting, nausea, abdominal cramps and low fever (Su & Liu 2007). In some cases vibrio infections can result in a very serious condition, septicemia. Septicemia, presence of bacteria in blood, can target people with a compromised immune system and can be fatal (Su & Liu, 2007). In the United States, there are roughly 200,000 deaths per year associated with sepsis (Wang et al., 2010). Examples are people with liver disease, cancer, inflammatory bowel disease, and many others (Martinez-Urtaza et al., 2010).

The viability of this bacterium depends on its optimal growth conditions, most importantly temperature and salinity. Water temperature can range from less than 10°C to as warm as 37°C and salinity can range from 10 parts per thousand (ppt) to 34ppt (Martinez-Urtaza et al., 2010). Vibrios can live and grow in any favorable place as long as these conditions are met.

Vibrios are found in the water column, sediment, and various types of seafood (Noriea III et al., 2010). Mollusks can build up this bacterium in their digestive systems due to their filter feeding ability (Su & Liu, 2007). People are susceptible to becoming ill due to vibrios when they consume raw seafood that have a concentrated amount of pathogenic vibrios (Fleming et al., 2006). Consumption of raw oysters is one of the most common ways to ingest vibrios and therefore is associated with a high risk of becoming ill (Fleming et al., 2006).

With over 60 species found in the *Vibrionaceae* family, ten species are known to be human pathogens, and three cause thousands of illnesses and fatalities annually (Adeleye, Daniels, & Enyinnia, 2010). *Vibrio cholera*e, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* are the main pathogenic vibrios that cause illness in people. *Vibrio cholerae*, the causative agent of cholera, has been a leading cause of deaths in developing countries because of human consumption of untreated drinking water or consumption of contaminated food (WHO, 2011**)**. The most recently publicized cholera outbreak occurred in Haiti after a catastrophic earthquake rattled the country in 2010 (Ceccarelli et al., 2011**)**. According to the World Health Organization (2011), there have been roughly 500,000 reported cholera cases in Haiti as of July 31, 2011. Of the 500,000 reported cases, there have been approximately 6,000 fatalities (WHO, 2011).

Vibrio vulnificus can cause numerous fatalities and commonly enters through open wounds, not necessarily through drinking water or eating raw seafood (Jones & Oliver, 2009**)**. According to Dechet et al. (2008), there were 4,754 vibrio cases reported to the CDC between the years of 1997 and 2006. A quarter of the reported cases were associated with non-food-borne *Vibrio* illnesses. Of this group, 35% were associated with *Vibrio vulnificus* infections. Out of all the *Vibrio vulnificus* cases reported over nine years, 72% were reported from the Gulf of Mexico region. Out of all of the *Vibrio* non-food-borne fatalities during a span of nine years, *Vibrio vulnificus* made up 78% of those deaths (Dechet et al., 2008).

Vibrio parahaemolyticus (*Vp*) is primarily associated with foodborne illnesses, whereas *Vibrio vulnificus* infections are associated with both foodborne and nonfoodborne illnesses that can be responsible for illnesses when people consume raw seafood, particularly oysters (Jones & Oliver, 2009). *Vp* has an enterotoxin that causes gastronintestinal problems as previously stated. A famous preliminary study by Johnson and Calia looked at enterotoxicity of *Vp* in rabbit ileal

loop tests (1976). Male rabbits were injected with various concentrations of *Vp* in the small intestine of look at any adverse effects associated with *Vp*. It was concluded that *Vp* reacts more in vivo rather than in vitro, and bacteria do harm to the intestinal epithelium of the rabbits, which would lead to the hypothesis that it might cause the same effect in humans (Johnson & Calia, 1976). *Vp* creates fluid secretion in the GI tract and is the reason for classic symptoms, such as diarrhea, abdominal cramps, nausea, and other gastrointestinal problems in humans after consumption. In addition, *Vp* can also incubate in an organism's system for as little as four hours to as much as eight days (Wong et al., 2000).

1.2 Pathogenicity Factors

Vibrios contain two chromosomes, and there is a pathogenicity island (PAI) on chromosome two that carries genes that encode for two hemolysins (*tdh* and *trh)*. The *tdh* gene encodes themostabile direct hemolysin. The *trh* (*tdh*-related hemolysin) gene has 70% nucleotide sequence relatedness with the *tdh* gene (Matsumoto et al., 2000). Also, studies have proven that *trh* is associated with the enzyme urease, which is a catalyst in the hydrolysis of urea to carbon dioxide and ammonia (Okuda et al., 1997).

From studies that have examined pathogenicity factors, it has been concluded that *Vp* are pathogenic when *tdh* and/or *trh* are present. However, there are still unsolved mysteries concerning pathogenicity factors in *Vp*. For example, a study in Chile assessed *Vp* O3:K6 serotype clinical cases from 2006 to 2009. Thirty-six percent of all clinical cases were negative for both *tdh* and *trh.* There is still no explanation for how people can become ill due to *Vp* if the *Vp* lack both the *tdh* and *trh* genes (Garcia et al., 2009).

Other pathogenicity factors have been examined in previous studies. Mahoney et al. (2010) examined *tdh-/trh-* clinical strains and environmental strains from New England estuaries

to determine other virulence factors. Both the clinical and environmental strains were cytotoxic and possessed virulence-related factors such as protease, siderophore, and motility. In addition, *tdh*- environmental strains can regulate *tdh* when temperature and cell densities are increased (Mahoney et al., 2010).

According to Hyoshi et al. (2010), all *Vp* are presumed to possess one type three secretion system (TTSS) that is present on chromosome one (TTSS1). Most reports have indicated that TTSS1 is not related to factors that make *Vp* pathogenic because it is present in both pathogenic and non-pathogenic isolates, with the exception of a groundbreaking study published in early 2010 (Hyoshi et al., 2010). The study concluded that TTSS1, which originally had no pathogenic characteristics, is cytotoxic to more cells than TTSS2. The study also discovered TTSS2 is a contributor to enterotoxicity (Hyoshi et al., 2010). Finally, TTSS2 is on the second chromosome and is found on the same mobile PAI as *tdh* and *trh* (Noriea III et al. (2010).

More specifically, there have been studies that reflect two TTSS2 lineages. The TTSS2α is a lineage that is present when an isolate is *tdh*+/*trh*– (Noriea III et al., 2010). The second lineage, TTSS2β, has only been recently discovered and is present when an isolate is *tdh*–/*trh*+. Lack of knowledge about the pathogenicity of *Vp* is further illustrated by the fact that scientists have not discovered a TTSS that is present when both *tdh* is positive and *trh* is positive (Mescas & Strauss, 1996; Noriea III et al., 2010).

In addition to pathogenicity, the PAI is made up of many uncharacterized and unidentified genes that contribute to the fitness and adaptation of the microbe (Hacker & Kaper, 2000). There is evidence that the PAI can move in response to environmental stresses (Mescas & Strauss, 1996). The PAI island helps organisms survive during an environmental stress because it

consists of genes that help *Vp* adapt to changes (Hacker & Kaper, 2000). Using horizontal gene transfer, the PAI can be moved to *Vp* bacteria that do not carry these genes. This transfer contributes to the overall fitness of the population and ensures survival during an environmental change (Hacker & Kaper, 2000; Hacker & Carniel, 2001).

1.3 *Deepwater Horizon* Oil Spill

In April of 2010 an environmental crisis occurred in the Gulf of Mexico about 45 miles southeast of Venice, Louisiana. An oil rig exploded and killed 11 workers that were on the platform at the time of explosion. From late April to early August, approximately 4.9 million barrels of oil were released into the Gulf and roughly one fifth of it (~ 1.1 million barrels) recovered out of the Gulf (Unified Command's Joint Information Center, 2011).

The magnitude of this spill was large enough to affect the entire Gulf region: Texas, Louisiana, Alabama, Mississippi, and Florida. There were detrimental effects to both the native and migratory birds along the Gulf region. The aquatic life was especially at risk, ranging from animals as large as dolphins to crustaceans as miniscule as copepods. In addition, the seafood industry, a vital component to the Gulf region's economy, was put on an indefinite hiatus. There were numerous fishing area closures all along the Gulf coast that put fishermen out of business for months (Oil Spill Commission, 2011). The oil spill impacted the ecology of many autochthonous organisms in the Gulf, including vibrios.

1.4 *Vibrio parahaemolyticus* and Oil

Natural oil seeps are ubiquitous throughout the world. About 47% of crude oil that enters the environment is caused by seeps. The remaining 53% is caused by anthropogenic sources, such as refining and extraction of oil which can lead to oil spills. The Gulf of Mexico has much higher seepage rates than other parts of the world with roughly 63 seeps (Kvenvolden & Cooper,

2003). The fact that vibrios are also found naturally in the marine environment suggests that this bacterium has been interacting with oil over a long period of time.

Sindermann (1982) discussed the impact of an environmental stress, such as oil pollution, on marine organisms and defined stress using three stages: alarm, resistance, and exhaustion. Alarm is a short term response whereas resistance is a long-term response in which organisms adapt to their environment to help them survive and resist a particular environmental pressure. Exhaustion is a term for the eventual death of an organism or population due to the stress.

It is possible that vibrios have been interacting with oil for many years due to natural oil seeps. This chronic interaction could have given vibrios the ability to resist and utilize polyaromatic hydrocarbons, a trait that may be a result of its ability to biodegrade. However, a significant increase in oil found in a marine environment could be an initial stress to vibrios, causing the alarm response as described by Selye (Sindermann, 1982).

The *Deepwater Horizon* oil spill has raised questions concerning the impact on *Vp* and the vibrio community. The oil spill may be a stress to *Vp* that could trigger the PAI to cause an increase in pathogenic *Vp*. Also, the vibrio community may be affected by this change in the environment. Bijilsma and Loeschcke (2005) defined stress as some intrinsic or extrinsic force that could shape adaptation of an organism or population to its changing environment. Therefore, the genetic response from *Vp* or the vibrio community, the biological component, in response to the oil spill, the environmental component could be classified as a stress.

For example, MacNaughton et al. (1999) analyzed the microbial population changes using a block field experiment located at Fowler Beach, Delaware. The plots were treated with oil or oil and nutrients and were compared to controls, which were treated with neither. They concluded that there was no significant change in the plots that included both oil and nutrients.

However, analysis of the plots that contained only oil revealed that there was a significant change in the microbial community.

CHAPTER 2. IMPACT OF THE *DEEPWATER HORIZON* OIL SPILL ON THE *VIBRIO PARAHAEMOLYTICUS* TYPE THREE SECRETION SYSTEM

2.1 Purpose and Hypotheses

To date no published studies have looked at changes in pathogenicity of *Vp* to determine if polyaromatic hydrocarbons are an environmental stressor. Analyzing the presence of TTSS2 in environmental samples before and after the *Deepwater Horizon* oil spill will give better insight as to whether oil as an environmental stressor should be studied in greater detail with respect to its potential to select for *Vp* containing the PAI on chromosome two. If oil does increase the prevalence of pathogenicity factors it would become a public health issue. The hypothesis for the TTSS study is that there will be a change in pathogenicity of *Vp* after the *Deepwater Horizon* oil spill.

H_0: There will be no change in the presence of TTSS2 in environmental samples after the *Deepwater Horizon* oil spill.

H_1: There will be a change in the presence of TTSS2 in environmental samples after the *Deepwater Horizon* oil spill.

2.2 Materials and Methods

2.2.1 Sample Collection

Sample collections occurred from April through August 2010. Water, oyster, and sediment samples were collected once a week from sampling sites at Port Fourchon, Louisiana or Cocodrie, Louisiana. Approximately six liters of water were collected in autoclaved plastic containers. Approximately 15 live oysters were collected and placed in plastic bags and a minimum of 100 grams (g) of sediment were collected in a pre-weighed autoclaved plastic container. All samples were placed in coolers with ice packs and processed within three to five hours after collection. Oyster, water, and sediment samples were collected and processed as

described by the Food and Drug Administration Bacteriological Analytical Manual (FDA, 2004).

2.2.1.1 Sample Locations

LF (Louisiana Fourchon) samples were collected in Port Fourchon, Louisiana (N 29° 15.231', W 90° 39.825') and LC (Louisiana Cocodrie) samples were collected at the LUMCON site in Cocodrie, Louisiana (N 29° 07.128', W 90° 11.575'). Port Fourchon is located southwest of Grand Isle, Louisiana and is the southernmost port in the state of Louisiana (Figure 2.1). The sampling location at Cocodrie, Louisiana is approximately 75 miles by automobile to the west of Port Fourchon, off of Terrebone Bay (Figure 2.2).

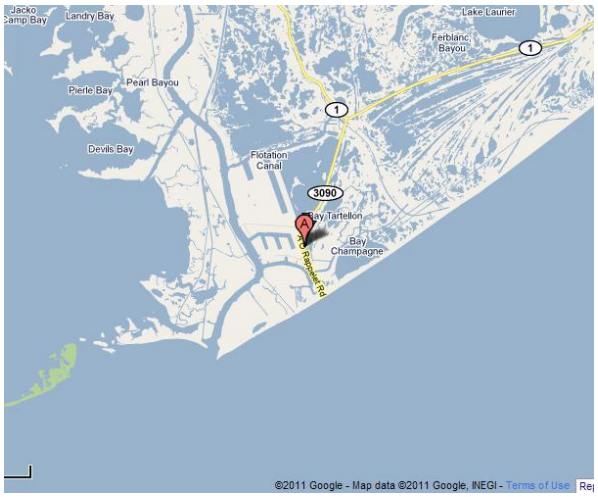

Figure 2.1: Sampling location at Port Fourchon, Louisiana (courtesy of Google Maps).

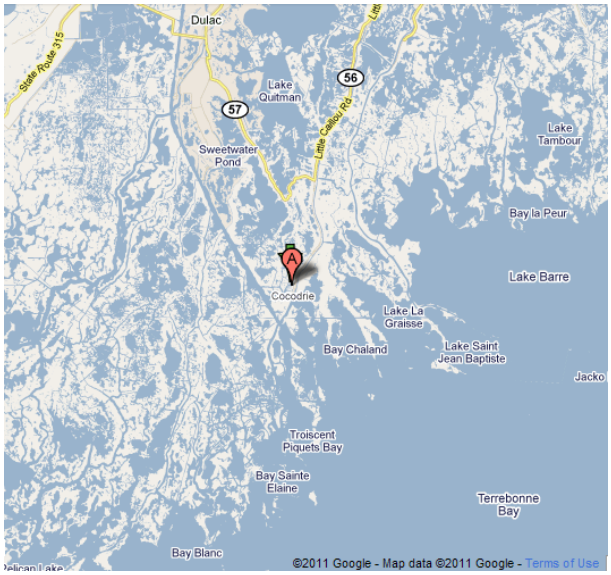

Figure 2.2: Sampling location at Cocodrie, Louisiana (courtesy of Google Maps).

2.2.2 Sample Preparation and Plating

2.2.2.1 Oyster Preparation and Plating

Oysters were scrubbed using a sterilized brush under cold water to remove sediment and shucked using sterile oyster knives on a sterile cutting surface. Oysters were shucked from the opposite side of the hinge to minimize contamination with sediment. At least 250g of oyster meat and liquor were placed in a sterile blender. A 1:1 dilution of oyster and sterile phosphate buffer saline (PBS, 3.72 mM $NaH_2PO_4 \cdot 2H_2O$, 14.0 mM $Na_2HPO_4 \cdot 2H_2O$, 0.145 M NaCl, pH 7.4) were mixed in the blender and homogenized for 90 to 120 seconds.

The homogenate was used for making enrichments and for plating onto T_1N_3 (10% tryptone, 3% NaCl) and *Vibrio vulnificus* (VVA) agar plates. A total of 0.2g of homogenate was spread onto the 0.1g agar plates. For the 0.01g oyster plates, 100µL taken from bottle E (20g oyster homogenate and 80mL PBS) and spread on the 0.01g agar plates. For each plate a sterile spreader was used to spread the homogenate or diluted homogenate equally on the plate until it was absorbed. Six T_1N_3 and two VVA plates were used for 0.1g and 0.01g oyster plating, a total

of 12 T_1N_3 plates and four VVA plates (Table 2.1). The plates were incubated for 16-18 hours at 33℃ and used for analysis.

Table 2.1: Summary of the oyster plating and spreading process.

Amount on Plate	Plate Type	Final Yield	AP-probe
0.2g (Homogenate)	T_1N_3	0.1g Oyster	*Tlh*
0.2g	T_1N_3	0.1g Oyster	*Tlh*
0.2g	T_1N_3	0.1g Oyster	*Tdh*
0.2g	T_1N_3	0.1g Oyster	*Tdh*
0.2g	T_1N_3	0.1g Oyster	*Trh*
0.2g	T_1N_3	0.1g Oyster	*Trh*
0.2g	VVA	0.1g Oyster	*Vvh*
0.2g	VVA	0.1g Oyster	*Vvh*
100µL (bottle E)	T_1N_3	0.01g Oyster	*Tlh*
100µL	T_1N_3	0.01g Oyster	*Tlh*
100µL	T_1N_3	0.01g Oyster	*Tdh*
100µL	T_1N_3	0.01g Oyster	*Tdh*
100µL	T_1N_3	0.01g Oyster	*Trh*
100µL	T_1N_3	0.01g Oyster	*Trh*
100µL	VVA	0.01g Oyster	*Vvh*
100µL	VVA	0.01g Oyster	*Vvh*

2.2.2.2 Water Preparation and Plating

Water was shaken 25 times for seven seconds to ensure a homogenous mixture before aliquoting into other containers. The sample seawater was aliquoted into enrichment containers and to a sub-sample bottle. The seawater from the sub-sample bottle was used for plating onto six T_1N_3 plates and two VVA plates. One mL of seawater was spread evenly using a sterile spreader onto each of the T_1N_3 and VVA plates. The water plates were incubated for 16–18 hours at 33°C.

