MONITORING OF *VIBRIO* SPECIES IN OYSTERS *Crassostrea virginica* AND SEAWATER OF DELAWARE BAY AND MOLECULAR CHARACTERIZATION OF *Vibrio parahaemolyticus*

By

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A THESIS

Submitted in partial fulfillment of the requirements for the degree of
Masters of Science in the Food Science and Biotechnology
Graduate Program of Delaware State University

DOVER, DELAWARE
August 2017

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DEDICATION

This thesis is dedicated to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future. Also, I dedicate this thesis to my wife, Albadar, and daughters who have always been there for me. I would not be where I am today without my wife's love, understanding, prayers, sacrifices and continuing support to complete this research work.

ACKNOWLEDGEMENTS

First, I thank God, the Almighty, for His blessing and grace throughout my research, helping me to complete my research successfully**.**

I would like to express my deep and sincere gratitude to my research advisor, Dr. Gulnihal Ozbay for giving me the opportunity to do research and providing guidance throughout this research. I would like to extend my thanks and gratitude as well to my research mentor, Dr. Lathadevi Karuna Chintapenta for her great and continuous support to carry out and to present the research works successfully. I am extremely grateful to Dr. Samuel Besong for providing an opportunity in the program, and his valuable advice and enthusiasm that has deeply inspired me. I would also like to thank my committee members Dr. Bettina Taylor, Dr. Alberta Aryee, Dr. Salina Parveen, and Dr. Bertrand Hankoua for providing their support, assistance and advice with my thesis. I would like to thank our research technician, Amanda Abbott and all the other lab members of Aquatic Sciences Program and Molecular Genetics and Epigenomics Laboratory who helped me with my research, sharing their laboratory tools and equipment, aiding, and giving advice. I am also very thankful to the funders of USDA-NIFA (2014-38821-22430), NSF-EPSCoR Program (EPS-1301765), and CIBER at Delaware State University.

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ABSTRACT

Delaware Bay is the prime oystering ground in Delaware. Oysters naturally inhabit marine environments and, as filter-feeding mollusks, they tend to accumulate different microorganisms from seawater during their filtration process. Most of the illnesses caused by the consumption of raw and undercooked oysters are strongly associated with the oysters contaminated with *Vibrio* spp. *Vibrio* spp. are Gram-negative pathogenic bacteria from the family Vibrionaceae that negatively impact aquatic systems and human health. This study aimed to identify and differentiate *Vibrio* spp. in oyster and seawater samples from the Delaware Bay using CHROMagar™ Vibrio medium which provides the means for easy differentiation of *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio cholerae,* and *Vibrio alginolyticus* based on the colony color. Pathogenic *Vibrio parahaemolyticus* was characterized using conventional PCR which was applied for the identification of total and pathogenic *V. parahaemolyticus* by screening the species-specific *tlh* gene, and the virulent *tdh*, *trh*, *toxR*, and *vpm* genes.

Vibrio spp. were differentiated and isolated, and total and pathogenic *V. parahaemolyticus* from oyster and seawater samples were identified. Oysters and seawater samples were collected once a month from June through October 2016, from Bowers Beach, and

Lewes, Broadkill in Delaware Bay. A third site, Slaughter Beach, was added to this study from August through October 2016. Physico-chemical water quality parameters recoded on-site during samples collection include water and air temperature, salinity, pH, dissolved oxygen, turbidity, chlorophyll *a*, depth, conductivity, weather, wind direction, wind speed, and tide.

