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Assessment of the human pathogen *Vibrio vulnificus* in Louisiana Eastern oysters *Crassostrea virginica* harvested from Barataria Bay at different salinities with subsequent depuration treatment

by

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Undergraduate honors thesis under the direction of

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Abstract

Vibrio vulnificus is a highly virulent human pathogen commonly found in oysters that is responsible for many deaths related to seafood consumption. The Food and Drug Administration (FDA) has issued protocols to rid oysters of this bacterium, one being the process of depuration. The number of *V. vulnificus* in oysters, with and without subsequent depuration treatment, as well as in the surrounding estuarine environments was detected at two locations, Hackberry Bay and Grand Isle, within Barataria Bay estuary that differed in salinity using colony hybridization and quantitative PCR techniques. The microbial flora that inhabits oysters, sediment, and seawater was also investigated. Colony hybridization identified 90% of microorganisms grown on *Vibrio* species-specific agar to be *V. vulnificus.* Quantitative PCR identified a 10,000-fold higher total *V. vulnificus* abundance in oysters and seawater when compared to colony hybridization. Furthermore, oysters that were treated with depuration saw a 10-fold decrease in microorganism growth. Finally, salinity did not greatly affect the genera of microorganisms that inhabit oysters, sediment, and seawater as genera such as *Bacillus* and *Pseudomonas* were identified in the multiple environments from both Hackberry Bay and Grand Isle. These findings may further the understanding of the conditions under which *V. vulnificus* can reach harmful concentrations.

Introduction

Shellfish have been recognized as a very important source of food and a valuable economic export for centuries. Shellfish have also been recognized as a cause of viral and bacterial infections for over 100 years, with one of the first outbreaks reported in 1816 [5]. Since the 19th century, over 14,000 cases of infection due to ingestion of shellfish have been reported in the United States alone [23]. The majority of these cases have been linked to oysters, followed by clams and mussels, with Hepatitis A virus leading in shellfish-associated viral infections and *Vibrio* species leading in bacterial infections [21].

Two major species of oysters are produced in the United States: *Crassostrea virginica* and *C. gigas* [11]. *Crassostrea virginica* is an oyster found along the Atlantic and Gulf of Mexico. Approximately 75 million to 80 million servings of these raw

oysters are consumed annually in the United States with Louisiana being the top producer of oysters in the nation [2, 8, 17]. *C. virginica* has been found to house a number of bacterial genera including *Pseudomonas, Flavobacterium, Micrococcus*, *Aeromonas*, and *Vibrio* [4], the most harmful being the human pathogen *Vibrio vulnificus*.

V. vulnificus is a Gram-negative, curve-shaped bacterium with a fast generation time of approximately 15 minutes [1]. This bacterium is a halophile with previously observed optimal salinity ranges at both low salinity values and high values. A study by Randa et al found the optimal salinity of the bacterium to be from 5 parts per thousand (ppt) to 10 ppt. Another study found high values of *V. vulnificus* at higher salinity ranges of 25 ppt to 27 ppt [6, 15, 22]. *V. vulnificus* can be found in and on a variety of marine animal environments including the digestive tract and skin surface of plankton, flatfish, jackmackerel, salmonids, larval and juvenile sea bream, blue crabs, shrimps, and oysters. *V. vulnificus* can also be found in temperate coastal and estuarine waters [14, 17, 19, 20].

V. vulnificus has been identified as the leading cause of death related to seafood consumption in the United States [2, 7, 17]. The consumption of any contaminated seafood as well as exposure to *V. vulnificus* via an open wound may have serious health effects on humans. *V. vulnificus* infections can cause gastroenteritis and primary septicemia. Wound infections caused by *V. vulnificus* can result in necrosis due to the bacterium's production of many endotoxins [10, 12, 13, 18]. Individuals with underlying diseases such as chronic liver disease, alcoholism, and diabetes, along with any immunocompromised individuals are at a much higher risk of *V. vulnificus* infections [17].

The U.S. reported 113 cases of *V. vulnificus* infection between 2000 and 2003. Ninety-six percent of those cases involved the consumption of raw oysters [17]. Oysters are filter feeds and as a result contain many bacteria including *V. vulnificus*. A widely used method to rid the oysters of this pathogenic bacterium is called depuration. Depuration is a process of placing harvested oysters in tanks with high quality cleaned water. The oysters are fed alga and kept in the tanks for at least one week to naturally purge themselves of any harmful bacteria. Bacteria such as *E. coli* and *Salmonella* are relatively easily removed with this treatment; however, depuration has not proven to be

as effective for *Vibrio* species. There are concerns that at an optimal salinity and temperature, depuration may actually increase the concentrations of these bacteria [9].

Along with depuration, the Food and Drug Administration (FDA) has implemented a protocol to detect and monitor the number of *V. vulnificus* within oysters. The FDA's *Bacteriological Analytical Manual* recommends a direct plate method of the bacterium along with molecular techniques of detecting one of *V. vulnificus*'s virulence factors, *vvhA*. The *vvhA* gene is unique to *V. vulnificus* and encodes a hemolytic protein specific to *V. vulnificus* and present only once in its genome. *V. vulnificus* is enumerated by directly plating homogenized oysters onto *V. vulnificus* agar (VVA) plates. Colony lifts are performed on countable plates and an alkaline phosphatase-labeled oligonucleotide probe is used to quantify *V. vulnificus* for the *vvh* gene [24, 25, 26].

In this study, we view the culturable diversity of microorganisms within oysters, sediment, and seawater from two sites within the Barataria Bay estuary during the month of July. We also examine the abundance of *V. vulnificus* in differing salinities through a direct plating method and through a quantification method. Finally, we investigate the role of depuration in decreasing total bacterial counts and specifically *V. vulnificus* counts in *C. virginica.*

Method Overview

Sample collection:

Two locations, Hackberry Bay and Grand Isle, were chosen in Barataria estuary based on their differences in salinity. Temperature and salinity were determined at both locations using the YSI 85 meter (Fischer Scientific). All sample collection was done on July 15, 2013. Sixteen oysters were collected from each site and placed in burlap bags and stored at room temperature. Sediment samples were collected using a Ponar grab dredge (Wildco); approximately 50g was sampled twice from each site. Six liters of seawater was collected from both sites and wrapped in bubble wrap then placed in ice chests according to the Environmental Protection Agency protocol for water sampling found in the Volunteer Estuary Monitoring Manual. Samples were transported back to the lab approximately 7 hours post collection.