Table 2.2: Summary of the water plating and spreading process.

Amount on Plate	Plate Type	Final Yield	AP-probe
1mL	T_1N_3	1mL Water	*Tlh*
1mL	T_1N_3	1mL Water	*Tlh*
1mL	T_1N_3	1mL Water	*Tdh*
1mL	T_1N_3	1mL Water	*Tdh*
1mL	T_1N_3	1mL Water	*Trh*
1mL	T_1N_3	1mL Water	*Trh*
1mL	VVA	1mL Water	*Vvh*
1mL	VVA	1mL Water	*Vvh*

2.2.2.3 Sediment Preparation and Plating

Sediment was processed after water and oyster samples were processed to prevent cross-contamination, as we have demonstrated previously that it is the richest source of vibrios (Johnson et al., 2010). Excess water from the sediment bottle was removed before weighing the bottle to get the sample weight. A 1:1 dilution of sediment and PBS was made for enrichments and dilutions. Various dilutions were made for plating purposes. Exactly 0.2g of the appropriate dilution was plated with sterile spreaders on T_1N_3 and VVA plates. The plates were incubated for 16–18 hours at 33°C. Table 2.3 shows the plates used during the sediment sample workups.

Table 2.3: Summary of the sediment plating and spreading process.

Amount on Plate	Dilution	Plate Type	Final Yield	AP-probe
0.2g	1:3	T_1N_3	0.05g	*tlh*
0.2g	1:3	T_1N_3	0.05g	*tlh*
0.2g	1:3	T_1N_3	0.05g	*tdh*
0.2g	1:3	T_1N_3	0.05g	*tdh*
0.2g	1:3	T_1N_3	0.05g	*trh*
0.2g	1:3	T_1N_3	0.05g	*trh*
0.2g	1:19	T_1N_3	0.01g	*tlh*
0.2g	1:19	T_1N_3	0.01g	*tlh*
0.2g	1:19	T_1N_3	0.01g	*tdh*
0.2g	1:19	T_1N_3	0.01g	*tdh*
0.2g	1:19	T_1N_3	0.01g	*trh*
0.2g	1:19	T_1N_3	0.01g	*trh*
0.2g	1:19	VVA	0.01g	*vvh*
0.2g	1:19	VVA	0.01g	*vvh*
0.2g	1:39	T_1N_3	0.005g	*tlh*
0.2g	1:39	T_1N_3	0.005g	*tlh*
0.2g	1:39	VVA	0.005g	*vvh*
0.2g	1:39	VVA	0.005g	*vvh*
0.2g	1:59	T_1N_3	0.003g	*tlh*
0.2g	1:59	T_1N_3	0.003g	*tlh*
0.2g	1:59	VVA	0.003g	*vvh*
0.2g	1:59	VVA	0.003g	*vvh*

2.2.3 Colony Lifting and Hybridization

After 16–18 hours of incubation, bacterial colonies on the agar plates were transferred to an appropriately labeled 85mm Whatman filter paper disk. The filters were labeled with two orientation lines. Then the labeled filters were placed face down on the matching agar plate and evenly spread using a sterilized spreading rod to ensure all colonies were transferred to the filter. Orientation lines were drawn on the backs of the agar plates to match the orientation lines on the filter. The filters were removed using sterile forceps and placed in 1 mL of lysis solution (0.5M NaOH, 1.5M NaCl) per filter. DNA was fixed to the filters by microwaving for 10 seconds per filter.

For each filter, 4mL of ammonium acetate was added to a washing container. The washing container was shaken at 125 revolutions per minute (rpm) at room temperature for five minutes and decanted. After five minutes the washing container was shaken (~125rpm) and rinsed twice with 10mL of 1X saline sodium citrate (SSC) per filter for two minutes (four minutes total) at room temperature to get rid of ammonium acetate residue. The filters were dried on paper towels and placed in Whirl-Pak bags until probing.

2.2.3.1 Proteinase-K (Pro-K)

All of the filters to be probed were combined in one washing container and rinsed with 10mL of 1X SSC and 20µL of stock Pro-K per filter. After ensuring that each filter was saturated with the 1X SSC and stock Pro-K in the washing container, each filter was placed in a warm water bath (42°C) with shaking (~50rpm) for thirty minutes. Then, the filters were rinsed three times with 10mL of 1X SSC per filter for 10 minutes at room temperature with shaking (~125rpm) in a washing container.

After thirty minutes of shaking in the water bath, the filters were rinsed three times in 10mL of 1X SSC per filter for 10 minutes at room temperature. The filters were dried and placed in Whirl-Pak bags or continued to the hybridization step.

2.2.3.2 Hybridization

In a labeled 4.5" by 9" Whirl Pak bag, three important components were added: one to five filters to be probed for a specific gene, a control strip, and 10mL of warm hybridization buffer. Once added, the bags were tightly closed with as few air bubbles as possible. The labeled bags were submerged in a 54°C water bath with approximately 50rpm for thirty minutes.

After thirty minutes of shaking in the water bath, the filters were put in new, appropriately labeled Whirl-Pak bags. In addition to 10mL of warm hybridization buffer, five

picamoles of AP-probe (*tlh, trh, tdh, vvh*) was added and thoroughly mixed to have consistency in each bag. The bags were closed with as few air bubbles as possible and placed in the same 54℃ water bath for one hour.

After one hour, each filter was washed with 10mL of pre-warmed 1X SSC/SDS (sodium dodecyl sulfate) or 3X SSC/SDS, depending on which probe was used. Only 3X SSC/SDS was used for *tdh*. The remaining probes were washed with 1X SSC/SDS. The filters were washed in the appropriate washing container two times for 10 minutes at 54℃ with shaking (~50rpm) in the water bath. After this step, it was no longer necessary to wash the filters separately if *tdh* was used. All filters were combined in a single washing container and rinsed with 1XSSC five times for five minutes at room temperature on the orbital shaker (~125rpm).

After the rinsing steps the filters were ready to be developed. For five filters, 20mL of nitro-blue tetrazolium chloride 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) was used for development. The filters and the NBT/BCIP solution were combined in a coated washing container to prevent any development disruption by light emission. The filters were shaken for approximately one to two hours at room temperature until they were fully developed. Development was determined when the control strips showed the appropriate dark purple colonies. To stop the reaction the filters were washed in a washing container with distilled water three times for 10 minutes with shaking (~125rpm).

Finally, the filters were dried by placing them between two paper towels. After the filters completely dried the positive colony forming units (CFU) were counted and recorded. Positive colonies had a dark purple or brown color whereas the negative colonies were either colorless or had a vague yellow tint. The filters and control strips were taped on 8''x11'' paper and placed in plastic sleeves in a labeled binder.

2.2.4 Pathogenic *Vp* Isolation

Isolating pathogenic *Vp* was a process that took multiple days to determine if particular colonies were pathogenic. Using this method, 133 'potentially pathogenic' colonies were isolated and used for the determination of type three secretion systems.

After probing sampling filters, the colonies that were presumed positive in reference to the control strips were the colonies of interest to be isolated. The filters with positive colonies were matched with the appropriate sampling agar plate. After the filter and plate were aligned, a colony was picked with a toothpick and streaked on a TCBS plate using the three streak phase method. For each phase a fresh toothpick was used. Each colony and TCBS plate was assigned a number for recording purposes. The plates were grown overnight until a significant number of colonies were present on the TCBS plate.

The isolated green colonies from the TCBS plate were selected and picked. If the plate was too heavy with colonies without well isolated colonies the plate was subcultured on another TCBS plate. Each picked colony was placed in approximately 100µL 1X APW (10% peptone, 10% NaCl, pH 8.5 ± 0.2) in 96 well plates. Only the first six columns of the plate were used to coincide with the 48 prong replicator. The plate was labeled with a specific label and was placed in the incubator until evidence of turbidity (12–24 hours).

The 48-prong replicator was used to stamp the bacteria grown in the first six wells of the 96-well plate to four T_1N_3 plates, one plate each for permanent storage, *tdh*, *trh*, and *tlh*. The 48-prong replicator was ethanol-flamed and cooled in PBS before each placement into the 96-well plate. It was essential to cool the prongs before placing in the bacteria to prevent cell death due to heat. The plates were incubated at 33°C overnight.

The following day, the plates were lifted, as described previously, except for the permanent storage plate. The *tdh, trh*, and *tlh* plates were lifted and probed as described earlier. After hybridization positive colonies from the confirmation plates were assigned a permanent storage number, picked from the permanent storage plate, and placed in long term storage.

2.2.5 DNA Extraction

DNA was extracted using the Chowdhurry method (1991). Before extraction proceeded, the 'potentially pathogenic' isolates that were placed in long-term storage were streaked onto their own T_1N_3 plates and grown overnight in the 33°C incubator. The next day a fresh toothpick was used to collect bacteria from each T_1N_3 plate, and the bacteria were knocked off in a labeled microfuge tube with 500µL of 1X APW. The microfuge tubes were placed back in the incubator and were grown overnight or until turbid. Once the microfuge tubes looked turbid, DNA extraction could proceed.

The first step in DNA extraction was to add 500µL of a 25:24:1 ratio of phenol saturated with TE (10 mM Tris, 7.5, 1 mM EDTA), chloroform, and isoamylacohol to each turbid microfuge tube. The tubes were vortexed for one minute and spun on a centrifuge at 12,000 g for five minutes. During the five minutes of centrifuging, fresh microfuge tubes were labeled with corresponding labels from the original tubes. A total of 500µL of isopropanol was added to the fresh tubes. After centrifugation, 450µL of the upper aqueous phase from the original tubes was added to the new tubes with isopropanol, with care taken to leave the middle and lower aqueous phase untouched. The new tubes were mixed well and centrifuged at 12,000 g for five minutes. The supernatant was poured off, and a pellet remained in the tube. The tubes were washed twice with 500µL of 70% ethanol, with care taken to leave the pellet undisturbed. The pellet was dried, and 100µL of TE buffer was added. The DNA tubes were placed in the 33°C incubator for

approximately 10 minutes and were eluted. After elution the DNA tubes were placed in the refrigerator for further analysis.

2.2.6 Multiplex Polymerase Chain Reaction (PCR) Screening

To determine the amount of DNA template in each sample, the samples were quantified using a nanodrop analyzer. The samples ranged from 7.2 to over 1000ng/µL. There were a total of five gene targets for TTSS1 and nine gene targets for TTSS2 (Table 2.4).

Table 2.4: List of gene targets for both TTSS1 and TTSS2 with their associated product sizes.

Gene	Product Size
TTSS1 Targets	
VP 1669	326
VP 1670	392
VP1686	283
VP 1689	192
VP 1694	96
TTSS2 Targets	
VPA 1327 (α)	97
VPA 1335 (α)	174
VPA 1339 (α)	343
VPA 1362 (α)	250
VPA 1354 (α)	553
vscS2 (β)	224
vopC (β)	594
vopB2 (β)	942
vscC2 (β)	1400

The primers were mixed with other PCR components which included PCR H2O, buffer, dNTPS, and Go-Taq polymerase. Per each PCR 25µL reaction, there were 8.8µL of PCR water, 5X Buffer, 2.5 mM $MgCl_2$, 10 µM for each primer used, 5.0 units/µL Go Taq polymerase, and 2 µL of target DNA.

The isolates were screened for TTSS1, TTSS2α, and TTSS2β according to the conditions as described by Noriea et al. (2010) (Table 2.5). The entire PCR cycle lasted approximated 90

minutes. Once the PCR run was complete the PCR products were taken out of the PCR instrument and placed in a 4℃ refrigerator until agarose gel electrophoresis.

Table 2.5: PCR amplification conditions for TTSS.

PCR Amplification Conditions			
Step	**Cycle(s)**	**Temperature (°C)**	**Duration (seconds)**
Initiation	1	95	120
Denaturation	33	95	45
Annealing		60	45
Extension		72	40
Final Extension	1	72	180

2.2.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze PCR products after amplification. The gel contained 6g of agarose, 300mL of 1X sodium borate buffer, and 30µL of 1X Sybr Safe. All contents were mixed in a 500mL glass bottle and heated until there were no bubbles. The gel was cooled slightly and poured into a gel rig. Gel teeth were placed in the liquid gel before solidifying to make wells for the products. An aliquot of roughly 5–7µL of each PCR product was injected into its own well. The products and a reference 50 base pair ladder were run on a 2% agarose gel at 200 volts for one to two hours. The gel was placed under a UV transilluminator hood and a photo was taken to determine the presence or absence of bands (Figure 2.3).

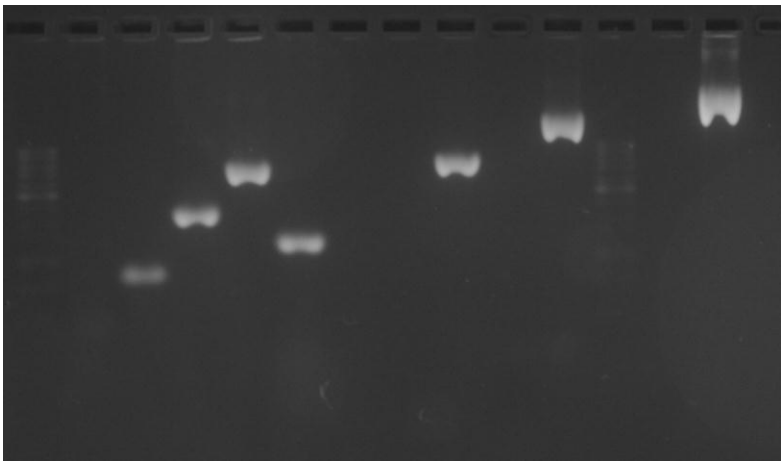

Figure 2.3: Individual amplicons for TTSS2α and TTSS2β.

2.2.8 Statistical Analysis

Chi-square analysis was the statistical method used via Microsoft Excel to determine a change in *Vp* pathogenicity with isolates collected before and after May 20, 2010.

2.3. Results

2.3.1 EID Data

There were a total of eight sample collections to gather isolates for the TTSS project. As stated earlier, at the time of sample collection water temperature and salinity were recorded (Figure 2.4 and Figure 2.5). The salinity was 5.8–8.2ppt at LC. In contrast, salinity was 24.5–29.2ppt at LF. The temperature was comparable at both sampling locations, ranging between 21.9–29.2℃ at both locations.

Table 2.6: Summary of sample locations and dates with the corresponding amount of isolates that were obtained from each sample collection.

Sampling location	Sampling date	*Vp* isolates	Temperature (°C) (Surface/Bottom)	Salinity (ppt) (Surface/Bottom)
LC	4/21/2010	21	21.9 / 21.9	5.8 / 5.8
LF	4/28/2010	14	22.0 / 21.9	27.1 / 29.2
LF	5/5/2010	16	28.1 / 28.2	24.5 / 24.5
LF	5/10/2010	5	25.2 / 25.5	24.9 / 24.7
LC	5/19/2010	5	28.3 / 28.1	7.7 / 8.2
LF	6/9/2010	72	30.2 / 30.1	21.3 / 21.3

Of the 133 isolates screened for TTSS, 64.5% came from oyster samples (86/133), 4.5% from water samples (6/133), and 30.8% from sediment samples (41/133). Table 2.6 shows the sample collections during the TTSS project. The majority of positive isolates were from LF (80.5%), whereas 19.5% of all isolates came from LC. In addition, the LC isolates that were used for the project was before the oil spill reached the region of coastline where samples were collected, which was around May 20, 2010 (Figure 2.4).

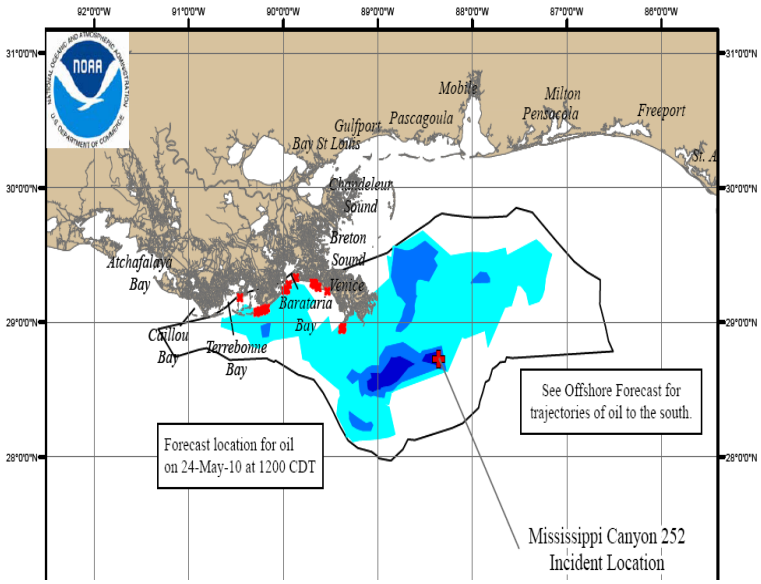

Figure 2.4: Oil spill trajectory map issued by NOAA for May 24, 2010.

2.3.2 Presence/Absence TTSS Chi-Square Analysis

Isolates were screened for TTSS1, TTSS2α, and TTSS2β genes. All 133 *Vp* confirmed isolates tested positive for the TTSS1 genes. There were a total of 61 isolates before May 20, 2010 and 72 after May 20, 2010. Of the 61 isolates prior to the oil spill reaching the geographic vicinity of the sample collections, a total of nine isolates were positive for TTSS2 (Table 2.7). After the oil spill, 17 out of 72 isolates tested positive for TTSS2 (Table 2.8). All positive isolates after the oil spill were from LF. There were a total of 22 positive TTSS2β isolates found in the study, nine before 5/20/2010 and 13 after 5/20/2010. Finally, there was a total of four positive TTSS2α isolates confirmed in the study and belonged to the post-spill numbers (Table 2.8).