Oyster and seawater samples were serially diluted up to (10^{-6}), and 100 µL of each dilution from each sample were spread plated on CHROMagar™ Vibrio medium. *V. parahaemolyticus, V. vulnificus/V. cholerae*, and *V. alginolyticus* colonies were identified based on color development (mauve, green/blue, and colorless, respectively). Average *Vibrio* spp. in oyster ranged from <10 CFU g^{-1} (non-detectable) to 2.8×10^{4} CFU g^{-1}, while the average of *Vibrio* spp. in seawater samples ranged from 1.7×10 CFU mL^{-1} to 4.47×10^{3} CFU mL^{-1}. More *Vibrio* colony forming units were observed during the warmer months (June through September). As anticipated, the total colony forming units of *Vibrio* spp. were proportional in both oyster and seawater samples. The multiple regression model indicated a significant positive relationship between *Vibrio* spp. and *V. parahaemolyticus* Log_{10} CFUs and water temperature, ($p < 0.05$). The one-way ANOVA analysis showed no statistical significant association between *Vibrio* spp. Log_{10} CFUs and the sample type, oysters and seawater ($p > 0.05$).

A total of 165 bacterial isolates of *Vibrio parahaemolyticus* (mauve colonies) from oyster and seawater samples were examined for the presence of *tlh*, *tdh*, *trh*, *toxR*, and *vpm* genetic markers. Only 19% of the samples tested were positive for *tdh* and 24% were positive for *trh* while 83%, 65.5%, and 67% of the samples were positive for *tlh*, *toxR*, and *vpm*, respectively. This confirmed the specificity of *tlh* gene for *V. parahaemolyticus* species. Screening for *tdh* and *trh* genes is not sufficient for the surveillance of pathogenic or potential pathogenic *V. parahaemolyticus* but the reliability of *toxR* and *vpm* as gene markers is notably higher.

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CHAPTER 1: INTRODUCTION

1.1. Background

Marine environments host millions of pathogens and toxins (Ralston et al., 2011). In the United States (U.S.), between 1995 -2010, bacterial pathogens were responsible for 25% of seafood-related outbreaks (Ralston et al., 2011). The increasing human consumption of marine products increases the potential threat for bacterial infections. Illnesses caused by marine bacterial pathogens are strongly associated with both seafood consumption and direct exposure to the contaminated marine water (Ralston et al., 2011). Risk assessment of the indigenous pathogenic bacteria depends solely on targeting a specific pathogen and understanding their potential occurrence (Thompson et al., 2005). Unlike most bacterial pathogens that are introduced to the water through fecal contamination, *Vibrio* spp. are endemic pathogens in the marine environment (Ralston et al., 2011). Seafood origin disease outbreak data between 1990-2002 indicates that *Vibrio* is the leading cause of food-borne illness (Ralston et al., 2011). In the United States, the estimated mean of *Vibrio* foodborne illnesses, hospitalization, and death cases each year are 52408, 278, and 48 cases, respectively (Scallan et al., 2011). Although vibriosis is mostly associated with the consumption of contaminated seafood, direct exposure to the coastal recreational waters causes 12-40% of *Vibrio* infections (CDC 1999–2014). Of the 12 *Vibrio* pathogenic species, *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* are the most important clinical pathogens for public surveillance (Siboni et al., 2016). *V. cholerae* is considered as the main cause of diarrhea while *V. vulnificus* is responsible for 95% of deaths caused by seafood consumption in the U.S. (Oliver, 2015); and *V. parahaemolyticus* is the leading cause of gastroenteritis associated with the consumption of seafood, particularly oysters, and accounted for 59.5% of laboratory-confirmed Vibrionaceae Infections (CDC, 2017).