Upon arrival at the lab, oysters were scrubbed and washed to remove residual debris. Eight oysters from each site were placed in depuration tanks that matched the salinity from their respective locations. Three oysters (2.47g – 13.63g) from each site were individually shucked and homogenized (BIOSPEC PRODUCTS INC.) with equal volumes of 1x PBS. Five grams of sediment from each site was vortexed with equal volumes of 1x PBS, performed in triplicates. One hundred milliliters (mL) of seawater from each site was filtered onto a 0.2μm polycarbonate filter in triplicates 27 hours post collection. The filter was cut in half and placed in 5mL of 1x PBS. Figure 1 provides an overview of the sampling and following protocols.

Direct plating on VVA and 10% R2A:

Oyster homogenates were serially diluted and plated on both *Vibrio vulnificus* agar (VVA) and 10% R2A. VVA is selective for *Vibrio* species due to the presence of cellobiose, which can be fermented by *Vibrio* species, and 10% R2A is a low nutrient media. The sediment slush was vortexed for 30 seconds to resuspend bacteria then serially diluted and plated on VVA and 10% R2A. The ½ seawater filter was vortexed for 30 seconds, serially diluted, and plated on VVA and 10% R2A. Both VVA and 10% R2A plates were incubated at 30°C for 2-4 days.

I. Culturable Microorganism Abundance and Diversity

Isolation and identification of prokaryotic organisms from samples:

Colonies that grew on the 10% R2A plates from oyster, sediment, and seawater samples were randomly chosen and transferred to 10% R2A liquid medium. A total of 118 colonies were grown in liquid 10% R2A at 30°C until turbid (overnight – 1 week). DNA extraction was performed using Sigma GeneElute kit according to manufacturer's protocol.

PCR was performed for the 16S rRNA gene using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reaction: hot start Jumpstart*Taq*^{TM} DNA polymerase (2.5U), 10x PCR buffer, 27F and 1492R (20μm each), DNA (range 4ng – 300ng) and deoxyribonucleotides (dNTPs, 200μm). Thermocycler conditions were as follows: (a) 95°C for three minutes, (b) 95°C for forty-five seconds, (c) 51°C for thirty seconds, (d) 72°C for forty-five seconds, and a

final extension step (e) 72°C for five minutes. The cycle (b-d) was repeated thirty times. PCR products were run on 1% agarose gel. Bands corresponding to 1.5kb were cut out and cleaned using MP Bio GeneClean Spin Kit. PCR products were then sequenced using BigDye sequencing kit. The nucleotide sequences were inputted into the Standard Nucleotide Basic Local Alignment Search Tool (BLAST) to compare the genomic context with known organisms. Matches of 95% and above were used to determine the genus.

II. Colony Hybridization

Colony hybridization for *Vibrio vulnificus*:

Colony hybridization was performed on the countable VVA plates from oyster homogenates, sediment, and seawater according to the FDA protocol. VVA plates were lifted after 48 hours of growth using Whatman Nytran and Amersham Hybond –N^{+} nylon membranes. The membranes were analyzed for presence of *vvh* using a *vvh*-specific alkaline phosphatase probe (DNA Technology) as previously described [24].

III. Quantitative PCR of *vvh* gene

Genomic DNA isolation from samples:

Genomic DNA extractions were performed using PowerSoil (MO Bio) by adding 250µL of oyster homogenate and 200µL of sediment slush to the PowerSoil extraction tubes. For the seawater samples, the second half of the filter was cut into thirds and each third was placed into a separate extraction tube. All extractions were performed in triplicates, and the genomic DNA was stored at -20°C.

Quantification of *vvh* gene through qPCR:

The extracted genomic DNA of each sample was used for SYBR Green based quantitative PCR of the *vvhA* gene. The qPCR was performed using primers Vvh-334F (5'-TTCCAACTTCAAACCGAACTATGA-3') and Vvh-533R (5'-ATTCCAGTCGATGCGAATACGTTG-3'). A standard curve was generated to calculate the quantity of the *vvh* gene by comparing known Ct values of *V. vulnificus* genomic DNA to unknown samples.

IV. Depuration Treatment of *C. virginica*

Depurated oyster treatment:

After the sample collection from Hackberry Bay and Grand Isle, eight oysters were randomly chosen from each site, scrubbed with water, and placed in depuration tanks that mimicked the salinity of each site for one week. Three oysters from each site were then randomly chosen for plating and DNA extraction. The homogenization process, DNA extraction, serial dilutions, plating on to VVA and 10% R2A, and colony hybridization processes for the depurated oysters all involved the same protocols used for the non-depurated oysters. These plates were placed in a $30^{o}C$ incubator. The VVA plates were counted 48 hours later and then underwent colony hybridization. The 10% R2A plates were counted 96 hours after plating due to slow microbial growth, and the colonies were isolated, placed in 100% R2A media in a $30^{o}C$ incubator until turbid, and sequenced.

Statistical tests to evaluate variance:

Statistical analysis tests were used to determine variance between all values. Both the F Test and T Test for variance were used. The significance or alpha level was set to 0.05.

Results

Environmental parameters:

Gulf of Mexico water temperatures between the two sites were $29^{o}C$ degrees for both sites. Salinities showed ranges of 10.5ppt at Hackberry Bay and 20ppt at Grand Isle. The meter's accuracy range was ± 0.1ppt.

I. Culturable Microorganism Abundance and Diversity

A. Culturable microorganisms on 10% R2A

Hackberry Bay:

Culturable microorganisms on 10% R2A were found in all three samples: oysters, sediment, and seawater. Oysters from Hackberry Bay were found to contain significantly more culturable microbes than sediment ($p < 0.03$) (Figure 2). Hackberry Bay oysters

contained an average of $2.85 \pm 1.43 \times 10^5$ cfu/g, n=7. Hackberry Bay sediment was found to house approximately $1.23 \pm 0.51 \times 10^5$ cfu/g, n=6 and seawater approximately $6.93 \pm 3.47 \times 10^4$ cfu/mL, n=7.

Grand Isle:

Overall, less culturable microorganisms were found in samples from Grand Isle. Oysters from Grand Isle contained approximately $6.8 \pm 2.3 \times 10^3$ cfu/g, n=7 of cultural microbes (Figure 2). Within the Grand Isle sediment, approximately $8.96 \pm 2.89 \times 10^4$ cfu/g, n=5 of culturable microbes were found, and within the seawater, approximately $5.43 \pm 4.96 \times 10^3$ cfu/mL, n=6. Between these environments within Grand Isle, the highest culturable colony counts were found in sediment. This value was statistically different from oysters ($p < 0.004$).