Table 2.7: Number of present TTSS2 isolates found before and after May 20, 2010.

	Before 5/20/2010	After 5/20/2010
Positive	9	17
Negative	52	55

Table 2.8: Number of present TTSS2α and TTSS2β isolates found before and after May 20, 2010.

	Before 5/20/2010	After 5/20/2010
TTSS2α	0	4
TTSS2β	9	13
Total	9	17

Chi-square analysis was used via Microsoft Excel to determine if there was a significant change in TTSS2 before and after the oil spill. After analyzing the data from both Table 2.7 and 2.8, there was a lack of statistical significance in the change of TTSS2. The p-values from Table 2.7 and 2.8 were 0.19 and 0.11.

2.4 Discussion and Future Research

2.4.1 Discussion

As stated earlier, the purpose and hypothesis was to determine if there was a change in pathogenicity in the environmental *Vp* isolates due to an environmental stress, the oil spill. The results proved that there was an increase in the pathogenicity of the *Vp* isolates but there was not statistical significance (P=0.19,P=0.11). Therefore, the null hypothesis that stated there would not be a change in in TTSS2 before and after the oil spill was not rejected because the type I error rate was greater than 0.05.

Interestingly, there were more isolates positive for TTSS2β than TTSS2α before and after the oil spill. According to Noriea III et al. (2010) TTSS2α (*tdh+/trh–*) is more likely to be seen than TTSS2β (*tdh–/trh+*). After testing 146 environmental isolates there were 27 isolates positive for TTSS2α and three isolates positive for TTSS2β (Noriea III et al., 2010). However, Robert-Pillot et al. (2004) obtained results that differed from those of Noriea III et al. (2010). Robert-Pillot et al. (2004) tested 135 environmental isolates for *tdh* and *trh*. There were 13 isolates positive for *tdh–/trh+* and only one isolate positive for *tdh+/trh–* (Robert-Pillot et al., 2004). It should be noted that the two compared studies were in different geographic areas. Noriea III et al. (2010) collected environmental samples from the northern Gulf of Mexico, very close to sample collections at LF and LC for this study. Robert-Pillot et al. (2004) collected environmental samples off of the northwest and southwest coasts of France. One would assume that the results from the TTSS study in discussion would have similar results with the samples collected in the Gulf of Mexico. However, the results were similar to the results from France.

There are several interesting points to discuss based on the TTSS results. The most important point to discuss is the lack of oil concentration measurements of the sample

collections. The *Vp* isolates after the oil spill were assumed to have interacted with the oil based on the trajectory maps issued by NOAA. If the TTSS project were to be replicated with environmental samples it would be beneficial to confirm oil presence. For example, Sette et al. (2006) looked at changes in the bacterial community when exposed to oil. Oil samples were collected from a petroleum field, and the oil concentrations were measured using various methods. The four methods were medium pressure liquid chromatography, gas chromatography, biomarkers, and gamma-ray intensity. Depending on available resources and funding, one of the mentioned methods would be an ideal solution. However, because sampling was taking place at two different locations, LF and LC, months before the oil spill, it would not have been ideal to change sampling locations where the oil concentrations were much higher because there would have been a lack of data before the oil spill for comparison.

In addition to not measuring the oil concentrations, the sample size was not as large as originally planned. Initially, there were 322 isolates, but after further *Vp* confirmation it was concluded that they were not *Vp*. This conclusion reduced the sample size from 322 to 133 isolates. When this finding occurred the oil spill in the Gulf had ceased. Therefore, it would have been impossible to know if the environmental samples collected had interacted with oil or not, especially since the samples were not measured for oil concentrations. This reduction in isolates lowered statistical accuracy and may have been responsible for accepting the null hypothesis.

It is difficult to say that the oil spill was the only reason for the increase in TTSS2+ isolates after the oil spill. There are many other environmental parameters that could be contributing factors. As stated earlier, temperature and salinity are key determinants of the abundance and prevalence of *Vibrio* spp. Since the environmental samples were collected as the water temperatures got warmer it is a possible that temperature was a factor. Salinity was

consistent throughout three months of sampling at both locations. The lack of isolates from LC could be due to the fact that the salinity at LC was low. As stated earlier, the optimal level for salinity ranges between 10ppt to 34ppt (Martinez-Urtaza et al., 2010). At the LC location salinity did not get higher than 8.2ppt and it was as low as 5.8ppt at one time. The levels of salinity recorded at LC were not at the optimal level for vibrio growth.

The freshwater diversions called for by the state of Louisiana on 5/10/2010 could have influenced salinity levels as well (GOHSEP, 2010). For example, the Mississippi River was diverted to the Davis Pond, which eventually flows through Louisiana to the Gulf of Mexico (Figure 2.7). Figures 2.8 and 2.9 from the USGS shows the change in the water discharge and velocity after the state of Louisiana decided to divert the freshwater to the Gulf of Mexico. There is a drastic increase in discharge and velocity between 5/1/2010 to 5/12/2010, a change from a daily mean of 4,570 cubic feet per second (ft^3/s) and 1.16 feet per second (ft/s) to 9,850 ft^3/s to 2.21ft/s. However, there was not enough substantial data to confirm that salinity was significantly lowered at LF or LC due to the diversions.

Figure 2.5: Davis Pond impact zone in Louisiana once freshwater is diverted from the Mississippi River. The LF location is within the box located at the bottom of figure (courtesy of nemwuppermiss.blogspot.com).

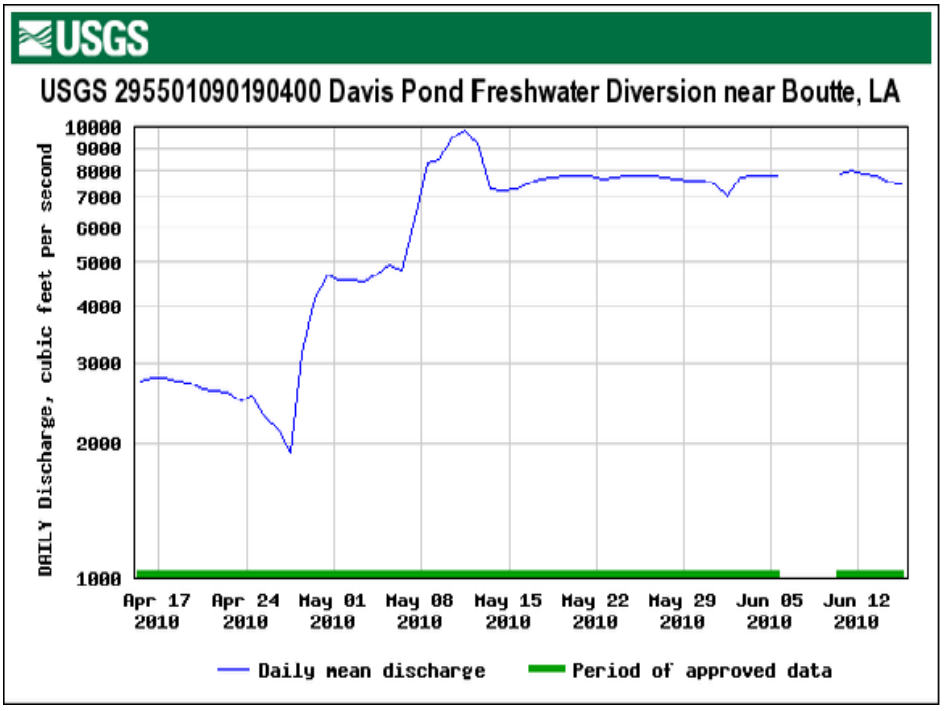

Figure 2.6: Daily discharge (cubic feet per second) readings of the Davis Pond freshwater diversion near Boutte, Lousiana from 4/15/2010 to 6/15/2010 (waterdata.usgs.gov).

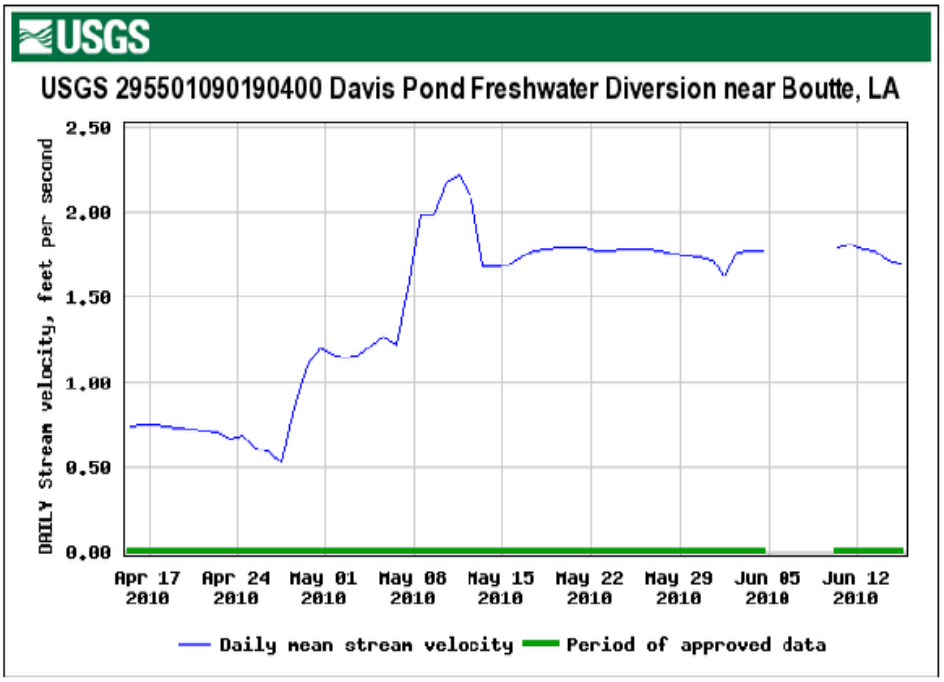

Figure 2.7: Daily stream velocity (feet per second) readings of the Davis Pond freshwater diversion near Boutte, Lousiana from 4/15/2010 to 6/15/2010 (waterdata.usgs.gov).

There were two sample collections after freshwater was diverted to the coast. The salinity at the LC location on 5/19/2010 was low (average 8.0ppt) but was comparable to the other sampling at LC on 4/21/2010 (average 5.8ppt). The samples collected at LF on 6/9/2010 had an average salinity of 21.3, which was 3ppt less than the average sample reading on 5/5/2010 at LF (24.5ppt). Even though there was a 3ppt decrease in salinity, the salinity was still at an optimal salinity condition after the freshwater diversion.

Another parameter of interest is the chemical dispersants used to disperse the oil being released into the Gulf. From mid-May to mid-July 2.1 million gallons of two different dispersants (Corexit 9500A and Corexit 9527) were released to disperse oil on the sea surface and at the wellhead (Kujawinski et al., 2011). There has not been an in-depth study of the effect of dispersants on *Vp* pathogenicity. If oil could be considered an environmental stressor due to large amounts released from the oil spill, dispersants could also be studied.

Two researchers from AEA Technology studied how oil-metabolizing bacteria interacted with various dispersants and different levels of nutrients (Swannell & Daniel, 1999). *Vibrio* spp. have proven to utilize elements in crude oil (Moxley & Schmidt, 2010). Swannell and Daniel (1999) analyzed various dispersants to determine if there was bacterial growth inhibition during interaction. They used four different types of dispersants: Enersperse 1583, COREXIT 9500, Finasol OSR-51, and Dasic Slickgone LTSW. They determined that after oil-metabolizing bacteria interacted with crude oil, dispersants, and nutrients, the bacteria utilized dispersants, and growth was not inhibited. This was especially the case when there were microcosms with low nutrient levels. The most important aspect of the study was the interaction with bacteria and Corexit 9500, the dispersant used for the Gulf oil spill. Out of the four dispersants used for the study, Corexit 9500 was 2nd in stimulating bacterial growth (Swannell & Daniel, 1999).

Hamdan and Fulmer (2011) also analyzed the prevalence and abundance of certain bacteria when there was interaction with a chemical dispersant, Corexit EC9500A. They determined that out of eight identified isolates, *Vibrio* sp. were the least inhibited by the dispersant and appear to have a tolerance for the dispersant. Both studies showed that oil-metabolizing bacteria highly interact with this dispersant, which could lead to further studies in relation to the *Deepwater Horizon* oil spill.

In conclusion, there are many different factors that could have affected the results. For example, temperature and salinity are constant factors that can inhibit or stimulate vibrios. The other two factors are the Davis Pond freshwater diversion and the chemical dispersants. The latter factors are the most interesting because vibrios do not naturally interact with large influxes of water or chemical dispersants.

2.4.2 Future Research

For future studies that evaluate the presence and absence of TTSS in *Vp*, some components need to be considered. There needs to be a sample size large enough to give an accurate representation of any findings. In addition, water or soil samples collected at sampling time would need to be measured for oil concentrations to confirm that oil is present.

This study brought up many relevant questions regarding oil, dispersants, and a dominance in TTSS2β. There have not been significant studies that analyzed the pathogenicity of *Vp* with oil, much less dispersants. However, there have been many studies that look at hemolysin expression, which has relevance to TTSS. A designed lab-controlled experiment would be an ideal solution to determine *Vp* pathogenicity with both oil and dispersants. Plus, the experiment could be replicated many times and one would not have wait for another oil spill to occur along a coast. In regards to the TTSS2β, more environmental isolates from LF and LC

need to be analyzed with the TTSS2 primers to determine if the dominance of TTSS2β was a coincidence or not. The lab-controlled experiment would also give better insight.

CHAPTER 3. IMPACT OF THE *DEEPWATER HORIZON* OIL SPILL ON THE VIBRIO COMMUNITY

3.1 Purpose and Hypotheses

In addition to determining if oil is an environmental stressor for *Vp*, it is useful to determine if oil can in fact change the vibrio community structure. As stated previously, three important *Vibrio* species, *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, can be fatal pathogens. To gain knowledge of the changes in the vibrio community that may occur when there is interaction with oil, a microcosm experiment was performed to get a better picture of the outcome. If there is a change that leads to an increase in one of the three mentioned species it would be a cause for human health concern. The second hypothesis is that there will be a change in the vibrio community, particularly *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, when various concentrations of *Deepwater Horizon* oil are present in the microcosm samples.

H_0: There will be no change in the vibrio community in the microcosm samples with oil.

H_1: There will be a change in the vibrio community in the microcosm samples with oil.

3.2 Materials and Methods

3.2.1 Water Collection

Water sample collections occurred from April through July 2011. Water samples were collected once a week from sampling sites at Port Fourchon, Louisiana and two sites at Sabine National Refuge, Louisiana. Approximately one to two liters of water were collected in autoclaved plastic containers. The water samples were placed in coolers with ice packs and stored at room temperature. The samples were processed either on the day of sampling or the following day.

3.2.1.1 Sample Locations

Samples were collected at Port Fourchon, Louisiana (29°15.231'N, 90°39.825'W) Figure 3.1) and two sites at Sabine National Wildlife Refuge, Louisiana (29°54.547'N, 93° 22.955'W and 29°53.372'N, 93°24.102'W) (Figure 3.2 and Figure 3.3).

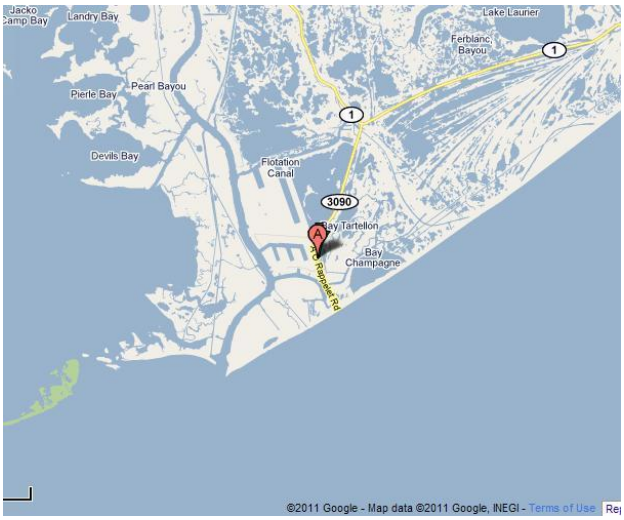

Figure 3.1: Sampling location at Port Fourchon, Louisiana (LF) (courtesy of Google Maps).

Figure 3.2: Sampling location at Sabine National Wildlife Refuge, Louisiana (LH) (courtesy of Google maps).

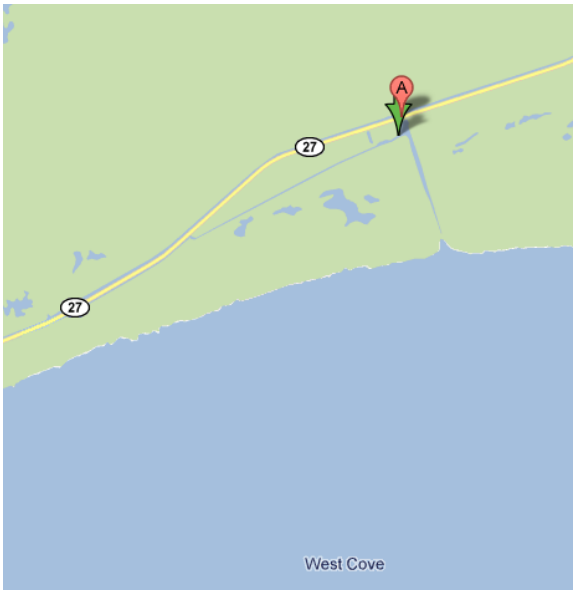

Figure 3.3: Sampling location at Sabine National Wildlife Refuge, Louisiana (LS) (Courtesy of Google maps).