Vibrio species can be identified using either phenotype or genotype examination. The phenotype test depends on the cell morphology, color, biochemical or physiological properties. Of the different conventional media used to identify *Vibrio* species, thiosulfate-citrate-bile salts-sucrose (TCBS) and CHROMagar™ Vibrio are the most common, relying on the color development of colonies for identification. Further identification through conventional biochemical tests include Oxidase, Arginine Dihydrolase, Ornithine Decarboxylase, Lysine Decarboxylase, salt tolerance, and acid from Sucrose, D-Cellobiose, Lactose, Arabinose, D-Mannose, D- Mannitol. In general, most of the culture-based methods used in *Vibrio* identification are supplemented by 0%, 3%, 6%, 8%, or 10% of sodium chloride (Kaysner and DePaola, 2004). Genotype characterization relies on molecular approaches developed for identification and rapid detection of *Vibrio* spp. Due to its higher reliability and specificity it is preferred to culture-based methods (Bisha et al., 2012). The nucleic acid amplification technique using Polymerase Chain Reaction (PCR) is the most common method used for *Vibrio* identification and detection (Amalina and Ina-Salwany, 2016). PCR can identify species-specific and pathogenic genes among *Vibrio* species, and several genes have been used to design primers for specific amplification of target genes of *Vibrio* (Amalina and Ina-Salwany, 2016). *V. parahaemolyticus* has various pathogenic gene factors of which the virulent genes *tdh* and *trh* are most frequently used for its detection by PCR (Luan et al., 2007b). However, according to reports, many of the clinical isolates possess neither of *tdh* and *trh* genes indicating the potential presence of other virulent gene factors (Garcia et al., 2009). Hence, there is a great need for identifying more reliable genetic markers that can increase the accuracy of pathogenic *V. parahaemolyticus* detection methods. Following section outlines my research objectives and hypotheses.

1.2. Statement of Problem and Hypotheses

1.2.1. Problem Statement

In the United States (U.S.), infections caused by *Vibrio* spp., particularly *V. parahaemolyticus,* are rising and threatening public health. Consumption of raw and undercooked oysters from the U.S. coastal areas, specifically during warmer months, is strongly associated with *Vibrio* spp., and particularly *V. parahaemolyticus* infections (Bisha et al., 2012). The Delaware Bay is a prime oystering ground on the Atlantic coast (Canzonier, 2005); however, to the best of our knowledge there are no published studies on the prevalence and occurrence of total *Vibrio* spp. in general, and pathogenic *V. parahaemolyticus* in particular in Delaware Bay's oysters. This emphasizes the importance of understanding the prevalence of *Vibrio* spp. and particularly *V. parahaemolyticus* in Delaware Bay environments and of identifying effective virulent factors of *V. parahaemolyticus.* Detection of *Vibrio* spp. in oysters and seawater from the Delaware Bay will provide important data on temporal and spatial variation in the abundance of *Vibrio* spp., total and pathogenic *V. parahaemolyticus* in Delaware Bay's oyster and seawater samples. Furthermore, using the CHROMagar™ Vibrio medium and molecular characterization of *V. parahaemolyticus* will contribute in the development of *Vibrio* spp. detection methods.

In this study, oyster and seawater samples were collected from Bowers Beach, Broadkill, Lewes and Slaughter Beach in the Delaware Bay. *Vibrio* spp. were identified using CHROMagar™ Vibrio medium. Further characterization of total and pathogenic *V. parahaemolyticus* was achieved using conventional PCR and targeting the five genetic markers previously reported: *tlh* (species-specific factor), *tdh*, *trh*, *toxR*, and *vpm* (virulent factors).

1.2.2. Hypotheses

$H_0$1: Levels of *Vibrio* spp. and *V. parahaemolyticus* in Delaware Bay's oysters and seawater samples do not correlate significantly with temperature change.

H_A1: Levels of *Vibrio* spp. and *V. parahaemolyticus* in Delaware Bay's oysters and seawater samples correlate significantly with temperature change.

$H_0$2: *Vibrio* levels in the oyster samples will not be significantly higher than in seawater samples.

H_A2: *Vibrio* levels in the oyster samples will be significantly higher than in seawater samples.

$H_0$3: Reliability of *toxR*/*vpm* genes of *V. parahaemolyticus* in Delaware Bay's oysters and seawater samples will not be significantly higher than of *tdh*/*trh* genes.

H_A3: Reliability of *toxR*/*vpm* genes of *V. parahaemolyticus* in Delaware Bay's oysters and seawater samples will be significantly higher than of *tdh*/*trh* genes.

1.3. Specific Objectives

1) Detection of *Vibrio* species, and the determination of total and pathogenic *V. parahaemolyticus* levels in oyster and seawater samples from the Delaware Bay.