Hackberry Bay v. Grand Isle:

Comparing the amount of microorganisms cultured from Hackberry Bay and Grand Isle, counts in oysters and seawater were found to be statistically different between the two sites ($p < 0.003$). Hackberry Bay values for both oysters and seawater were higher than those values from Grand Isle.

B. Culturable microorganisms on VVA

Hackberry Bay:

Oysters contained the most culturable microbes on VVA medium from both Hackberry Bay and Grand Isle. Oysters from Hackberry Bay housed an average of $2.88 \pm 0.88 \times 10^5$ cfu/g, n=8 (Figure 3). Hackberry Bay sediment contained an average of $8.23 \pm 7.18 \times 10^4$ cfu/g, n=9, and the seawater an average value of $2.37 \pm 1.04 \times 10^4$ cfu/mL, n=9. The numbers of culturable microorganisms found within Hackberry Bay oysters were found to be statistically different from sediment ($p < 0.0002$).

Grand Isle:

The Grand Isle oysters also showed the most culturable microorganisms as compared to the sediment and seawater from the same site (Figure 3). Grand Isle oysters contained an average of 3.29×10^5 cfu/g, n=2. Sediment samples contained $9.47 \pm 3.88 \times 10^4$ cfu/g, n=9, and seawater contained $2.30 \pm 2.76 \times 10^3$ cfu/mL, n=9.

Hackberry Bay v. Grand Isle:

The number of cultuable organisms found in samples from Hackberry Bay and Grand Isle were compared. A statistical difference was not found between the oysters ($p > 0.6$) or sediment ($p > 0.6$) from both sites; however there was a significant difference in seawater samples ($p < 0.0003$) with Hackberry Bay having higher values.

C. Isolated colonies from 10% R2A:

Several genera were found from isolates of the 10% R2A medium from oysters, sediment, and seawater samples. Within the Hackberry Bay site, thirty-three isolates were examined between the three environments (Table 1). Within the oysters, *Aeromonas*, *Bacillus*, *Elizabethingia*, *Klebsiella*, and *Pseudomonas* genera were found. Within sediment, only the *Bacillus* genus was found and within seawater, *Acinetobacter*, *Aeromonas*, *Bacillus*, *Cronobacter*, *Pseudoalteromonas*, *Pseudomonas*, and *Rhizobium* genera were found. In addition, twenty-nine isolates were examined between the three environments of Grand Isle (Table 2). Within oysters, *Aeromonas*, *Bacillus*, *Denitromonas*, *Elizabethkingia*, *Pseudomonas*, *Rhodococcus*, *Shewanella*, *Sphingopyxis*, and *Streptomyces* genera were found. Within sediment, *Bacillus* and *Pseudomonas* genera were found, and within seawater, *Bacillus*, *Brevibacillus*, and *Pseudomonas* genera were found. Within these sixty-two isolates, the *Bacillus* genus was the most common found in all three environments from both Hackberry Bay and Grand Isle. The *Pseudomonas* genus was the next common, found in five of the six environments. Overall, multiple genera were isolated from each environment with similar findings between the three environments as well as between the two sites.

<u>II. Colony Hybridization</u>

Hackberry Bay:

Oyster, sediment, and seawater environments from both Hackberry Bay and Grand Isle were found to house *Vibrio vulnificus*, but with very little statistical difference. The culturable colony counts of *V. vulnificus* in oysters housed an average of $9.67 \pm 5.99 \times 10^3$ cfu/g, n=6 (Figure 4). An average of $1.29 \pm 1.30 \times 10^4$ cfu/g, n=7 in sediment samples and an average of $4.76 \pm 4.57 \times 10^4$ cfu/mL, n=8 in seawater samples

were found. There was not a statistical difference between oyster and sediment counts ($p > 0.5$).

Grand Isle:

The samples collected from Grand Isle were more difficult to compare due to uncountable plates. Oysters from Grand Isle contained 3.8×10^4 cfu/g, n=1 (Figure 4). A standard deviation was unable to be calculated due to the few countable plates. The average amount of *V. vulnificus* found in sediment was $1.04 \pm 0.64 \times 10^4$ cfu/g, n=9, and the average amount found in seawater was much lower at $7.88 \pm 5.98 \times 10^2$ cfu/mL, n=5.

Hackberry Bay v. Grand Isle:

Although the culturable colony counts of *V. vulnificus* from oysters of Grand Isle do not elicit comparable data, those counts from sediment of Hackberry Bay and Grand Isle were not found to be statistically different ($p > 0.6$) while those counts from seawater of the two sites were found to be statistically different ($p < 0.02$). Seawater from Hackberry Bay was found to house more *V. vulnificus* than seawater from Grand Isle.

<u>III. Quantification of *vvh* gene</u>

Hackberry Bay:

For all three samples at both Hackberry Bay and Grand Isle sites, *V. vulnificus* values found through qPCR were higher than those values determined from the colony hybridization. The oysters housed *V. vulnificus* averages of $1.54 \pm 0.86 \times 10^8$ cfu/g, n=9 from Hackberry Bay (Figure 5). The sediment samples from Hackberry Bay contained an average of $2.41 \pm 1.84 \times 10^8$ cfu/g, n=8, not significantly different from the oyster values. The seawater samples contained lower averages at $1.55 \pm 1.22 \times 10^7$ cfu/mL, n=8. Within Hackberry Bay, a 4-log difference between qPCR values and colony hybridization values of *V. vulnificus* were seen in the oysters and sediment samples, while a 3-log difference was seen in seawater samples.

Grand Isle:

Within the oysters, *V. vulnificus* averages were $3.38 \pm 3.40 \times 10^8$ cfu/g, n=8 from Grand Isle (Figure 5). Sediment *V. vulnificus* averages from Grand Isle contained $8.89 \pm 5.23 \times 10^7$ cfu/g, n=9. Seawater averages contained $4.05 \pm 3.94 \times 10^6$ cfu/mL, n=9. A 4-log difference between these qPCR values and the colony hybridization values of *V.*

vulnificus were seen in both the oysters and seawater values. A 3-log difference was seen in sediment values.