3.2.2 Microcosm Preparation and Experimentation

Five experiments took place during the summer of 2011. Of the five experiments, four included 12 sterilized 100mL bottles with sample seawater and various concentrations of BP *Deepwater Horizon* crude oil (0.4 ppm, 0.2 ppm, and 0.02 ppm). The three oil concentrations were used for the microcosm experiment based on the findings from the NOAA R/V *WEATHERBIRD II* mission during May 22–28, 2010 (NOAA, 2010). The NOAA samples were collected from three locations ranging from 64.4 kilometers to 228.5 kilometers from the wellhead, the origin of the oil spill. The sample depths ranged from 50 meters to 1,400 meters, depending on the sampling location. The polyaromatic hydrocarbon readings at each location were less than 0.5 parts per million (NOAA, 2010). For this reason, low ppm concentrations were used for the microcosm experiment.

Prior to the addition of oil for experimentation, the oil stock bottle that was used for making oil dilutions was stirred for approximately five minutes. After five minutes the proper oil

dilutions were made in microfuge tubes. Each microfuge tube containing the oil was shaken or placed on the vortex mixer briefly before use.

Prior to adding 50mL of sample seawater to the 100mL glass bottles, the one liter container holding the sample seawater was shaken either 25 times or for seven seconds. Then the seawater was measured and poured into each bottle. Three bottles only contained 50mL of sample seawater and were labeled as the control bottles. Another three bottles contained 50mL of sample seawater and 50µL of 400ppm of *Deepwater Horizon* crude oil. The next three bottles also contained 50mL of sample seawater but 200ppm of the crude oil. The final three bottles contained 50mL of sample seawater and 20ppm of the crude oil. The fifth experiment only included six bottles: two control bottles, two bottles containing 50µL of 400ppm, and two bottles containing 50µL of 200ppm. In total, there were 54 samples to be analyzed for vibrio community differentiation.

For each experiment, the bottles were placed into an incubator with a temperature set between 29-31℃ with the bottle caps loosened. The bottles underwent a 24 period with 12 hours of light using a fluorescent light bulb and 12 hours of dark with shaking (~100rpm) for the entire 24 hour duration. After 24 hours of incubation the bottles were ready for filtration.

3.2.3 Syringe Filtration

Immediately after incubation, the samples were filtered via syringe filtration. Disposable sterile 60mL luer-lok tip syringes (Becton Dickinson #309653) were connected to autoclaved 25 mm filter holders (Swinnex 25 #SX0002500) that contained a 0.2µm 25mm plain filter (Supor PES Membrane Disc Filters #60309). In most cases only one filter was needed for each bottle. After each filtration, an ethanol flamed forcep was used to take the filter off the filter holder and was placed in a labeled test tube to be frozen at -20℃ until the sample DNA was extracted.

3.2.4 DNA Extraction

The DNA from each sample was extracted using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc. #12800-100). Each filter was taken out of its respective test tube with ethanol flamed forceps and cut in half with ethanol flamed scissors and placed in a labeled 2mL Bead Solution tube, using an aseptic technique. The Bead Solution tubes were placed on a vortex and mixed for up to five seconds. After mixing, 60µL of Solution S1 was pipetted into each Bead Solution tube. If S1 was precipitated, the solution was heated up to 60°C until completely dissolved. After the addition of S1 the Bead Solution tubes were mixed for up to five seconds on the vortex again. Since the samples were to be used for PCR, 200µL of Inhibitor Removal Solution (IRS) was added to each sample tube. For ten minutes the tubes were mixed thoroughly by securing the tubes horizontally and pressing down on the vortex. After the ten minute vortex step the tubes were placed in a microcentrifuge and were centrifuged at 10,000 x g for 30 seconds. After centrifugation, roughly 400-450µL of the supernatant from each tube was transferred to a clean microfuge tube.

In addition to the supernatant 250µL of Solution S2 was added to each microfuge tube and mixed for five seconds using the vortex. Immediately after mixing the tubes were placed in the 4°C fridge for five minutes. After the cooling step the tubes were centrifuged for one minute at 10,000 x g. The supernatant from each tube was transferred to a clean microfuge tube. Then, 1.3mL of Solution S3 was added to the microfuge tubes and mixed on the vortex for five seconds. After mixing the supernatant and S3, 700µL from each tube was placed on a fresh spin filter and centrifuged at 10,000 x g for one minute. The flow through was discarded and the spin filter was saved. To maximize yields, the entire tube containing supernatant and S3 was spun through its respective spin filter for another two times.

In step four 300µL of Solution S4 was added to each spin filter and centrifuged for 30 seconds at 10,000 x g. The flow through was discarded and the spin filters were centrifuged for another time for one minute at 10,000 x g. The spin filters were placed into a fresh microfuge tube and 50µL of Solution S5 was added. The tubes were centrifuged for 30 seconds at 10,000 x g. The spin filter was discarded and the solution remaining in the tube was the DNA final product ready to be analyzed. The products were placed in the -20°C until the PCR step.

3.2.5 PCR Amplification

To determine the amount of DNA template in each sample, the samples were quantified using a nanodrop analyzer. The samples ranged from two to 26.5ng/µL. Most samples were between 10-15ng/µL. The two primers used were Vibrio specific 16S rRNA primers, GC567F and 680R (Eiler, Johansson, & Bertilson, 2006). There was a GC clamp attached to the forward primer, 567F, so make sure the PCR products would not travel too far down the DGGE gel.

The two primers were mixed with the same PCR components used in the TTSS project. After the entire PCR cycle (Table 3.1), which lasted approximately three hours, the PCR products were taken out of the PCR machine and place in a 4°C refrigerator until agarose gel electrophoresis.

Table 3.1: PCR amplification conditions to target the 16s rRNA primers, GC567F and 680R.

PCR Amplification Conditions			
Step	**Cycle(s)**	**Temperature (°C)**	**Duration (seconds)**
Initiation	1	95	300
Denaturation	35	95	60
Annealing		64	60
Extension		72	120
Final Extension	1	72	300

3.2.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed on PCR products after amplification. The gel contained 6g of agarose, 300mL of 1X SBB, and 30µL of 1X Sybr Safe. All contents were mixed in a 500mL glass bottle and heated until there were no bubbles. The gel was cooled slightly and poured into a gel rig. Gel teeth were placed in the liquid gel before solidifying to make wells for the products. Roughly 2-3µL of each PCR product was injected into individual wells. The products and a reference 50 base pair ladder were run on a 2% agarose gel at 200 volts for one to two hours. The gel was placed under a UV transilluminator hood and a photo was taken to determine the presence and absence of bands and its relative intensity. If bands were too vague it was an indicator that the bands would also be vague on the DGGE gel. If this occurred, the PCR step was repeated until the bands were amplified to satisfaction.

3.2.7 Denaturating Gradient Gel Electrophoresis (DGGE) Analysis

DGGE was performed using the PCR products that were satisfactorily amplified. The DGGE gel was comprised of three different solutions: 0% Solution, Low Solution (38%), and High Solution (58%) (Table 3.2). The percentage was determined by the amount of denaturant solution added to the solution in relation to the other components. Once the gel plates were properly assembled, the low and high solutions were degassed and mixed in a gradient maker while simultaneously flowing into the stir plate via tubing with a syringe tip. Once the mixed solutions were one inch from the top of the plate, degassed 0% solution filled the remaining space until the solution overflowed. Gel teeth were placed in the gel and polymerization was the next step before adding the PCR products. One the gel polymerized the PCR products and a positive control ladder was added to the gel. The positive control ladder was a mixture of combing 5-7µL of three separate positive control PCR products into one tube for loading. The three positive control PCR products were *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*.

After loading 20-23µL of each PCR product and positive control ladder into a well and run 200 volts for 6.5 hours or 85 volts for 16 hours in a tank that contained 23L of 1X SBB at a constant temperature of 60°C.

Table 3.2: DGGE mixture components and amounts to make the three solutions: 0%, Low, and High.

DGGE Components	**0% Solution**	**Low Solution (38%)**	**High Solution (50%)**
Water (preferably chilled)	10.39mL	4.69mL	2.89mL
10X SBB Buffer	1.5mL	1.5mL	1.5mL
37:5:1 Acrylamide Solution	3mL	3mL	3mL
100% Denaturant Solution	---	5.7mL	7.5mL
Xylene Cyanol	Few Crystals	Few Crystals	---
10% Ammonium Persulfate Solution (APS)	103.5µL	103.5µL	103.5µL
Tetramethylethylenediamine (TEMED)	6.45µL	6.45µL	6.45µL

After the samples traveled through the gel it was stained with 1X Sybr Safe for 30 minutes hood and a photo was taken under a UV transilluminator hood with a digital camera (Figure 3.4). The photos were quantified and analyzed using image quantification software.

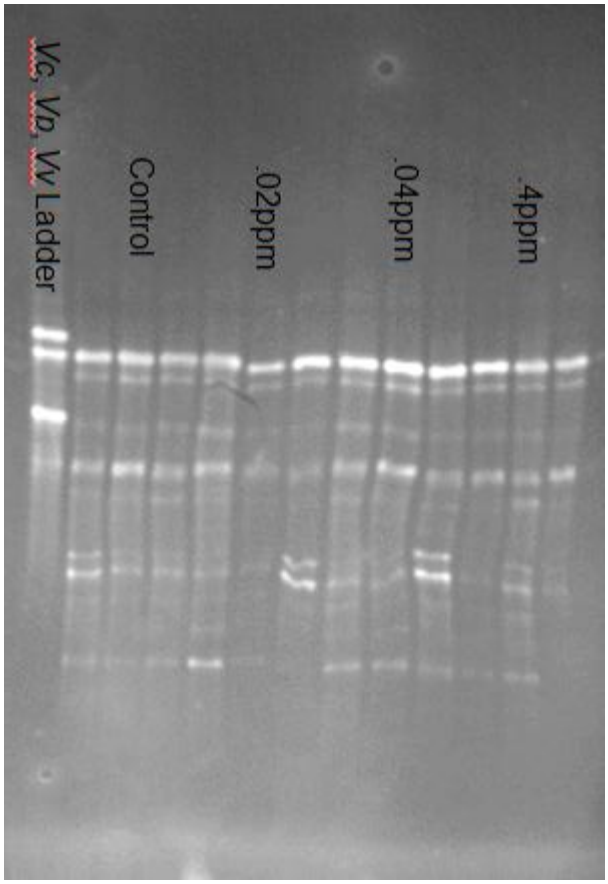

Figure 3.4: DGGE gel image picture of the first experiment on 8/3/2011.

3.2.8 ImageJ Software Quantification

The photos were analyzed using National Institute of Health ImageJ software (*rsbweb.nih.gov/ij/*). The photos were rotated to a vertical or horizontal position and cropped so the entire gel fit the ImageJ screen. The bands were enhanced and the photo was inverted to show the bands more clearly. Each lane was selected using the gel analyzer tool. Each peak shown in the peak analyzer window was sectioned off. The values were used for statistical analysis (3.5).

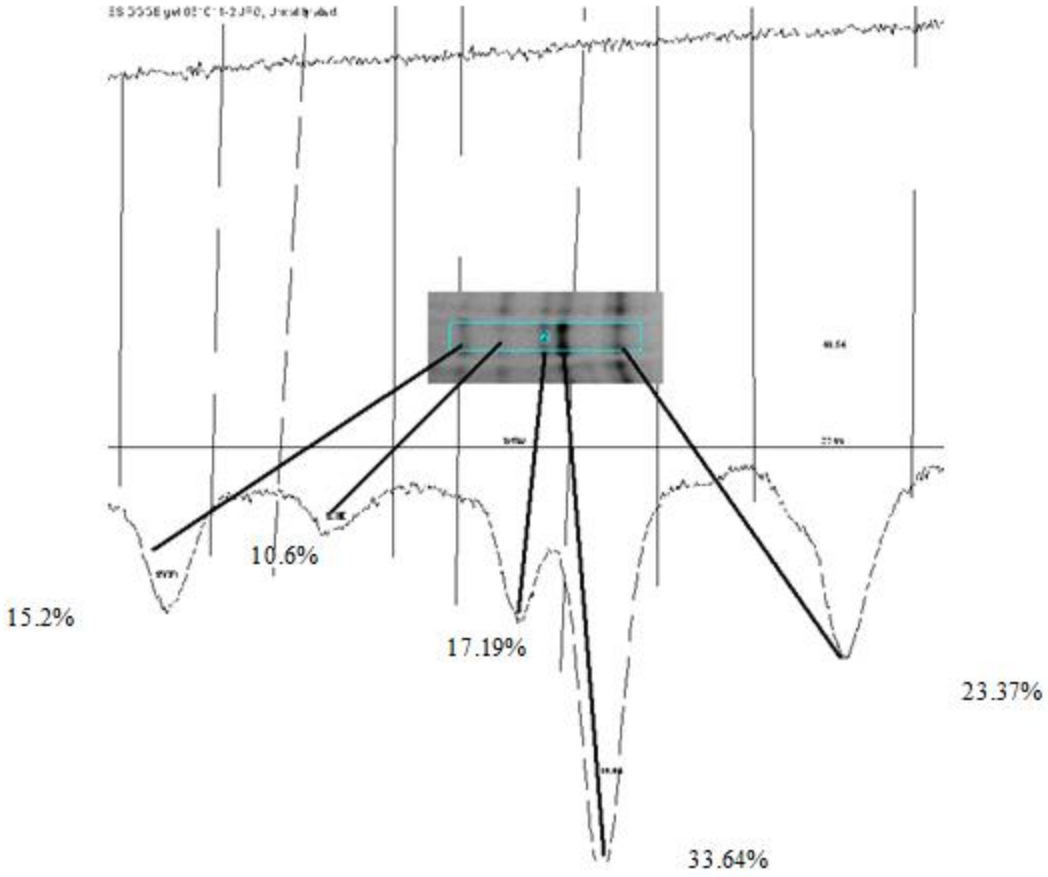

Figure 3.5: Values given for each peak using the peak analyzer in ImageJ software.

3.2.9 Statistical Analysis

Using Microsoft Excel, the Shannon and Simpson indices were calculated to quantify the vibrio community within each sample. In addition species evenness and richness were calculated for each sample. The calculated values were used for one-way analysis of variance (ANOVA) via SAS 9.3 statistical software.

3.3 Results

3.3.1 Diversity Indices

3.3.1.1 Shannon Index

The Shannon Index was one of the methods used to determine the diversity within each sample of the microcosm experiment,

$$H = -\sum_{i=1}^{S} p_i \ln(p_i)$$

Where *S* represents the number of DGGE bands present within each sample and p_i is the ratio of single band intensity (one species) compared to the total intensity (all species). The higher the value of the Shannon Index, the more diverse the community is.

3.3.1.2 Simpson's Index

Simpson's Index (D) was another method used to determine vibrio community within each sample,

$$D = \frac{N * (N - 1)}{\Sigma\ n_i * (n_i - 1)}$$

Where *N* is the total band intensity (all species and n_i is a given band intensity (one species). The Simpson Index (D) is the probability that two randomly selected individuals from a sample belongs to the same species (Crist et al., 2003).

3.3.1.3 Species Evenness

The Shannon Index values can be used to determine species evenness (E),

$E = H/\ln(S)$

Where *H* is the Shannon Index value and *S* represents the number of DGGE bands present within a sample. Species evenness is the measure of how well distributed the species are within a community (Wilsey & Polley, 2002).

3.3.1.4 Species Richness

Species richness (R) is the the number of species present in each sample,

$R = S$

Where *S* is the number of DGGE bands or the number of species found in the sample.

3.3.2 *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus* Band Intensities

In addition to determining various diversity indices, band intensities of three *Vibrio* spp. were evaluated: *Vibrio cholerae* (*Vc*), *Vibrio parahaemolyticus* (*Vp*) and *Vibrio vulnificus* (*Vv*). As stated previously, these three species comprised the DGGE ladder in each of the five experiments. The band intensity for each species in a given sample was divided by the sample's total intensity to calculate an intensity ratio.

3.3.3 Microcosm Experiments

3.3.3.1 Experiment One

A sea water sample from LF on 4/13/2011 was used for the first microcosm experiment (Table 3.3).

Table 3.3: Summary of EID data from the location site where seawater was collected for the first experiment.

Experiment 1	
Date	4/13/2011
Location	LF
Surface Temperature	23.9℃
Bottom Temperature	23.8℃
Surface Salinity	23.8 ppt
Bottom Salinity	23.8 ppt

Shannon Diversity

The highest Shannon Index value was calculated from one of the control samples (2.249) and the lowest Shannon Index value was recorded from one of the samples containing 0.04ppm (1.415). The samples containing 0.02ppm had an average Shannon Index of 2.2, slightly higher than the average of the controls samples, which was 2.18. The samples containing 0.04ppm had an average Shannon Index value of 1.705 (Table A.1). From the observation of Figure 13 and Appendix A.1, the control samples and samples containing 0.02ppm had very similar values. The values in the 0.04ppm samples dropped in comparison. The 0.4ppm samples had a higher diversity value than the 0.04ppm but it was not as high as the control samples and the 0.02ppm samples (Figure 3.6).