2) Examination of the correlation of *Vibrio* species levels in oyster and seawater samples with physico-chemical parameters, and of the correlation of *Vibrio* species levels with sample types, oysters and seawater.

3) Evaluation of the sensitivity and specificity of five genetic markers (*tlh, tdh, trh, toxR, vpm*) reported in the literature for the detection of *V. parahaemolyticus* to determine the most reliable gene factors for the detection optimization of total and pathogenic *V. parahaemolyticus.*

CHAPTER 2: LITERATURE REVIEW

2.1. The Delaware Bay and Its Water Quality

The Delaware Bay is the estuary outlet of the Delaware River, located in the Northeast of United States, and is bordered by the states of New Jersey and Delaware. It lies to the west of Cape May and north of Cape Henlopen on the Atlantic Ocean. The Delaware River, which flows into the Delaware Bay, provides about 60% of Delaware Bay's freshwater (Ford, 1997). River flow and other factors including tide, wind, and atmospheric pressure can have a significant impact on the water level of the Delaware Bay which consequently affects water quality including temperature, salinity, and dissolved oxygen (Delaware Bay Operational Forecast System, 2017). In regard to water quality, Delaware Bay's salinity ranges from 15 - 20 ppt, with the highest concentration of 30.7 ppt recorded near the ocean and the lowest of 1.0 ppt recorded near the river. The average water temperature of the bay ranges from 19.6 - 23.32 °C and the water depth from 5 to 8 meters contributing to the temperature changes throughout the year. The deeper areas warm more slowly in the spring and cool less rapidly in the fall than do the shallow areas. Dissolved oxygen in the water ranges from 5.45 - 8.19 mg/L (Neilan, 2015). Water quality is affected by contaminants and human activities, which produce gaseous, solid, and liquid wastes and have a great impact on surface water (Delaware Bay and Estuary Assessment Report, 2005). Agriculture utilizes 44% of Delaware Bay and Estuary Basin land area, with poultry and dairy being the primary agricultural products (Delaware Bay and Estuary Assessment Report, 2005). Impact of the bacteria from agricultural sources on surface water quality is of significant concern. The Department of Natural Resources and Environmental Control (DNREC, 2013) reported that the bacterial load of many of Delaware's estuarine and tidal waters exceeded the

acceptable limits, 70/100 mL of total coliform (DNREC, 2017), for the harvesting and consumption of shellfish during the warmer months.

Delaware Bay serves as nursery area and spawning and feeding ground of various shellfish species including oysters; hence it is considered a prime oystering ground (Canzonier, 2005). Oyster farming areas spread inside the bay from the artificial island of New Jersey through the Bombay Hook of Delaware and down to the estuary on the Atlantic Ocean (Figure 2.1) (Marenghi et al., 2017). Commercial harvest of oysters in Delaware Bay began at the early 1800s when trading oysters in the shell was the cornerstone in the growth of the oyster industry (Ford, 1997). Oyster reefs in Delaware Bay have provided a sustainable food supply over the past two centuries and contributed to the local economy of Delaware counties (Canzonier, 2005).