Hackberry Bay v. Grand Isle:

A statistical difference was not seen between the Hackberry Bay and Grand Isle oyster or sediment *V. vulnificus* values found through qPCR. A significant difference was seen in the seawater values of *V. vulnificus* between the two sites ($p < 0.04$) with Hackberry Bay having higher values than Grand Isle.

<u>**IV. Depuration Treatment of *C. virginica***</u>

A. Culturable microorganisms on 10% R2A post-depuration

Hackberry Bay:

The depurated oysters showed culturable colonies on 10% R2A medium and VVA medium at both Hackberry Bay and Grand Isle. Culturable microbes from the depurated oysters at Hackberry Bay were found at averages of $1.27 \pm 1.38 \times 10^4$ cfu/g, n=9 on 10% R2A and $2.96 \pm 3.64 \times 10^4$ cfu/g on VVA, n=5 (Figure 6). A statistical difference was not found between the depurated oyster values on 10% R2A or VVA of Hackberry Bay ($p > 0.3$).

Grand Isle:

Culturable microbes from the depurated oysters at Grand Isle were found to house $4.52 \pm 1.35 \times 10^3$ cfu/g, n=9 on 10% R2A and $4.93 \pm 0.23 \times 10^4$ cfu/g, n=3 on VVA (Figure 6). A statistical difference was found between the depurated oysters from Grand Isle on the two mediums ($p < 1 \times 10^{-12}$). The colony counts from Grand Isle were found to be higher on VVA medium rather than 10% R2A medium

Hackberry Bay v. Grand Isle:

A statistical difference was not found between the depurated oyster culturable colony counts on 10% R2A from Hackberry Bay and Grand Isle ($p > 0.1$) or between the counts on VVA from the two sites ($p > 0.2$). The quantity of culturable colonies did decrease with depuration treatment. The culturable colonies from the 10% R2A medium decreased by ten-fold when compared to the non-depurated oyster culturable colonies for both Hackberry Bay and Grand Isle ($p < 0.003$ and $p < 0.03$). Hackberry Bay culturable microbes on VVA also saw a ten-fold decrease with the treatment ($p < 0.0002$).

B. Culturable *Vibrio vulnificus* found through colony hybridization post-depuration

Culturable *V. vulnificus* found through colony hybridization saw a ten-fold decrease after the depuration treatment for both Hackberry Bay and Grand Isle sites (Figure 7) when compared to non-depurated oyster colony hybridization values. Depurated oysters from Hackberry Bay contained an average of $1.43 \pm 0.61 \times 10^3$ cfu/g, n=8 *V. vulnificus*, and those from Grand Isle contained an average of $2.89 \pm 2.66 \times 10^3$ cfu/g, n=9. A statistical difference was not observed between the two sites ($p > 0.1$).

C. Quantification of *vvh* gene through qPCR post-depuration

Hackberry Bay:

The depurated oysters contained an average of $2.05 \pm 2.17 \pm 10^7$ cfu/g, n=8, and were found to be statistically different from the non-depurated oysters ($p < 0.002$) with previously stated values of $1.54 \pm 0.86 \times 10^8$ cfu/g, n=9 (Figure 8). Within Hackberry Bay, a 4-log increase of *V. vulnificus* values was seen in qPCR when compared to colony hybridization values of the depurated oysters.

Grand Isle:

Depurated oysters from Grand Isle housed an average of $3.36 \pm 3.63 \times 10^7$ cfu/g, n=8, significantly less than non-depurated oysters ($p < 0.04$) with previously stated values of $3.38 \pm 3.40 \times 10^8$ cfu/g, n=8 (Figure 8). A 5-log increase was seen in qPCR values when compared to colony hybridization values of the depurated oysters.

Hackberry Bay v. Grand Isle:

A statistical difference was not seen between the depurated oyster *V. vulnificus* values between Hackberry Bay and Grand Isle.

Discussion

The goals of this study were to view culturable diversity of microorganisms within oysters, sediment, and seawater from two sites differing in salinity within the Barataria Bay estuary. In addition, *V. vulnificus* abundance in differing salinities was examined, and the role of depuration in decreasing total bacterial counts and *V. vulnificus* counts in oysters was investigated. Viewing the abundance as well as the diversity of

microbes within oysters, sediment, and seawater samples of high and low salinities enables an assessment of how *V. vulnificus* is cycled in the environment and inhabit oysters.

The high yield of culturable microbes on 10% R2A found in the oysters from both Hackberry Bay and Grand Isle provide evidence that 10% R2A is more suitable for diverse microorganism growth versus VVA. VVA is a selective media for *Vibrio* species, while 10% R2A is a non-selective media. The results also suggest that more culturable organisms exist within the oyster instead of the sediment and seawater. Previous studies support this result through findings of an association with gut bacteria and aquatic invertebrates whether by ingestion, parasitism, incubation, etc. This data could represent a mutualism effect occurring between the oysters and bacteria. The most common genera of gut bacteria include *Vibrio, Pseudomonas, Flavobacterium, Micrococcus*, and *Aeromonas* [4].

In order to examine the diversity of microorganisms found in oysters, sediment, and seawater from the two sites, 10% R2A media was used. This medium was previously shown to culture a diverse range of marine microorganisms [3]. Within the experimental parameters used in this study, the majority of genera isolated were Gram-negative bacteria. In addition, there were similar findings in each of the environments. It would appear from these results that the differences in salinity do not greatly affect the types of microbes present. Genera including *Aeromonas*, *Bacillus*, *Elizabethkingia* and *Pseudomonas* were found in both Hackberry Bay and Grand Isle. This parallels a study by Colwell and Liston, which examined the normal flora in oysters. They observed a high degree of similarity between the areas observed. These observations may be explained by the method of food intake by oysters. Oysters are filter feeders, so the bacteria found in the seawater would also be found within the oyster. In addition, the oyster beds rested directly on the sediment, so both shared very similar environments, allowing for easy transmission of the bacteria between the oyster and sediment. Thus, it would be expected that the three environments within each site are inhabited by similar bacterial communities. Since our methods involved random selection of isolated colonies, these findings represent those culturable microorganisms that were most

numerous on the 10% R2A plates as opposed to total diversity. Therefore, these findings represent only a very small amount of what naturally inhabits these samples and sites.

While the 10% R2A media is considered non-selective, it does not have a high salt concentration, which may have inhibited *V. vulnificus* colony growth. The pathogen was identified on the VVA plates, but it was not isolated from the 10% R2A plates. The bacteria that were able to grow on 10% R2A may have created an environment where *V. vulnificus* is unable to compete.