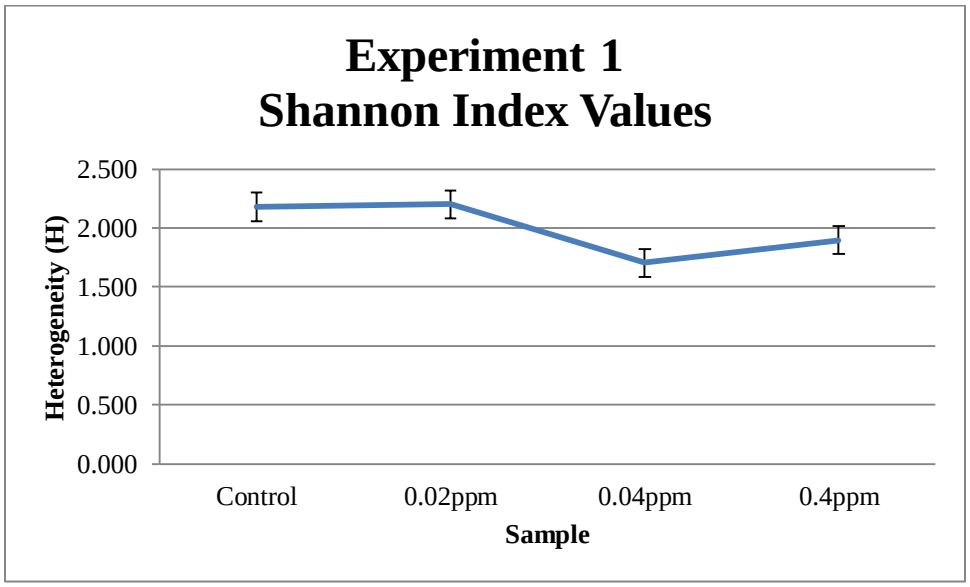

Figure 3.6: Shannon Index values calculated from the first microcosm experiment.

Simpson Index

The Simpson Index had similar values compared to the Shannon Index values from the first experiment. The lowest Simpson Index value was calculated from one of the control

samples (0.11) and the highest Simpson Index value was recorded from one of the samples containing 0.04ppm (0.21). The control samples had an average value of 0.119, slightly lower than the average of the 0.02ppm samples, which was 0.124. The samples containing 0.4ppm had the highest average Simpson Index value of 0.167 (Appendix A). Figure 3.7 and Table A.1 shows the similar values between the control samples and 0.02ppm samples. However, the 0.04ppm and 0.4ppm samples had lower values. Only two out of the six samples had values similar to the control and 0.02ppm samples (0.127 and 0.123). The remaining four samples hovered around a value of 0.20.

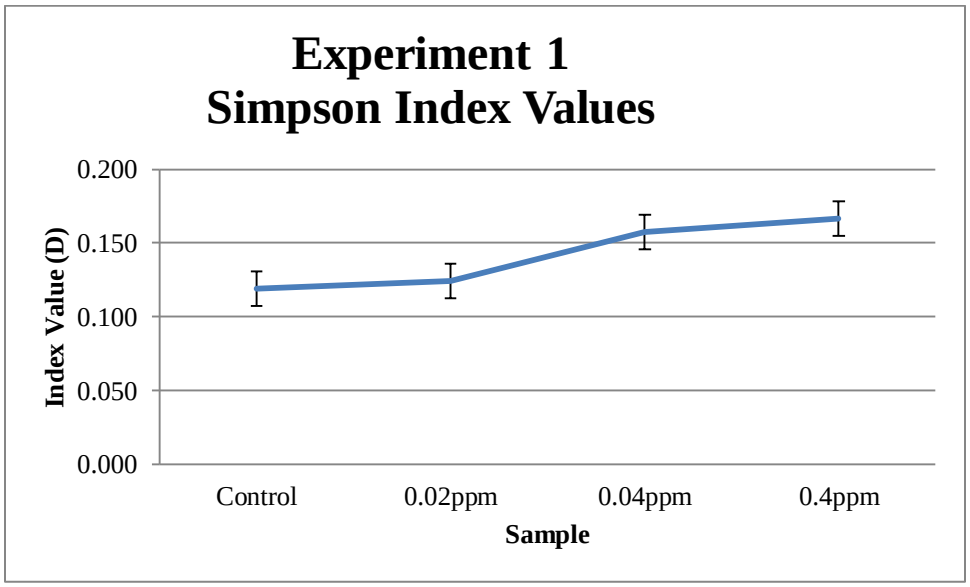

Figure 3.7: Simpson Index values calculated from the first microcosm experiment.

Species Evenness

The species evenness values ranged from 0.614 to 0.977 (Table A.1 and Figure 3.8). The average evenness of the samples from experiment one was 0.928 (Table A.1).

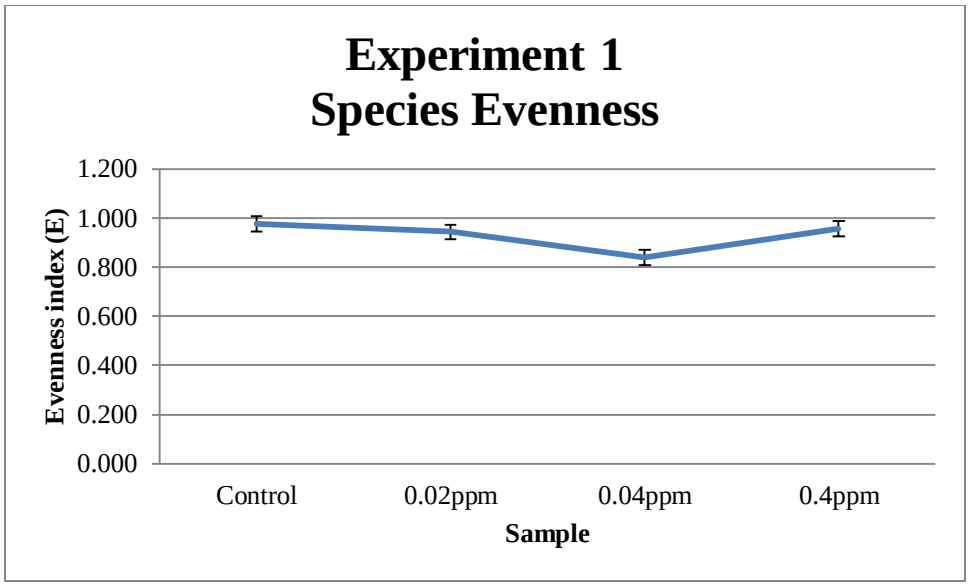

Figure 3.8: Species evenness values calculated from the first microcosm experiment.

Species Richness

The species richness values ranged from six to 11 species (Table A.1 and Figure 3.9). The greatest number of species was found in the control and 0.02ppm samples whereas the fewest number of species was found in the samples containing 0.04ppm and 0.4ppm.

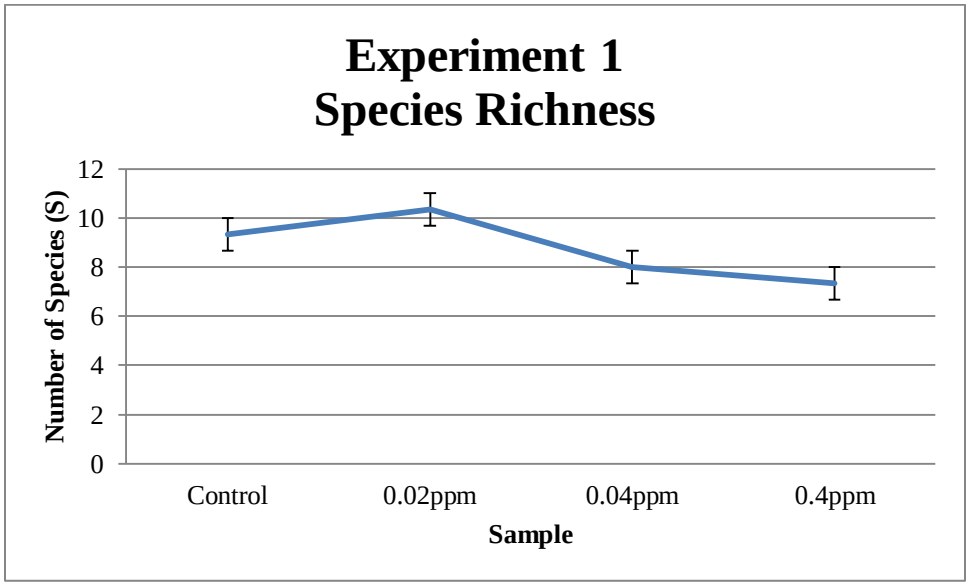

Figure 3.9: Species richness values from the first microcosm experiment.

Vc, *Vp*, and *Vv* Band Intensities

The first experiment did not have any detectable bands of *Vc* and *Vv* present in the 12 samples. However, *Vp* had detectable bands in all samples with various intensities (Table B.1). Figure 3.10 shows the steady upward trend of *Vp* intensity found in experiment one.

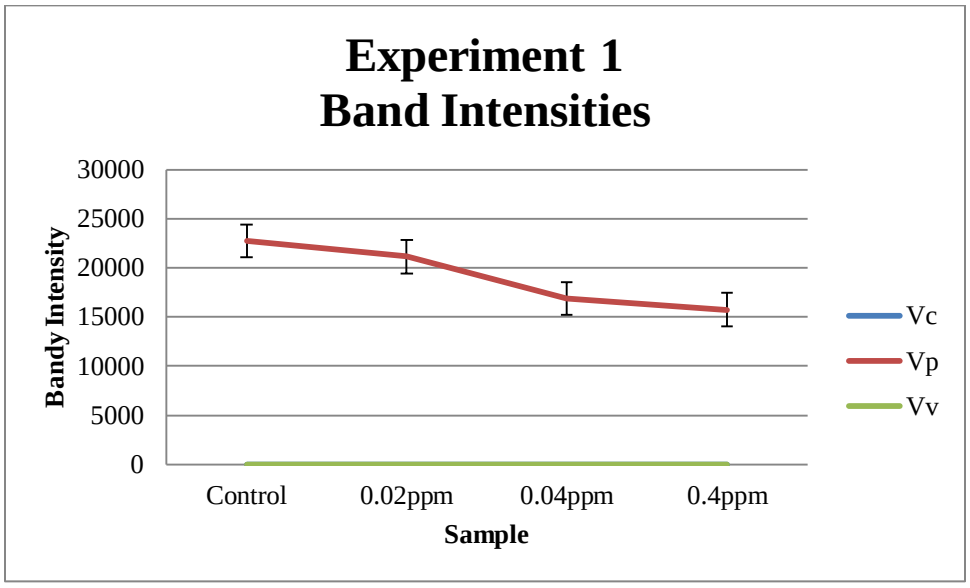

Figure 3.10: Band intensities of *Vc*, *Vp*, and *Vv* from experiment one.

3.3.3.2 Experiment Two

A sea water sample from LS on 4/27/2011 was used for the second microcosm experiment (Table 3.4).

Table 3.4: Summary of EID data from the location site where seawater was collected for the second experiment.

Experiment 2	
Date	4/27/2011
Location	LS
Surface Temperature	24.9°C
Bottom Temperature	24.6°C
Surface Salinity	18.7ppt
Bottom Salnity	16.1ppt

Shannon Index

The highest Shannon Index value was calculated from one of the control samples (2.811) and the lowest Shannon Index value was recorded from one of the samples containing 0.04ppm (2.067). The average Shannon Index values were very similar in the control samples and the samples containing oil. The average Shannon Indexes in order from the control samples to the 0.4ppm samples were 2.32, 2.503, 2.341 and 2.326 (Table A.2). Figure 3.11 shows that the Shannon Index was very consistent with little variability.

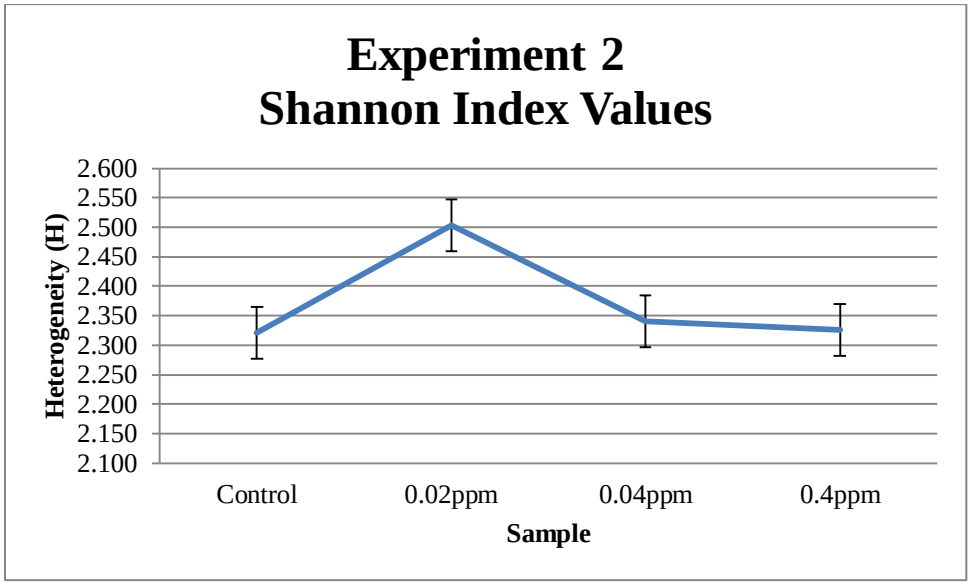

Figure 3.11: Shannon Index values calculated from the second microcosm experiment.

Simpson Index

The Simpson Index had similar values compared to the Shannon Index values from the second experiment. The lowest Simpson Index value was calculated from one of the control samples (0.085) and the highest Simpson Index value was recorded from one of the samples containing 0.04ppm (0.151). The average values, with the exception of 0.02ppm samples, were 0.12-0.11. The samples containing 0.02ppm had an average of 0.141. Figure 3.12 and Table A.2 shows that all samples from the second experiment had similar values.

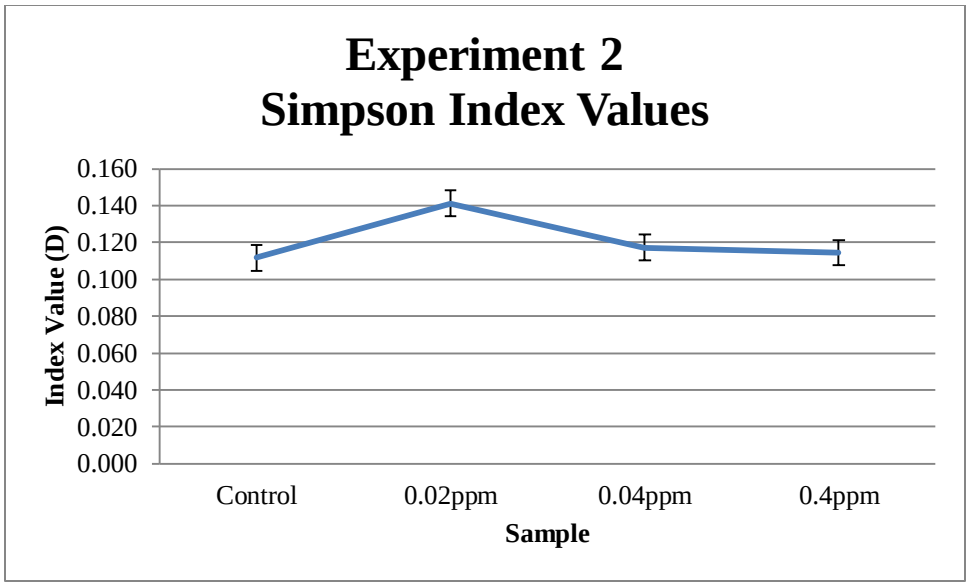

Figure 3.12: Simpson Index values calculated from the second microcosm experiment.

Species Evenness

The species evenness values ranged from 0.89 to 0.96 (Table A.2 and Figure 3.13). The average evenness of the samples from the second experiment was 0.931 (Table A.2).

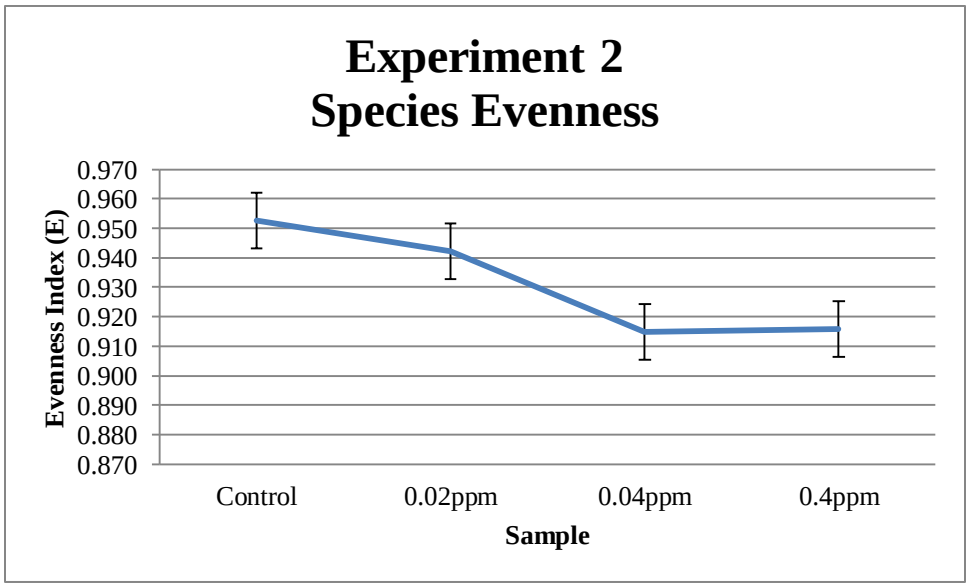

Figure 3.13: Species evenness values calculated from the second microcosm experiment.

Species Richness

The species richness values ranged from nine to 15 species (Table A.2 and Figure 3.14). Two out of three control samples had the lowest number of species compared to the other 10 samples.