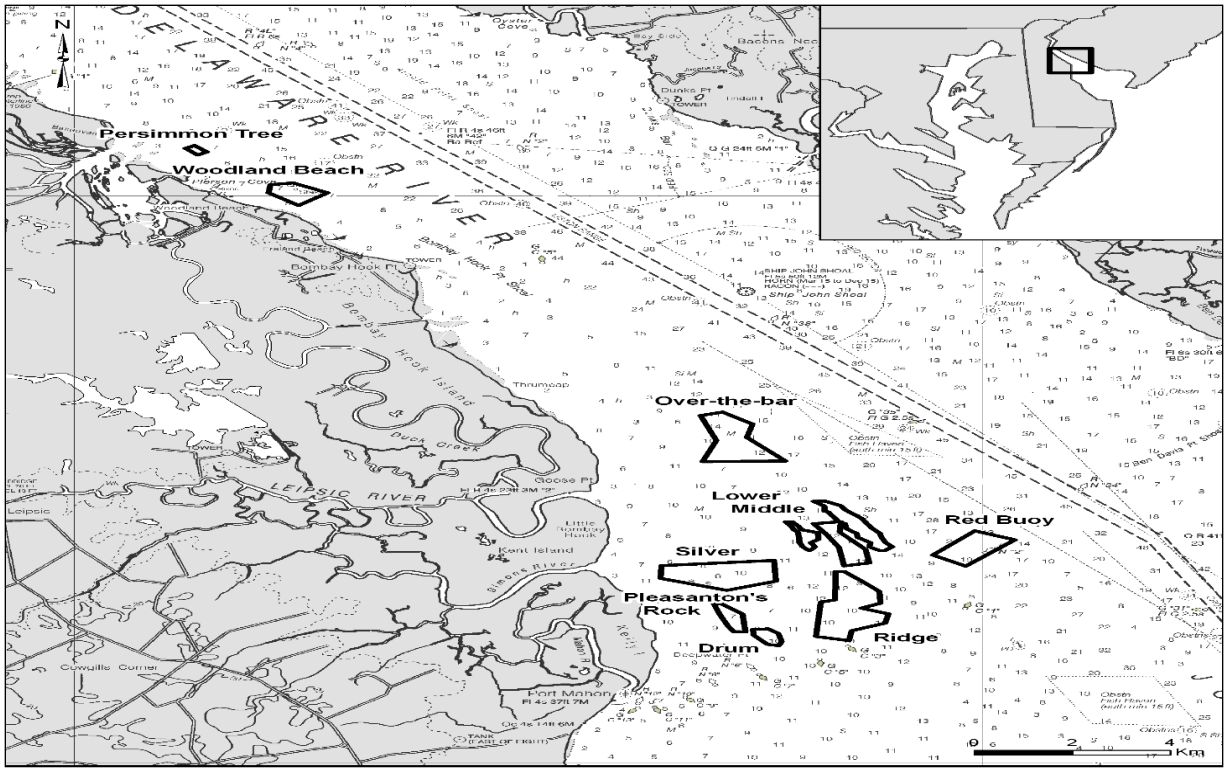

Figure 2.1. Shaded Areas in Delaware Bay Represent Natural Oyster Beds, DNREC (2008) cited by Marenghi et al., (2017).

2.2. Eastern Oysters (*Crassostrea virginica*)

Among the different oyster species in the United States, the two species of commercial value are Eastern oysters (*Crassostrea virginica*) and Olympia oysters *(Ostrea lurida*). *C. virginica* is the native and most important species which inhabit areas from northern New Brunswick down the coast to the Gulf of Mexico on the Atlantic coast (Mackenzie, 1996). The harvest of 18.2 million pounds of C. *virginica* in 2010 had an estimated value of $76.2 million. In 2011, however, the Delaware Bay industry of C. *virginica* landing declined to 94,470 bushels (1 bushel = 37 L) with estimated dockside value of $4.2 million (Munroe et al., 2013). Although oysters tolerate challenging environmental conditions by closing their shells and not feeding, their capability can be limited when for example shells became un-openable which consequently prevents expelling of toxic metabolites and regaining feeding for energy (Munroe et al., 2013). Oyster harvesting size ranges from 76-90mm, which may take 18-24 months of growth in the Gulf of Mexico and 4-5 years in Long Island Sound (Eastern Oyster Biological Review Team, 2007). Oyster size and growth depends on several environmental factors and the availability of food (Eastern Oyster Biological Review Team, 2007). Optimal growth conditions for oysters include a water temperature of 20-30°C; a salinity of 5-40 ppt; a depth of 0-11m; a pH of 6.75-8.75; and dissolved oxygen (DO) of 20-100% (Eastern Oyster Biological Review Team, 2007).