A significant relationship between *V. vulnificus* and its preferred environment was not identified through colony hybridization. The only evident trends seen were in the Grand Isle environment. The lack of comparable data may be due to errors in plating technique, as triplicates were unable to be collected from each environmental parameter. However, another study was also unable to identity a significant trend of *V. vulnificus* in oysters in varying salinity. When salinity varied over a narrow range, there was no identifiable trend between salinity and *V. vulnificus*, whereas when salinity varied over a wider range (3ppt to 35ppt) a positive relationship with salinity was identified [6]. Nonetheless, it was concluded through colonly hybridization that 90% of the culturable microorganisms on the VVA plates were *V. vulnificus* colonies.

The purpose of the direct plating method was to examine the number of culturable *V. vulnificus* found within oysters. *V. vulnificus* can enter a state of viable but non-culturable (VBNC) in which the organisms will not grow on solid media. By selecting for the *vvhA* gene through the qPCR technique, total *V. vulnificus* including VBNC were examined. The VBNC state may be caused my many external parameters including low temperature, high salinity, and light stress. For *V. vulnificus* a temperature stress of less than 10°C may cause the bacterium to enter the VBNC state [16]. While the water temperature at the time of sampling was not a temperature that would elicit a stress response, a fluctuation in salinity was observed during the week before sampling. According to the United States Geological Survey for the National Water Information System, Barataria Bay salinity was recorded at 14ppt on July 8, 2013. Four days later, the salinity dropped to nearly 6ppt followed by increase to 10.5ppt just three days later. In addition, Barataria Pass located at the Grand Isle sampling site recorded salinities of 11ppt seven days before sampling with a steady increase in salinity to 20ppt recorded on

the day of sampling. This fluctuation in salinity may have caused the VBNC state of the bacterium observed in the discrepancies between the qPCR values and colony hybridization values of *V. vulnificus*.

Depuration treatment did have an effect on the total number of culturable microorganisms present on both types of media; however, it did not eliminate the presence of all microorganisms, including *V. Vulnificus*, in oysters. A study by the Food and Agriculture Organization discusses the limitations of depuration treatment stating that the treatment has been ineffective in reducing pathogenic *Vibrio* species. Alternative methods for reduction of *V. vulnificus* should be used for cleansing oysters before consumption. The safest way to consume oysters, though, is by fully cooking them before serving.

This study has permitted the observation of the quantities of *V. vulnificus* in addition to other microorganisms that naturally inhabit oysters, sediment, and seawater of estuaries that differ in salinity. Many previously reported findings were supported by this study including the high quantity of microbes in oysters as opposed to sediment or seawater, the different genera present in each environment, and the effects of depuration treatment. Future studies might expand the parameters of this experiment to include a wider range of salinities. Overall, it has been found that *V. vulnificus* does inhabit *C. virginica* and its surrounding environment found in the Gulf of Mexico. Expanding upon these experiments may improve the information currently known about this human pathogen and prevent further illnesses and fatalities related to seafood consumption.

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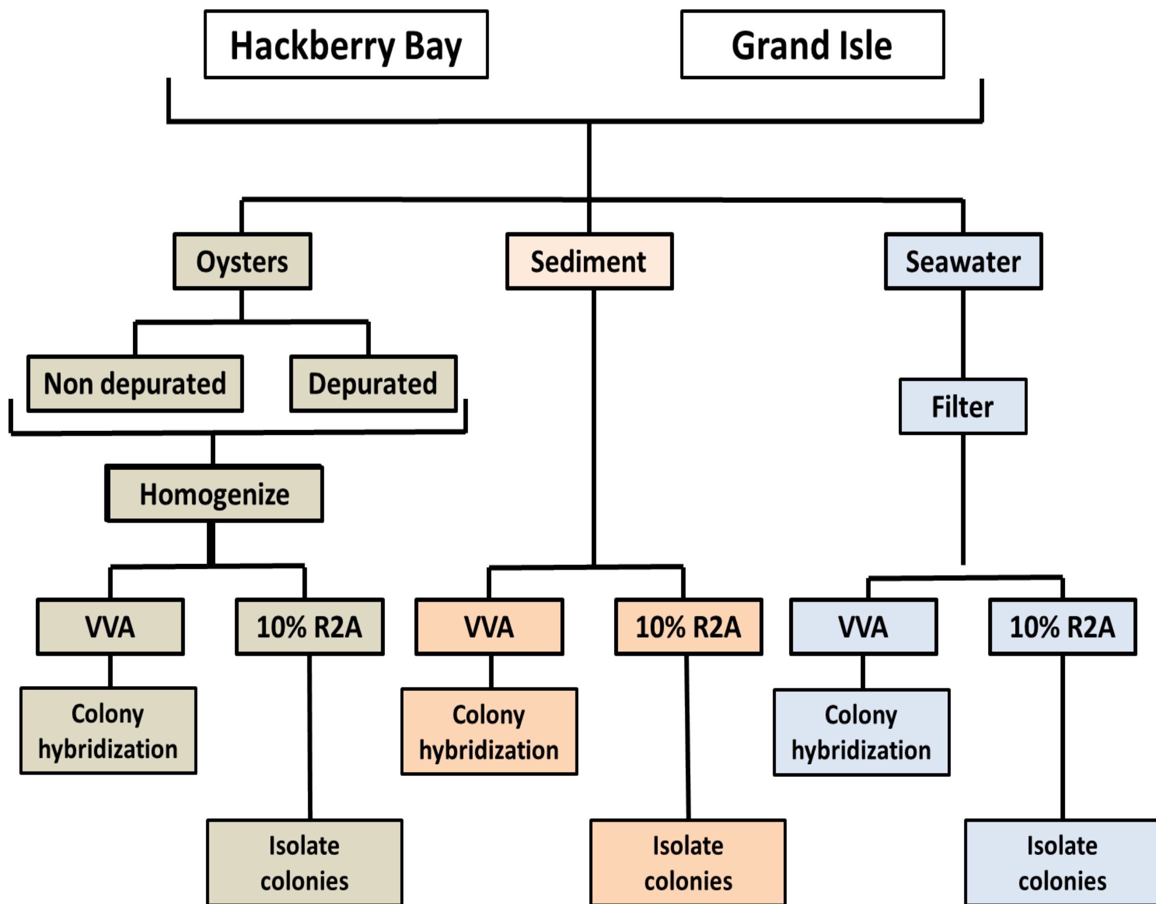

Figure 1. An overview of the experimental design. Hackberry Bay and Grand Isle were the two sites for sampling within the Barataria Bay estuary. Oyster, sediment, and seawater were sampled at each site. The collected oysters were divided into a non-depurated category that immediately underwent homogenization and a depurated category that underwent depuration treatment before homogenization. Oyster homogenates, sediment samples, and .2µm filtered seawater samples were plated on VVA and 10% R2A medium. The VVA plates were used for colony hybridization, selecting for *V. vulnificus*, and the 10% R2A plates were used to isolate colonies for sequencing. Each protocol was conducted in triplicates.