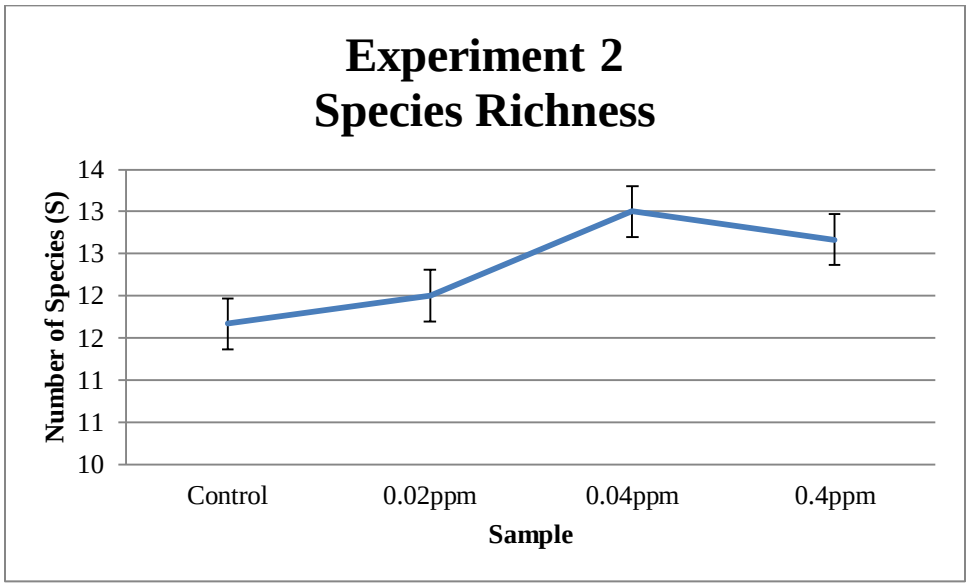

Figure 3.14: Species richness values from the second microcosm experiment.

***Vc*, *Vp*, and *Vv* Band Intensities**

The second experiment did not have any detectable bands of *Vc* and *Vv* present in the 12 samples. However, *Vp* had detectable bands in all samples with various intensities (Table B.2). Figure 3.15 shows the various intensities of *Vp* in experiment two.

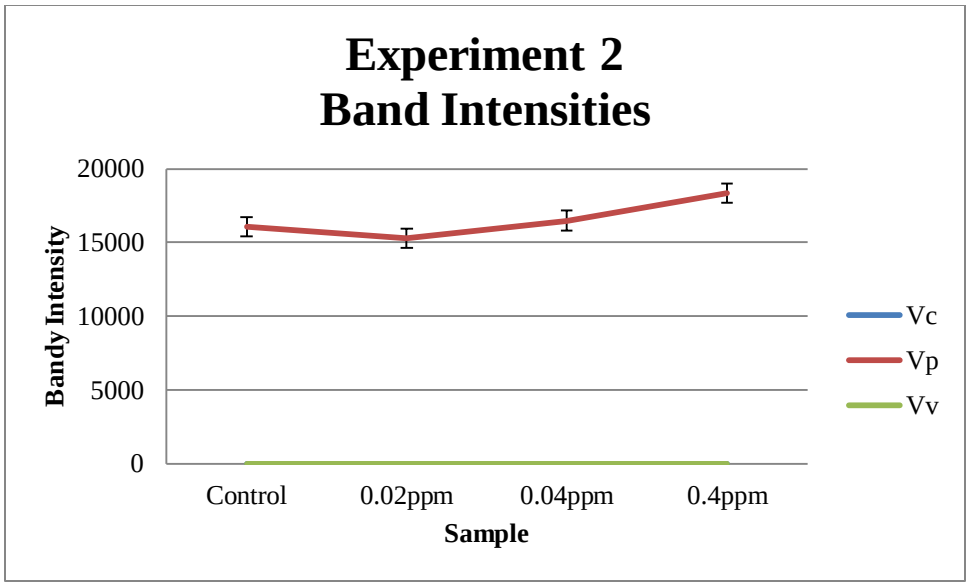

Figure 3.15: The band intensities of *Vc*, *Vp*, and *Vv* from experiment two.

3.3.3.3 Experiment Three

A sea water sample from LF on 5/18/2011 was used for the third microcosm experiment (Table 3.5).

Table 3.5: Summary of EID data from the location site where seawater was collected for the third experiment.

Experiment 3	
Date	5/18/2011
Location	LF
Surface Temperature	24.4℃
Bottom Temperature	24.1℃
Surface Salinity	29.0ppt
Bottom Salinity	28.9ppt

Shannon Diversity

The highest Shannon Index value calculated from one of the control samples (2.695) and the lowest Shannon Index value calculated from one of the samples containing 0.04ppm (1.453) Table A.3). The control samples from the third experiment had the highest average Shannon

Index value (2.239). The lowest average Shannon Index value was 1.453, the 0.04ppm samples. Figure 3.16 is a graphical representation of Shannon Index value variability between samples.

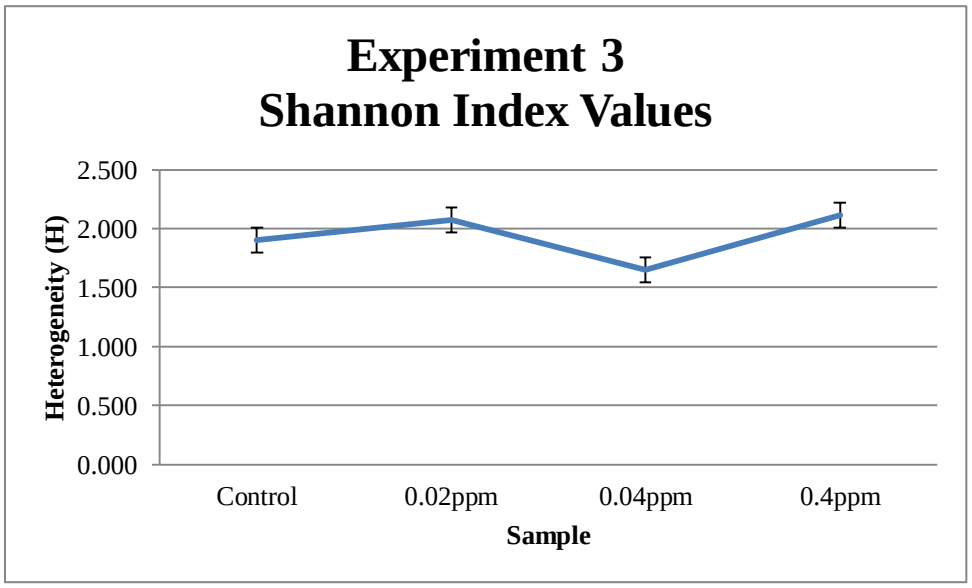

Figure 3.16: Shannon Index values calculated from the third microcosm experiment.

Simpson Index

The lowest Simpson Index value was calculated from one of the control samples (0.072) and the highest Simpson Index value was recorded from one of the samples containing 0.02ppm (0.267). The control samples had an average value of 0.122, slightly higher than the average of the 0.02ppm samples, which was 0.163. The samples containing 0.4ppm had the lowest average Simpson Index value of 0.237 (Table A.3). Figure 3.17 and Table A.3 shows that the control samples had the highest values compared to the values of the samples containing oil.

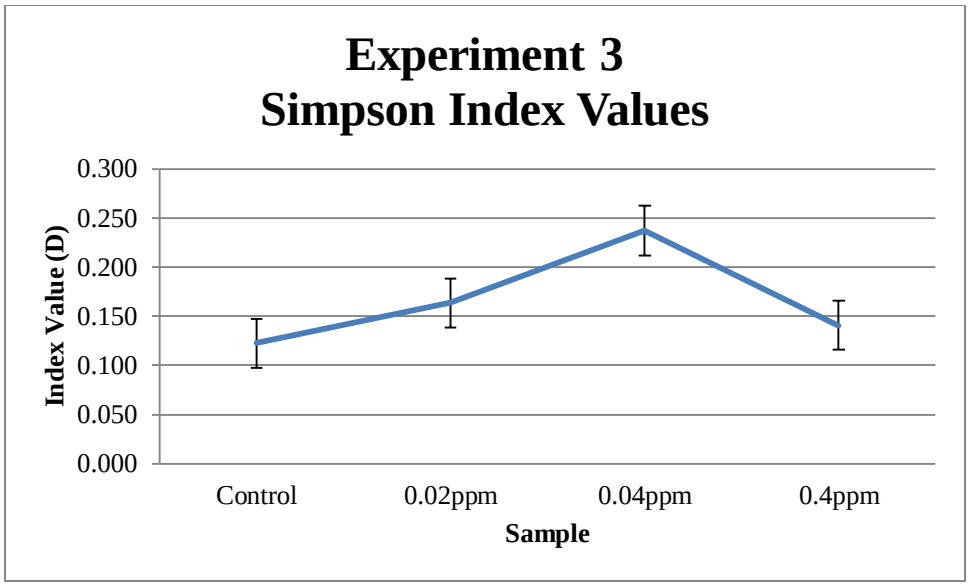

Figure 3.17: Simpson Index values calculated from the third microcosm experiment.

Species Evenness

The species evenness values ranged from 0.21 to 0.028 (Table A.3 and Figure 3.18). The average evenness of the samples from experiment one was 0.093 (Table A.3).

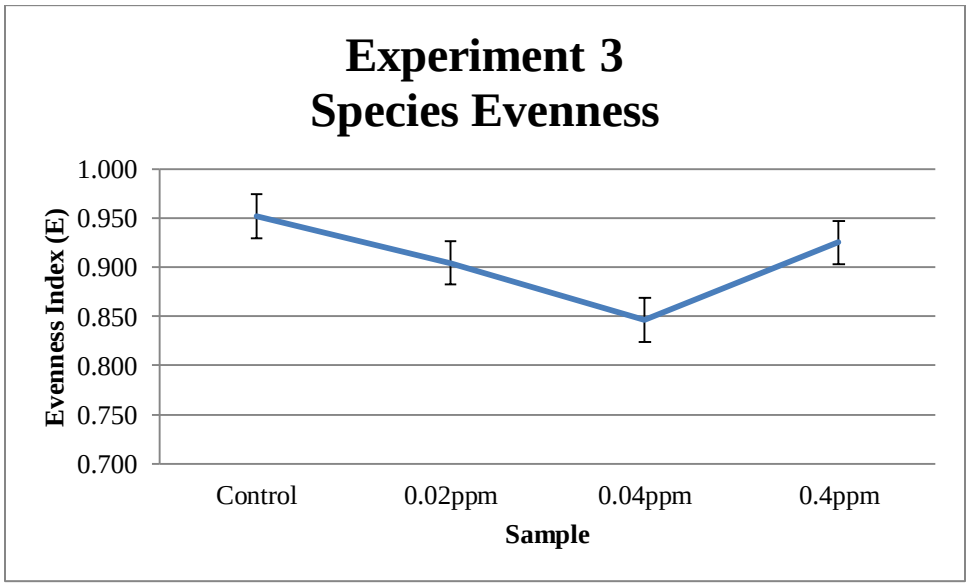

Figure 3.18: Species evenness values calculated from the third microcosm experiment.

Species Richness

The species richness values ranged from five to 16 species (Table A.3 and Figure 3.19), the largest belonging to one of the control samples and the smallest belonging one of the 0.04ppm samples.

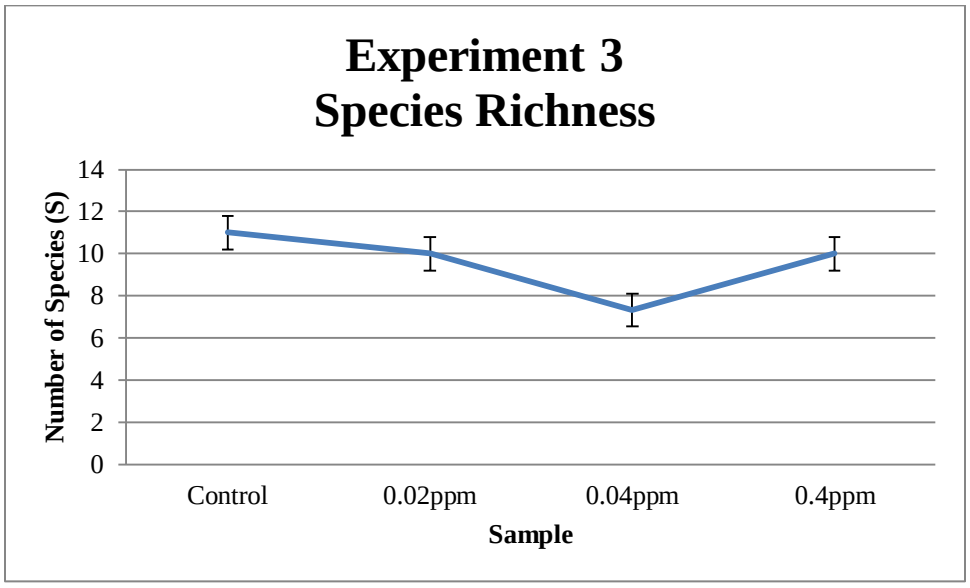

Figure 3.19: Species richness values from the third microcosm experiment.

***Vc*, *Vp*, and *Vv* Band Intensities**

The third experiment did not have any detectable bands of *Vc* in the 11 samples. However, *Vp* had detectable bands in all samples with various intensities (Table B.3). Figure 3.20 shows the various intensities in experiment three.

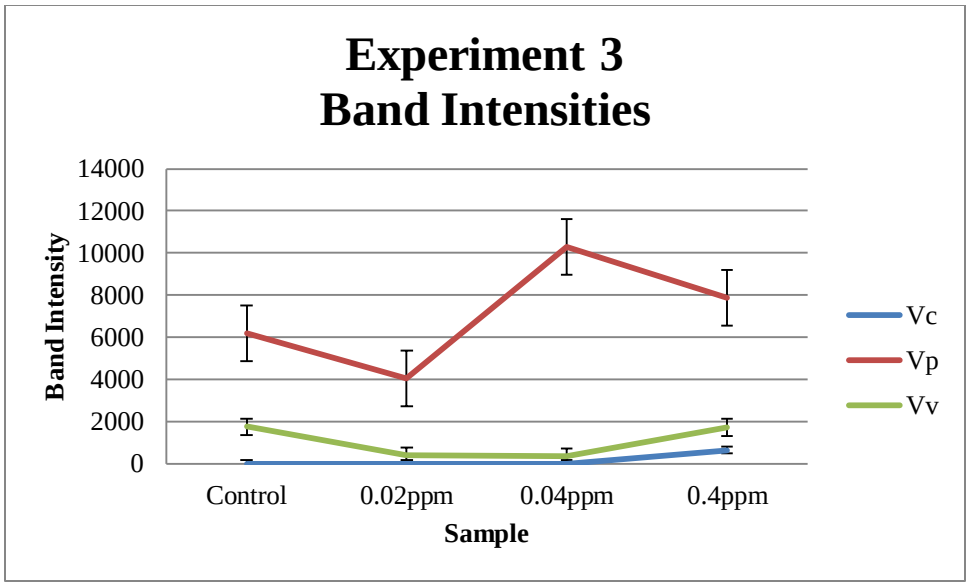

Figure 3.20: The band intensities of *Vc*, *Vp*, and *Vv* from experiment three.

3.3.3.4 Experiment Four

A sea water sample from LF on 5/18/2011 was used for the fourth microcosm experiment (Table 3.6).

Table 3.6: Summary of EID data from the location site where seawater was collected for the fourth experiment.

Experiment 4	
Date	5/18/2011
Location	LF
Surface Temperature	24.4℃
Bottom Temperature	24.1℃
Surface Salinity	29.0ppt
Bottom Salinity	28.9ppt

Shannon Diversity

The highest Shannon Index value calculated from one of the 0.02ppm samples (2.859) and the lowest Shannon Index value calculated from one of the samples containing 0.04ppm (1.656). The control and 0.02ppm samples from the fourth experiment had the highest average

Shannon Index values (2.601 and 2.597). The lowest average Shannon Index value was 1.967, the 0.04ppm samples. Figure 3.21 and Table A.4 shows that the control samples and the 0.02ppm samples had the highest values compared to the values of the samples containing 0.04ppm and 0.4ppm oil.

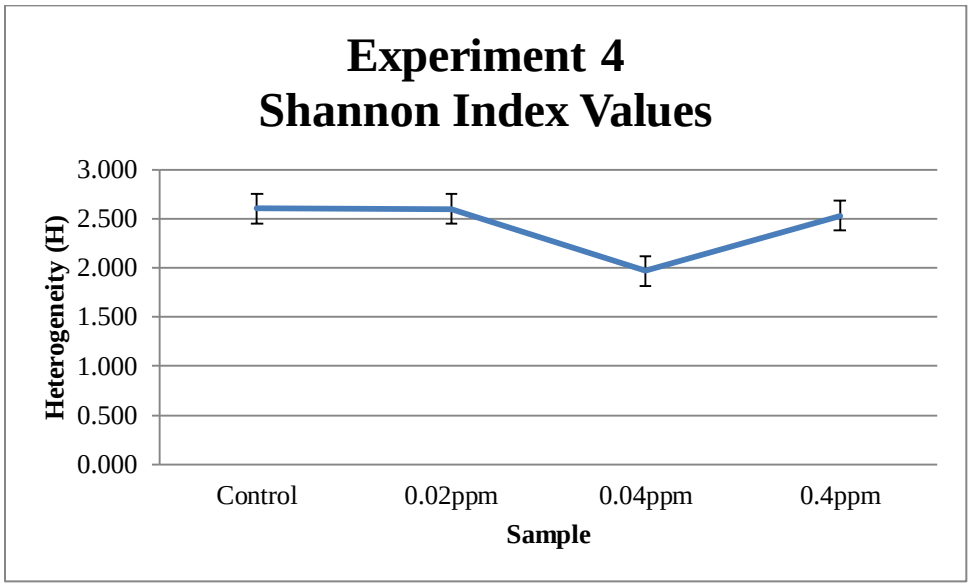

Figure 3.21: Shannon Index values calculated from the fourth microcosm experiment.