The *C. virginica* has distinct characteristics that make it marketable as a food item: It can be consumed throughout the year and has a long shelf life if handled properly (Canzonier, 2005). Many connoisseurs have described the oyster flavor in interesting terms. Michel de Montaigne said it tastes like violets, Eleanor Clark thinks of oyster flavor as a “shock of freshness”, and the French poet Leon-Paul Fargue described it as “kissing the lip of the sea” (Jacobsen 2007). McMurray (2007) suggested that *C. virginica* oyster has a “perfect balance of salt and sweet”.

Oyster is considered as a healthy meat choice for a low-fat, low-saturated-fat, and high-protein diet (Food and Nutrition Board, 2007). Also, oysters contain a decent amount of omega-3 fatty acids that are equivalent to the recommended daily intake by the Food and Nutrition Board (2004). Especially Eicosapentaenoic acid (EPA), and Docosahexaenoic acid (DHA), very long chain fatty acids, are precursors for anti-inflammatory prostaglandins in the human body. EPA, DHA, and other fatty acid levels in oysters vary according to the oyster's origin. Chen (2011) reported that oysters from Chesapeake Bay adjacent to the Delaware Bay have higher contents of EPA and DHA than those from New Brunswick, Canada. Oysters are also an excellent source of Iron, Zinc, Copper, and Vitamin B12 (Faye, 2009). However, consumption of raw and undercooked oyster is strongly associated with *Vibrio* infections.

2.3. Properties and Characteristics of *Vibrio* spp. and *Vibrio parahaemolyticus*

2.3.1. *Vibrio* spp.

Vibrios are Gram-negative, halophilic, pathogenic bacteria, that are straight or curved rods shaped, motile with a single polar flagellum belonging to the family Vibrionaceae that negatively impact aquatic ecosystems and human health (Morris and Blac,1985; Luan et al., 2007a; Lee et al., 2015). Most of the *Vibrio* spp. are oxidase and catalase positive with the ability of glucose fermentation with no gas production (Kaysner and DePaola, 2004). *Vibrio* abundance increases when water exceed 20°C and with salinity less than 10ppt (Takemura et al., 2014). Other environmental measures have also been associated with variations in *Vibrio* levels, including dissolved oxygen, turbidity, pH, and chlorophyll (Takemura et al., 2014). It is well documented that *V. parahaemolyticus, V. vulnificus,* and *V. cholerae* are human pathogens with a high frequency of occurrence respectively (Kaysner and DePaola, 2004). Vibriosis cases

reported in 2014 and deaths confirmed by Centers for Disease Control and Prevention (CDC) in the United States are listed in (Table 2.1).

Table 2.1 *Vibrio* infection: Human disease and deaths cases associated with common *Vibrio* spp.

***Vibrio* species**	**Clinical Presentation**		
	Cases	**Hospitalization**	**Deaths**
V. cholerae (excluding toxigenic O1 and O139)*	80	36/79	3/65
V. parahaemolyticus	605	86/575	4/389
V. vulnificus	124	97/123	21/117
V. alginolyticus	239	32/222	0/180

*Includes non-toxigenic *V. cholerae* non-O1, non-O139 (78 cases) and O1 (2 cases).

Among various virulence factors of pathogenic *Vibrio*, exotoxins (haemolysin) are the most virulent factors associated with *Vibrio* infection (Zhang and Austin, 2005). Hemolytic activities refer to red blood cell lysis causing the liberation of hemoglobin (Honda et al., 1980; Kelly and Stroh, 1989; Shirai et al., 1990; Garcia et al., 2009). Toxins associated with hemolytic activity work as pore-forming agents on the erythrocytes' walls and other cell types such as neutrophils, polymorphonuclear leukocytes, and mast cells causing tissue damage (Zhang and Austin, 2005). Haemolysin families in *Vibrio* spp. are listed in (Table 2.2). Intensive studies were conducted to understand TDH of *Vibrio parahaemolyticus* and HlyA of *Vibrio cholerae*; however, the role of TLH and δ-VPH are still elusive (Zhang and Austin, 2005).