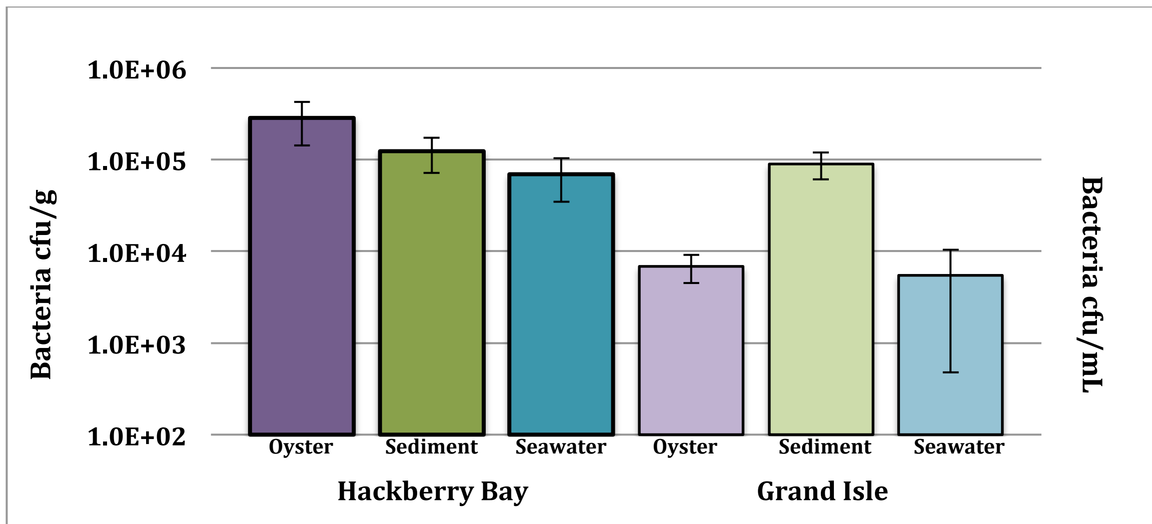

Figure 2. Total microbial counts on 10% R2A non-selective medium. Hackberry Bay (HB) oysters showed significantly higher values of culturable microbes than in sediment while Grand Isle (GI) sediment showed significantly higher values of culturable microbes than in oysters. Between the two sites, oyster and seawater values at HB were significantly higher than those values at GI. Error bars are from standard deviations of three studies in triplicates.

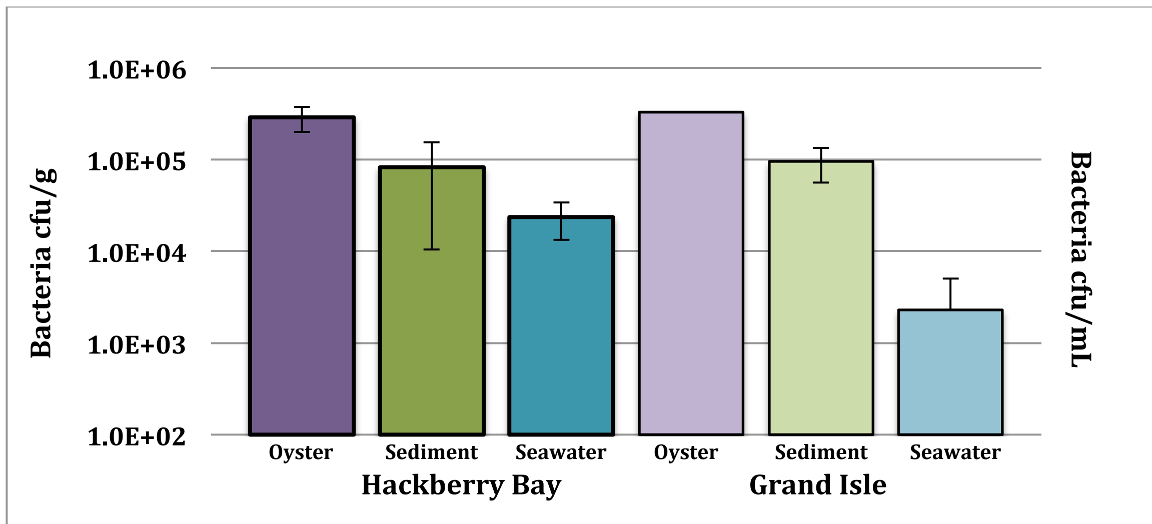

Figure 3. Total bacterial counts on VVA medium specific for *Vibrio* species. Hackberry Bay (HB) showed culturable microbes in oysters that were statistically higher than sediment values. A standard deviation was unable to be calculated for Grand Isle (GI) oysters, preventing further comparisons within that site. Between both sites, HB seawater values were significantly higher than seawater values at GI. Error bars are from standard deviations of three studies in triplicates.

Hackberry Bay	Genus
Oysters	*Aeromonas* (4) *Bacillus* (1) *Elizabethkingia* (1) *Klebsiella* (5) *Pseudomonas* (2)
Sediment	*Bacillus* (10)
Seawater	*Acinetobacter* (1) *Aeromonas* (1) *Bacillus* (2) *Cronobacter* (1) *Pseudoalteromonas* (2) *Pseudomonas* (2) *Rhizobium* (1)

Table 1. Culturable microorganisms isolated from Hackberry Bay on 10% R2A medium. Thirty-three isolates were sequenced and their genus determined using BLAST.

Grand Isle	Genus
Oysters	*Aeromonas* (2) *Bacillus* (2) *Denitromonas* (1) *Elizabethkingia* (1) *Pseudomonas* (7) *Rhodococcus* (2) *Shewanella* (1) *Sphingopyxis* (1) *Streptomyces* (1)
Sediment	*Bacillus* (3) *Pseudomonas* (1)
Seawater	*Bacillus* (5) *Brevibacillus* (1) *Pseudomonas* (1)

Table 2. Culturable microorganisms isolated from Grand Isle on 10% R2A medium. Twenty-nine isolates were sequenced and their genus determined using BLAST.