Simpson Index of Diversity

The lowest Simpson Index value was calculated from one of the 0.02ppm samples (0.067) and the highest Simpson Index value was recorded from one of the samples containing 0.04ppm (0.214). The control samples had an average value of 0.094, slightly higher than the average of the 0.02ppm samples, which was 0.089. The samples containing 0.4ppm had the highest average Simpson Index value of 0.171 (Table A.4). Figure 3.22 and Table A.4 shows that the control samples and the 0.02ppm samples had the highest values compared to the values of the samples containing 0.04ppm and 0.4ppm oil.

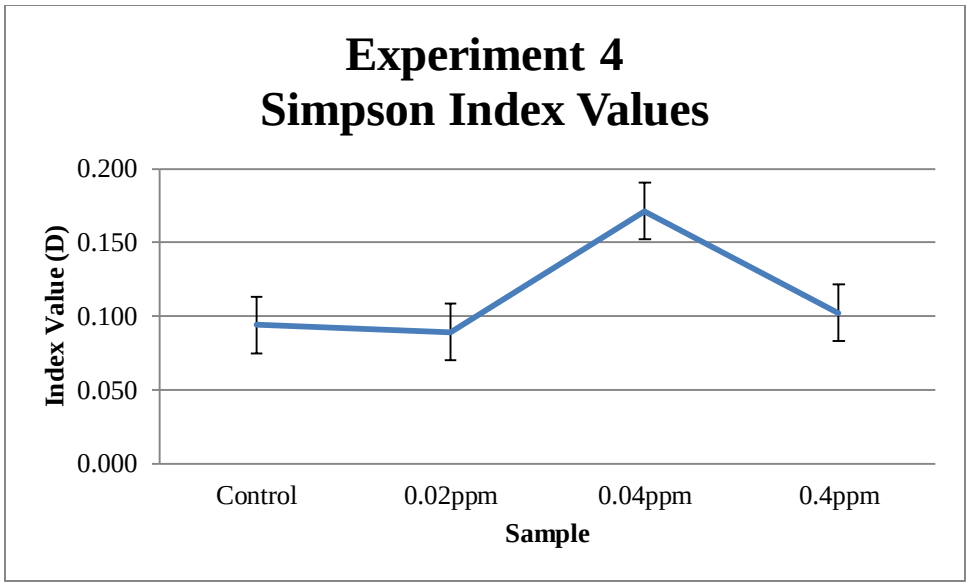

Figure 3.22: Simpson Index values calculated from the fourth microcosm experiment.

Species Evenness

The species evenness values ranged from 0.873 to 0.959 (Table A.4 and Figure 3.23). The average evenness of the samples from experiment one was 0.915 (Table A.4).

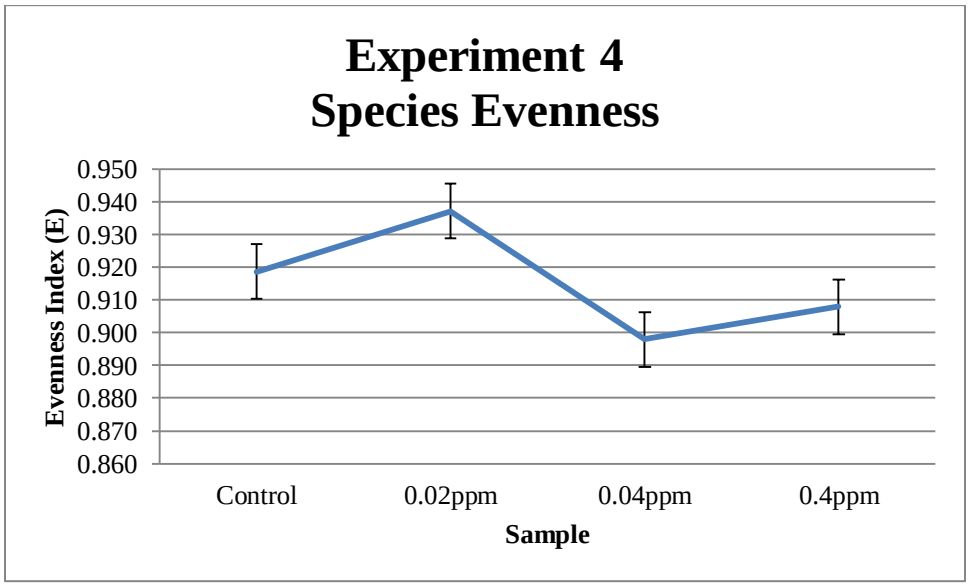

Figure 3.23: Species evenness values calculated from the fourth microcosm experiment.

Species Richness

The species richness values ranged from six to 21 species (Table A.4 and Figure 3.24), the largest belonging to one of the 0.02ppm samples and the smallest belonging one of the 0.04ppm samples. All three 0.04 ppm samples had the lowest richness compared to the other nine samples.

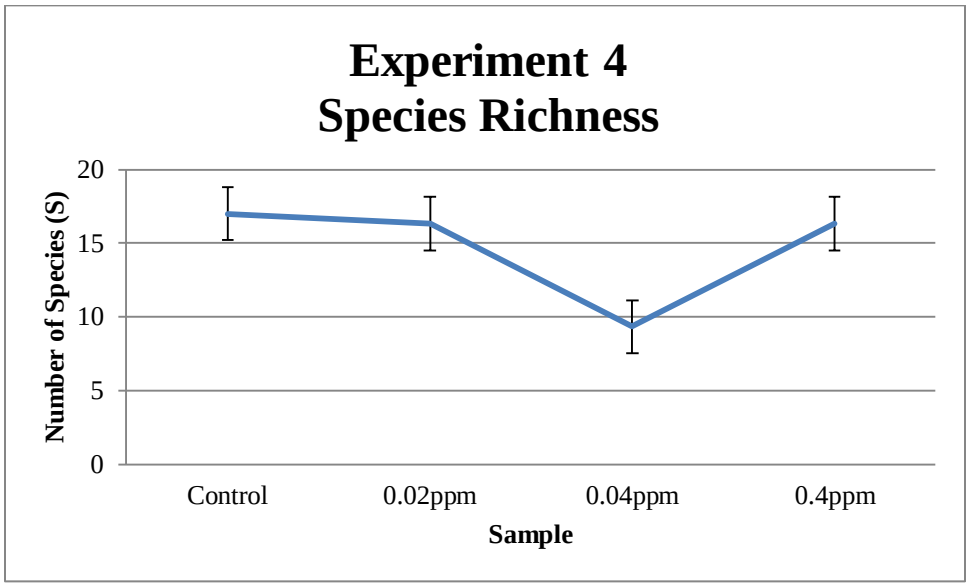

Figure 3.24: Species richness values from the fourth microcosm experiment.

***Vc*, *Vp*, and *Vv* Band Intensities**

Experiment four had detectable bands from all three species in 12 samples. Of the three, *Vp* had the highest intensities ratios, ranging from 8.80% to 29.85%. *Vv* was present in 11 out of 12 samples and *Vc* was present in the control samples, two 0.02ppm samples, and one 0.04ppm sample (Table B.4). Figure 3.25 is a graphical representation of the various band intensity ratios for the three mentioned species found in the samples of experiment four.

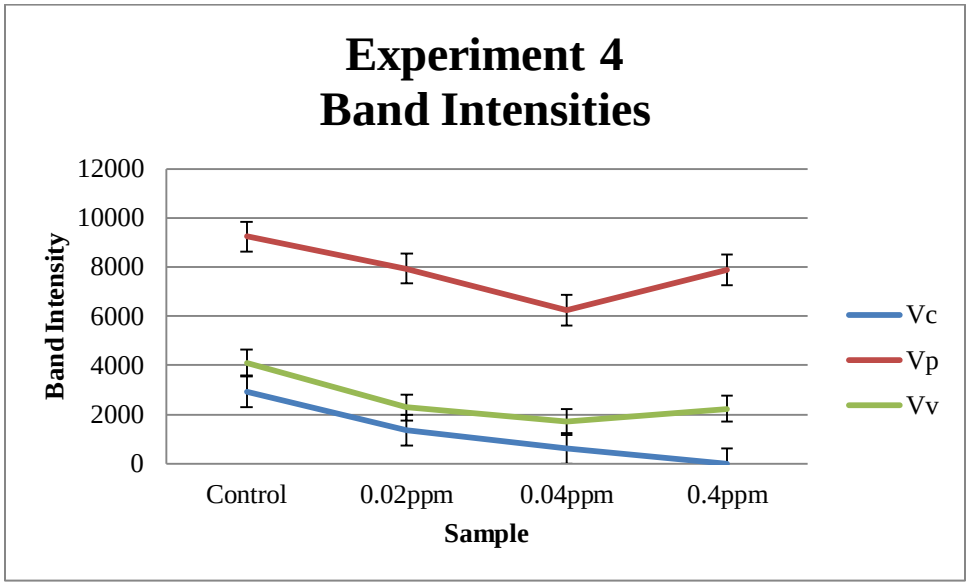

Figure 3.25: Band intensities of *Vc, Vp,* and *Vv* from experiment four.

3.3.3.5 Experiment Five

A sea water sample from LH on 7/26/2011 was used from the fifth microcosm experiment (Table 3.7).

Table 3.7: Summary of EID data from the location site where seawater was collected for the fifth experiment.

Experiment 5	
Date	7/26/2011
Location	LH
Surface Temperature	30.0℃
Bottom Temperature	29.7℃
Surface Salinity	22.2ppt
Bottom Salinity	21.9ppt

Shannon Diversity

Unlike the previous first four experiments, the highest Shannon Index value was from one of the 0.4ppm samples, 2.816. One of the two control samples had the lowest Shannon value

index, 2.326 (Table A.5). Figure 3.26 is a visual representation of the steady increase in the values from the control samples to the samples containing 0.4ppm.

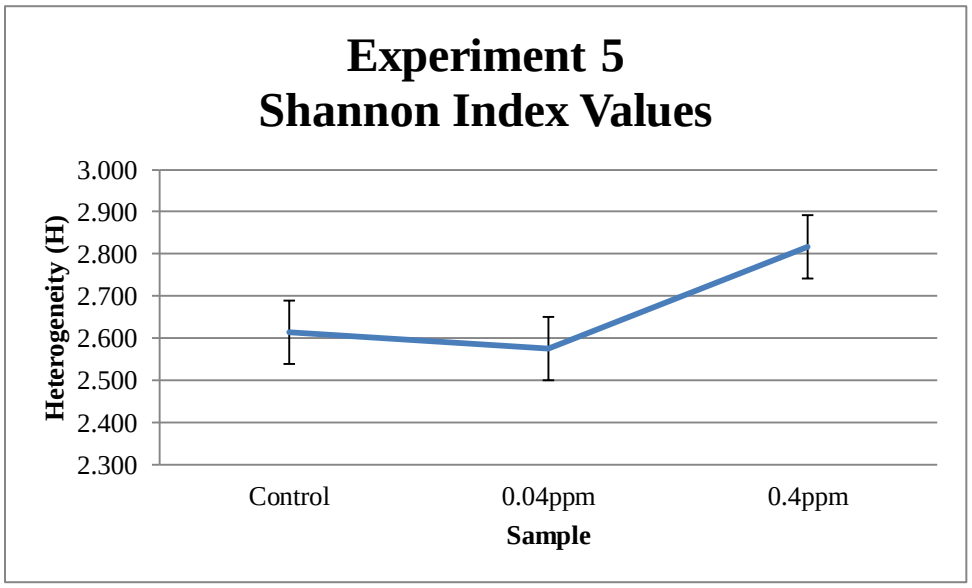

Figure 3.26: The Shannon Index values calculated from the fifth microcosm experiment.

Simpson Index

The lowest Simpson Index value was calculated from one of the 0.4ppm samples (0.07) and the highest Simpson Index value was recorded from one of the control samples (0.105). From the observation of Figure 3.27 and Table A.5, the two control samples had the highest values compared to the 0.04ppm and 0.4ppm samples.

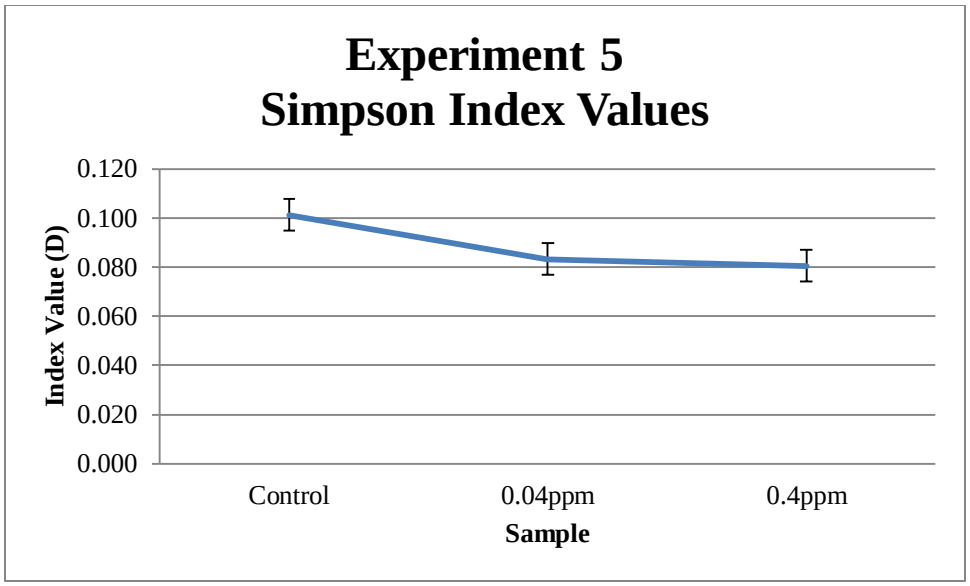

Figure 3.27: The Simpson Index values calculated from the fifth microcosm experiment.

Species Evenness

The species evenness values ranged from 0.928 to 0.97 (Table A.5 and Figure 3.28). The average evenness of the samples from experiment five was 0.945 (Table A.5).

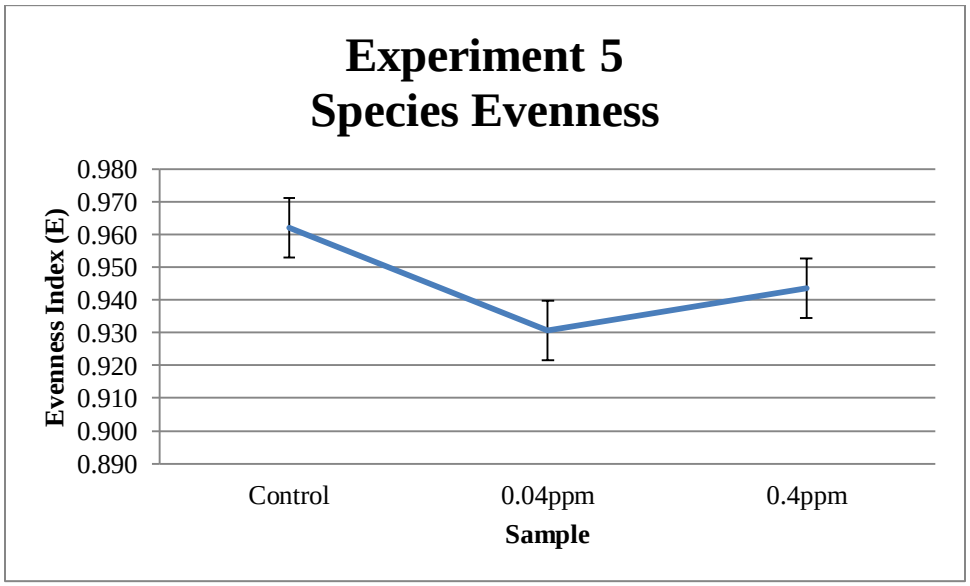

Figure 3.28: The species evenness values calculated from the fifth microcosm experiment.

Species Richness

The species richness values ranged from 13 to 20 species (Table A.5 and Figure 3.29), the largest belonging to one of the 0.02ppm samples and the smallest belonging one of the 0.04ppm samples. The two control samples had the lowest richness (11 and 13) whereas the lowest richness value from remaining four samples was 15.

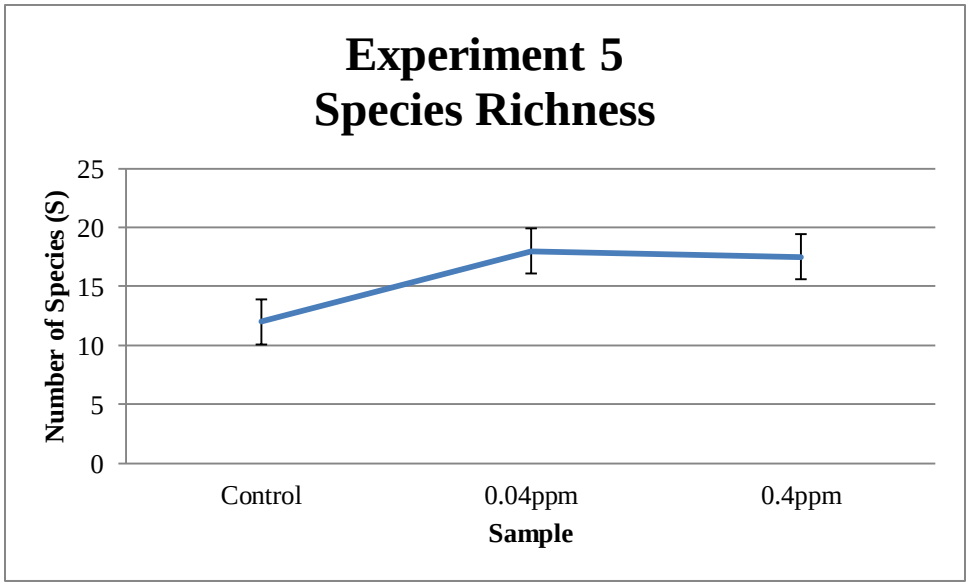

Figure 3.29: The species richness values from the fifth microcosm experiment.