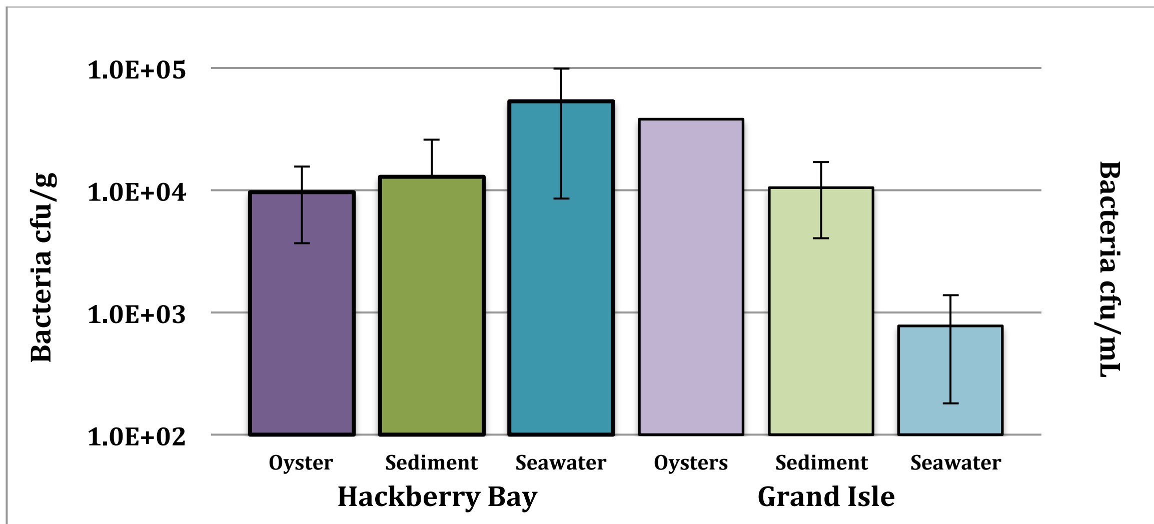

Figure 4. *Vibrio vulnificus* counts determined by colony hybridization without depuration treatment. Hackberry Bay (HB) oyster values show no significant difference between sediment values of culturable *V. vulnificus*. A standard deviation was unable to be calculated for oyster values at Grand Isle (GI). Between the two sites, HB seawater values were significantly higher than seawater values at GI. Error bars are from standard deviations of three studies in triplicates.

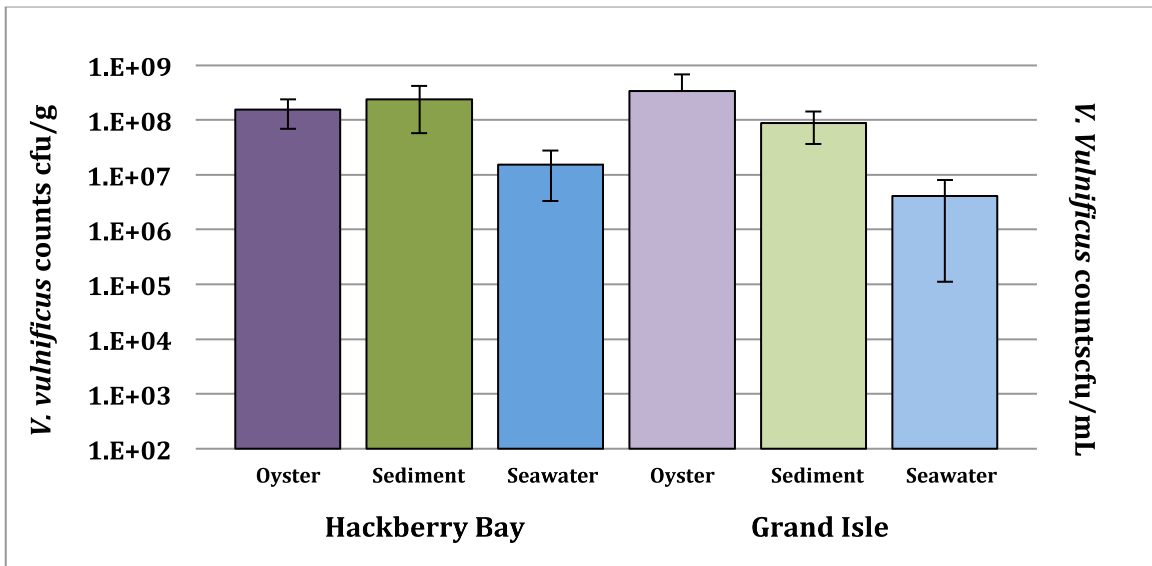

Figure 5. *Vibrio vulnificus* abundance determined by qPCR. Hackberry Bay (HB) oyster values show no significant difference between sediment values of *V. vulnificus*. Similarly, Grand Isle (GI) oyster values do not show a significant difference between sediment values. Between the two sites, HB seawater values were significantly higher than GI seawater values. Error bars are from standard deviations of three studies in triplicates.