***Vc*, *Vp*, and *Vv* Band Intensities**

In experiment five, *Vp* was present in all samples. The *Vp* band intensity ratio in all samples ranged from 7.49% to 14.37%. *Vv* was present in one 0.04ppm sample with a band intensity ratio of 1.58%. *Vc* was not detectable in the six samples from experiment five (Table B.5). Figure 3.30 is a graphical representation of the various band intensity ratios for the three mentioned species found in the samples of experiment five.

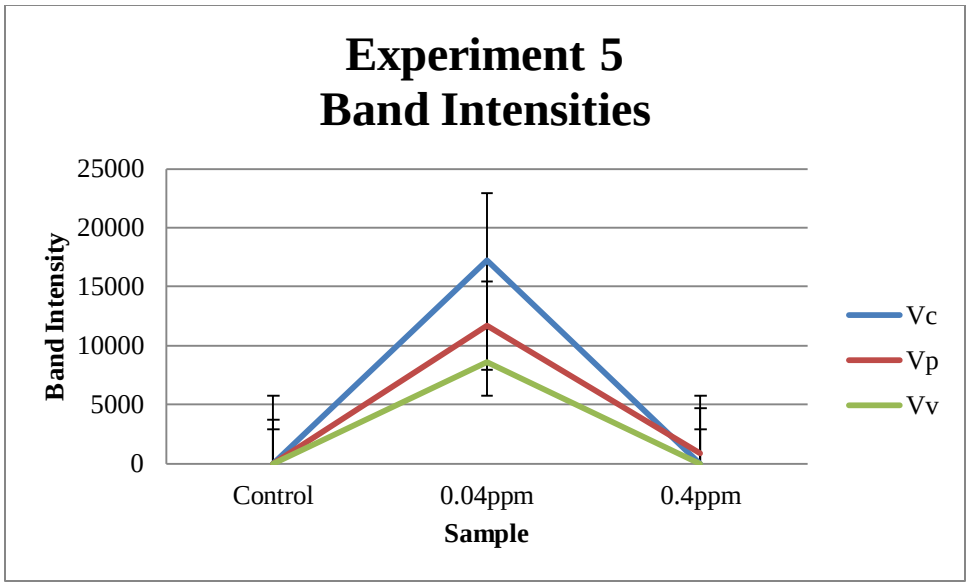

Figure 3.30: The band intensities of *Vc*, *Vp*, and *Vv* from experiment five.

3.3.4 Statistical Results

The results from one-way ANOVA with the five experiments showed a variability of results (Table 3.8). Experiments one and four had Shannon index p-values less than 0.05, which were statistically significant results. The majority of the Simpson index, evenness, and richness results were not statistically significant. Experiment four had significant Simpson index and richness results and experiment two had significant evenness results (Table 3.8).

Table 3.8: One-way ANOVA p-values for each experiment and diversity index.

Experiment	Shannon (H)	Simpson Index (D)	Evenness (E)	Richness (R)
Experiment 1	0.03	0.14	0.38	0.07
Experiment 2	0.71	0.20	0.03	0.85
Experiment 3	0.27	0.17	0.28	0.53
Experiment 4	0.02	0.02	0.48	0.04
Experiment 5	0.18	0.26	0.05	0.20

3.4 Discussion and Future Research

3.4.1 Discussion

As previously stated, the purpose and hypothesis was to determine if there was a change in the vibrio community in the samples containing various oil concentrations. All five experiments had relative changes in the vibrio community when comparing the control samples and the samples containing oil. Experiments two, three, and five had statistically insignificant changes in diversity between samples, with the exception of experiment two's evenness results (P=0.03). However, experiment one and experiment four had statistically significant Shannon index results (P=0.03,P=0.02). In addition, the Simpson index and richness results (P=0.02, P=0.04) further support the significant change in diversity in experiment four. Experiments one and four support the hypothesis that there would be a change in diversity of the vibrio community.

One interesting result was found in the samples containing 0.04ppm but not in the samples containing 0.4ppm. Based off observation, there was a noticeable decline in the diversity indices of the 0.04ppm samples. This finding is peculiar because the same result did not occur in the samples containing 0.4ppm, ten times the amount of oil concentration than the 0.04ppm samples.

The band intensity ratios of *Vc*, *Vp*, and *Vv* had similar trends in the five experiments. *Vp* was present in the 54 samples, *Vv* was present in 14 samples and *Vc* was present in seven samples. There was a noticeable presence of *Vv* and *Vc* in the last three experiments. The presence of these two species is most likely attributed to warmer temperatures when the sample seawater was collected for those three experiments. As stated earlier, the abundance of *Vibrio* spp. is higher when temperatures are warmer (Martinez-Urtaza et al., 2010). Finally, in all the

samples, *Vp* had a higher intensity than *Vc* and *Vv*. This finding shows that *Vp* had a higher dominance compared to *Vc* and *Vv* in the samples. When *Vp* had a lower intensity ratio is was because of a higher diversity. The more diverse the samples were, the lower the intensity of *Vp* the higher the intensity for *Vc* and *Vv*.

There were a few factors that could have influenced the results of this project. The first factor was digitizing the DGGE bands using ImageJ software. The digitization method used for this project can have its advantages and disadvantages. One advantage is the ability to quantify bands to have a numerical value of each species from DGGE even if the bands aren't visible. Quantifying the results using the digitization method is a better alternative than visual observation. However, to have accurate digitization results, the DNA extraction method needs to be as flawless as possible. Zhang and Fang (2000) stated that if the DNA was not lysed properly during the bead beating process then the amplification of the PCR products on the DGGE gel would not be at its optimal level. The PCR step is a crucial step as well. There is a risk when performing multiple experiments at different times because some DNA templates in one experiment could have a better amplified PCR product than DNA templates from another experiment (Zhang & Fang, 2000).

The second factor was the oil concentrations used for each experiment. Zhou et al. (2009) assessed changes within a microbial community in sediment samples contaminated with polyaromatic hydrocarbons (PAH) using DGGE analysis. The DGGE bands were analyzed and the species richness was calculated for each sample. It was determined that the concentration of exposure of the bacterial community with PAH lowered the community diversity. Even concentrations as low as 60ppm were toxic to the microbial community. However, if oil concentrations are low enough (less than 10ppm), oil can be used as a carbon source (Zhou et al.

2009). If oil can be used as a carbon source for the bacteria if the concentrations are low enough, it could explain why experiment five had an increase in diversity.

Finally, the last factor was the incubation period. In addition, Zhou et al. (2009) determined that a microbial community's diversity decreased when there is a longer exposure time with PAH. The lack of positive or negative trends present in three experiments could be due in part to an incubation time that was not long enough for change to occur.

3.4.2 Future Research

In future experiments of analyzing the vibrio community with various oil concentrations, it would be beneficial to use a quantification PCR method (QPCR) as described by Thompson et al. (2004). QPCR would be a way to quantify the bacteria before an experiment was performed so there could be a comparison of quantification before and after an experiment. Similarly, it would be beneficial to identify all species found in the samples. This project was limited due to only three *Vibrio* strains. However, the three *Vibrio* strains used for the DGGE ladder are very important vibrios to study. The three vibrios are foodborne pathogens and, as stated previously, are the main pathogenic vibrios that cause illness in people.

In addition, based off of the findings of Zhou et al. (2009), an increase in oil concentrations in each experiment to 60ppm would give better insight into the toxicity of oil on microbial communities. However, if one were to study the benefits of oil for biodegraders, the oil concentrations in each experiment should be less than 60ppm (Zhou et al., 2009). The microbial community's exposure with oil could be longer duration instead of 24 hours. Finally, the research of Hamdan and Fulmer (2011) as discussed in the TTSS project showed the high interaction between *Vibrio* spp. with one of the dispersants used in the *Deepwater Horizon*

incident. Adding dispersants to the experiment would give an interesting insight in the dominance or hindrance of certain vibrios.

CHAPTER 4. CONCLUSION

There are many unanswered questions regarding the pathogenicity of *Vp*. There are many factors, known and unknown, that can cause *Vp* to possess pathogenic traits. It has been proven that the *tdh* and *trh* hemolysin genes are contributors to the virulence of *Vp*. However, studies have proven that *tdh* and *trh* are not the only virulence factors. *Vp* isolates that lack *tdh* and *trh* have proven to be pathogenic as well (Mahoney et al., 2010). It is commonly known that temperature and salinity are driving factors in pathogenicity (Martinez-Urtaza et al., 2010). Discovering any other possible environmental factors that could cause pathogenicity in *Vp* is very important to determine from a human health perspective. Studying TTSS2, a trait found in pathogenic *Vp*, in isolates collected from the Gulf of Mexico during the *Deepwater Horizon* oil spill is a novel approach to determining pathogenicity. The TTSS study showed that there was not a statistical significance in the presence of TTSS2 after the oil spill. Having statistically significant results was not the intent of the study. The study gave an original approach to studying the pathogenicity of *Vp*.

In addition to the study of *Vp* pathogenicity, vibrio community changes in association with oil is another interesting perspective. If oil interaction with the vibrio community changed to where the most dominating species in samples were the most common vibrio foodborne species (*Vc*, *Vp*, and *Vc*), it would be a cause for human health concern. The importance of the vibrio community study was to see if such a change occurred. The results from the vibrio community study presented significant diversity results in two out of five experiments. Studying the vibrio community when interacting with oil is just as pertinent as the study of *Vp* pathogenicity when interacting with oil due to the lack of studies published. Even though the

results did not show a consistent trend in all five experiments of the study, it was an innovative evaluation of the vibrio community and various oil concentrations.

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APPENDIX A: DIVERSITY INDEX DATA

Table A.1: Summary of the diversity indices calculated from the samples in experiment one.

Experiment 1	Shannon (H)	Simpson Index (D)	Evenness (E)	Richness (R)
Control 1	2.249	0.110	0.977	10
Control 2	2.124	0.128	0.967	9
Control 3	2.162	0.119	0.984	9
0.02ppm 1	2.213	0.118	0.961	10
0.02ppm 2	2.216	0.130	0.924	11
0.02ppm 3	2.169	0.125	0.942	10
0.04ppm 1	1.882	0.162	0.967	7
0.04ppm 2	1.818	0.183	0.934	7
0.04ppm 3	1.415	0.127	0.614	10
0.4ppm 1	1.674	0.210	0.934	6
0.4ppm 2	2.146	0.123	0.977	9
0.4ppm 3	1.867	0.167	0.960	7

Table A.2: Summary of the diversity indices calculated from the samples in experiment two.

Experiment 2	Shannon (H)	Simpson Index (D)	Evenness (E)	Richness (R)
Control 1	2.591	0.085	0.957	15
Control 2	2.067	0.141	0.941	9
Control 3	2.303	0.109	0.960	11
0.02ppm 1	2.170	0.128	0.943	10
0.02ppm 2	2.811	0.151	0.941	14
0.02ppm 3	2.528	0.144	0.943	12
0.04ppm 1	2.304	0.116	0.927	12
0.04ppm 2	2.265	0.124	0.912	12
0.04ppm 3	2.454	0.112	0.906	15
0.4ppm 1	2.406	0.103	0.938	13
0.4ppm 2	2.359	0.112	0.920	13
0.4ppm 3	2.212	0.128	0.890	12

Table A.3: Summary of the diversity indices calculated from the samples in experiment three.

Experiment 3	Shannon (H)	Simpson Index (D)	Evenness (E)	Richness (R)
Control 1	2.695	0.072	0.972	16
Control 2	2.004	0.143	0.964	8
Control 3	2.019	0.152	0.919	9
0.02ppm 1	2.461	0.094	0.960	13
0.02ppm 2	2.117	0.130	0.964	9
0.02ppm 3	1.642	0.267	0.790	8
0.04ppm 1	1.834	0.214	0.835	9
0.04ppm 2	1.453	0.258	0.903	5
0.04ppm 3	1.667	0.239	0.802	8
0.4ppm 1	2.324	0.111	0.935	12
0.4ppm 2	1.699	0.205	0.873	7
0.4ppm 3	2.318	0.106	0.967	11

Table A.4: Summary of the diversity indices calculated from the samples in experiment four.

Experiment 4	Shannon (H)	Simpson Index (D)	Evenness (E)	Richness (R)
Control 1	2.631	0.089	0.929	17
Control 2	2.386	0.121	0.881	15
Control 3	2.786	0.072	0.946	19
0.02ppm 1	2.472	0.106	0.913	15
0.02ppm 2	2.859	0.067	0.939	21
0.02ppm 3	2.460	0.094	0.959	13
0.04ppm 1	1.656	0.214	0.924	6
0.04ppm 2	2.094	0.157	0.873	11
0.04ppm 3	2.150	0.143	0.896	11
0.4ppm 1	2.698	0.076	0.952	17
0.4ppm 2	2.338	0.122	0.886	14
0.4ppm 3	2.558	0.109	0.885	18

Table A.5: Summary of the diversity indices calculated from the samples in experiment five.

Experiment 5	Shannon (H)	Simpson Index (D)	Evenness (E)	Richness (R)
Control 1	2.326	0.105	0.970	11
Control 2	2.447	0.098	0.954	13
0.04ppm 1	2.781	0.076	0.928	20
0.04ppm 2	2.586	0.091	0.933	16
0.4ppm 1	2.565	0.091	0.947	15
0.4ppm 2	2.816	0.070	0.940	20

APPENDIX B: VIBRIO BAND INTENSITY DATA

Table B.1: Summary of the band intensities of *Vc*, *Vp*, and *Vv* from experiment one.

Experiment 1	***Vc***	***Vp***	***Vv***
Control 1	0.00	14.14	0.00
Control 2	0.00	17.85	0.00
Control 3	0.00	14.69	0.00
0.02ppm 1	0.00	15.51	0.00
0.02ppm 2	0.00	16.53	0.00
0.02ppm 3	0.00	19.63	0.00
0.04ppm 1	0.00	24.53	0.00
0.04ppm 2	0.00	30.02	0.00
0.04ppm 3	0.00	21.01	0.00
0.4ppm 1	0.00	32.17	0.00
0.4ppm 2	0.00	19.53	0.00
0.4ppm 3	0.00	23.86	0.00

Table B.2: Summary of the band intensities of *Vc*, *Vp*, and *Vv* from experiment two.

Experiment 2	***Vc***	***Vp***	***Vv***
Control 1	0.00	13.65	0.00
Control 2	0.00	20.04	0.00
Control 3	0.00	16.14	0.00
0.02ppm 1	0.00	21.61	0.00
0.02ppm 2	0.00	19.22	0.00
0.02ppm 3	0.00	18.53	0.00
0.04ppm 1	0.00	12.71	0.00
0.04ppm 2	0.00	22.36	0.00
0.04ppm 3	0.00	23.78	0.00
0.4ppm 1	0.00	17.31	0.00
0.4ppm 2	0.00	19.91	0.00
0.4ppm 3	0.00	21.00	0.00

Table B.3: Summary of the band intensities of *Vc*, *Vp*, and *Vv* from experiment three.

Experiment 3	***Vc***	***Vp***	***Vv***
Control 1	0.00	10.16	9.39
Control 2	0.00	11.78	0.00
Control 3	0.00	25.69	0.00
0.02ppm 1	0.00	16.14	0.00
0.02ppm 2	0.00	13.23	0.00
0.02ppm 3	0.00	4.32	4.18
0.04ppm 1	0.00	38.95	3.54
0.04ppm 2	0.00	24.07	0.00
0.04ppm 3	0.00	38.16	0.00
0.4ppm 1	0.00	16.30	2.89
0.4ppm 2	0.00	23.36	0.00
0.4ppm 3	4.14	16.50	8.44

Table B.4: Summary of the band intensities of *Vc*, *Vp*, and *Vv* from experiment four.

Experiment 4	***Vc***	***Vp***	***Vv***
Control 1	5.79	17.44	7.43
Control 2	4.89	22.42	11.21
Control 3	4.10	10.35	4.24
0.02ppm 1	4.39	19.68	7.27
0.02ppm 2	2.19	9.34	2.76
0.02ppm 3	0.00	8.80	0.00
0.04ppm 1	0.00	29.85	10.83
0.04ppm 2	3.90	13.50	3.52
0.04ppm 3	0.00	17.60	3.84
0.4ppm 1	0.00	11.10	5.25
0.4ppm 2	0.00	20.66	5.20
0.4ppm 3	0.00	11.60	1.87

Table B.5: Summary of the band intensities of *Vc*, *Vp*, and *Vv* from experiment five.

Experiment 5	***Vc***	***Vp***	***Vv***
Control 1	0.00	13.57	0.00
Control 2	0.00	14.37	0.00
0.04ppm 1	0.00	10.64	0.00
0.04ppm 2	0.00	10.02	1.58
0.4ppm 1	0.00	9.42	0.00
0.4ppm 2	0.00	7.49	0.00

VITA

Erica Leigh Stephens was born on September 22nd in Gainesville, Georgia. She is the daughter of Jean Hodges and Reno Simmons of Greenville, South Carolina, and has four siblings. In May of 2004, she graduated from Hillcrest High School in Simpsonville, South Carolina. In 2009, she received a Bachelor of Science degree in biological science with a minor in environmental studies from the University of South Carolina. During her undergraduate tenure, she was a laboratory intern at the Greenville Water System in Greenville, South Carolina and a research participant through the Oak Ridge Institute for Science and Education (ORISE) at Fort Jackson, South Carolina. Her internships consisted of studying various environmental factors, both in the laboratory and field setting. In January of 2010, she was offered a graduate research assistantship by the Department of Environmental Sciences, Louisiana State University, to work in Dr. Crystal Johnson's environmental microbiology laboratory. She plans to use the knowledge gained in her graduate course work and practical experience in Dr. Johnson's laboratory to become a successful scientist.