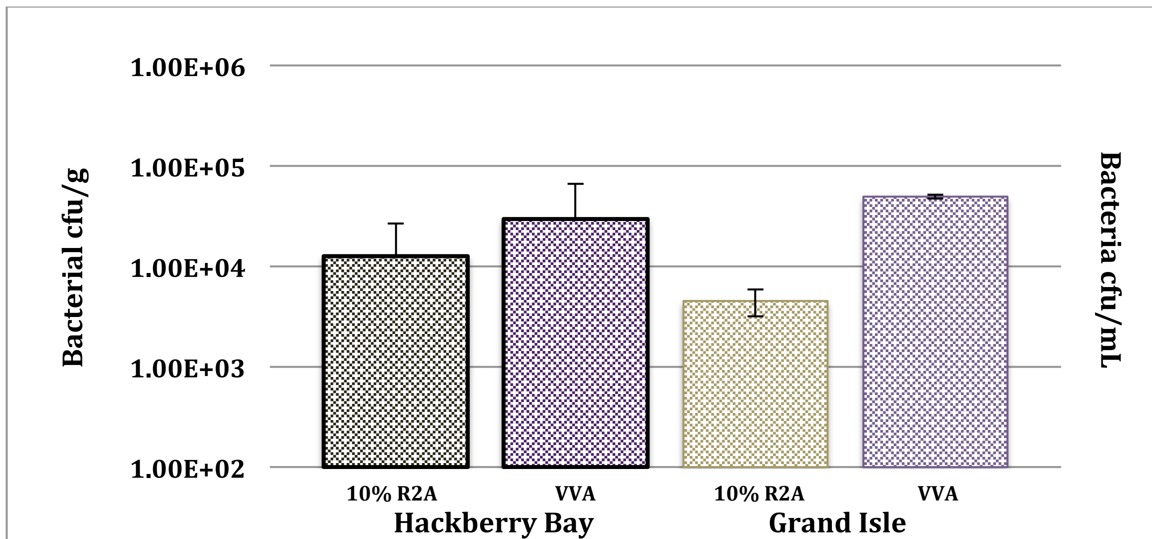

Figure 6. Total bacterial counts (cfu/g) on 10% R2A and VVA media post-depuration treatment. Bacterial counts from oysters sampled at Hackberry Bay (HB) were not significantly different between the two media, while bacterial counts from oysters sampled at Grand Isle (GI) were significantly different between the two media. Overall, bacterial counts dropped in oysters from both sites following depuration treatment. Error bars are from standard deviations of three studies in triplicates.

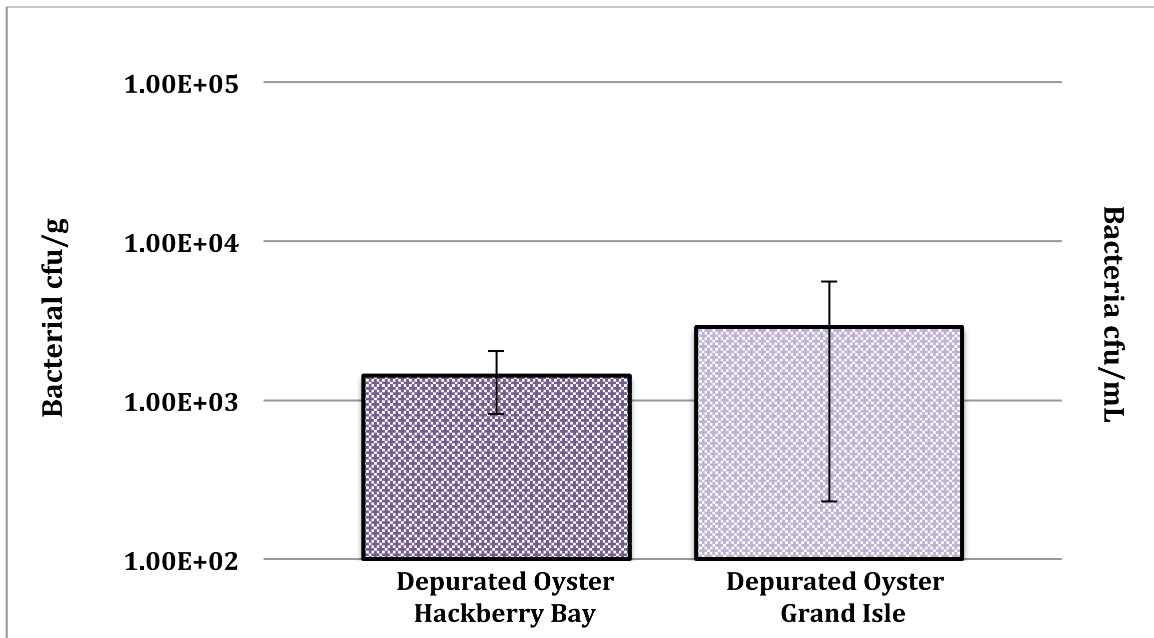

Figure 7. *Vibrio vulnificus* counts determined by colony hybridization post-depuration treatment. Oysters sampled at Hackberry Bay (HB) and Grand Isle (GI) showed no significant difference in the number of *V. vulnificus* post-depuration treatment. *V. vulnificus* counts (cfu/g) were significantly different between oysters pre- and post-depuration treatment from both HB and GI. Depuration can decrease the number of *V. vulnificus* in oysters. Error bars are from standard deviations of three studies in triplicates.

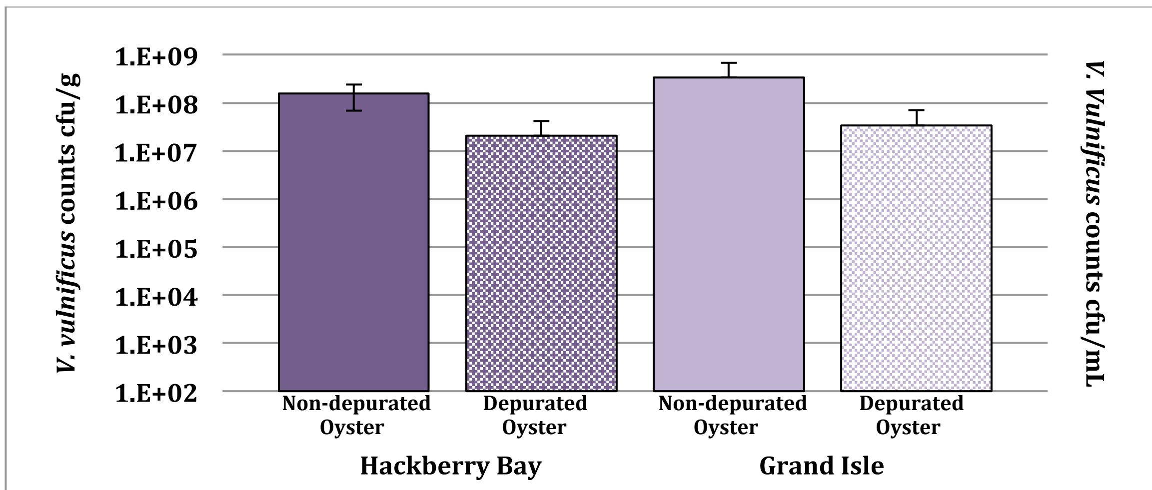

Figure 8. *V. vulnificus* abundance through qPCR in oyster pre- and post-depuration samples. Both Hackberry Bay (HB) and Grand Isle (GI) oyster samples pre-depuration were significantly higher than oysters post-depuration. Error bars are from standard deviations of three studies in triplicates.