Cholera enterotoxin is the causative factor of cholera infection, and group O1 and O139 are the only serogroup types that are associated with epidemic cholera cases. *V. cholerae* non-O1/O139 serogroups are strains that are identical or similar to O1/O139 serogroup but do not agglutinate in either anti-O1 or -O139 sera. While *V. cholerae* O1/O139 strains are the only serogroup that produce cholera toxin (CT), the other serogroup types cause diarrheal diseases of

varying levels of severity (Kaysner and DePaola, 2004). Unlike *V. cholerae*, several virulence factors are associated with *V. vulnificus* including a polysaccharide capsule, various extracellular enzymes, exotoxins, and iron acquisition from transferrin. Capsular polysaccharide expression was reported to be induced when cells were grown at 30°C compared to 37°C. Extracellular protease was found to be the most important enzyme *V. vulnificus* used to degrade native albumin (Drake et al., 2007). According to the scientific literature, hemolytic/cytolytic activity of *V. vulnificus* is the most common virulence factor and is coded by the *VvhA* gene. Hemolysin/cytolysin is a thermo-labile protein that causes red blood cell lysis and cytotoxicity to the tissue culture of cell line (Drake et al., 2007).

Detection of *Vibrio* spp. can be performed using conventional biochemical methods, but in the TCBS agar medium *V. cholerae* develop a yellow-colored colony while *V. vulnificus* and *V. parahaemolyticus* develop a green-colored colony which mean that TCBS is not suitable for *V. vulnificus* and *V. parahaemolyticus* identification. Furthermore, *V. vulnificus* and *V. parahaemolyticus* produce the same results in several biochemical tests including the Oxidase, Arginine Dihydrolase, Ornithine Decarboxylase, Lysine Decarboxylase, salt tolerance, and acid from sucrose and D-M-mannose tests (Kaysner and DePaola, 2004). Unlike the TCBS agar, the CHROMagar™ Vibrio medium is more suitable for presumptive identification of *V. parahaemolyticus*, *V. vulnificus*/*V. cholerae*, and *V. alginolyticus*; resulting in colonies which can be distinguished based on color development; mauve, green/blue, and colorless, respectively. Among *Vibrio* spp., *V. parahaemolyticus* possess several virulence factors, so that conventional biochemical detection methods cannot be used for the identification of pathogenic *V. parahaemolyticus,* emphasizing the importance of molecular-based identification method (Drake et al., 2007).

Table 2.2 Haemolysins produced by *Vibrios*[a].

Bacterial taxon	Haemolysin family	Specific haemolysin	Key reference
V. anguillarum	HlyA	VAH1	Hirono et al. (1996)
V. cholerae O1	HlyA	HlyA	Yamamoto et al. (1990a)
	TLH lecithinase	LEC	Fiore et al. (1997)
	δ-VPH	Vc-δ TH	Fallarino et al. (2002)
V. cholerae non-O1	HlyA	HlyA	Ichinose et al. (1987)
	TDH	NAG-TDH	Baba et al. (1991b)
V. fluvialis	HlyA	VFH	Han et al. (2002)
V. harveyi	TLH	VHH	Zhang et al. (2001)
V. hollisae	TDH	Vh-TDH	Nishibuchi et al. (1990)
V. mimicus	TDH	Vm-TDH	Terai et al. (1990)
	HlyA	VMH	Kim et al. (1997)
	TLH	Lecithinase PHL	Kang et al. (1998)
	–	HLX	Nagamune et al. (1995)
V. parahaemolyticus	TLH	TLH or LDH	Taniguchi et al. (1986)
	δ-VPH	δ-VPH	Taniguchi et al. (1990)
Kanagawa positive	TDH	Vp-TDH	Tsunasawa et al. (1987)
Kanagawa negative	TDH	Vp-TRH	Honda et al. (1988)
V. tubiashii	HlyA	Cytolysin	Kothary et al. (2001)
V. vulnificus	HlyA	VVH	Yamamoto et al. (1990b)
	TLH	VPL	Genbank AF291424
	–	HLYIII	Chen et al. (2004)
	–	VLLY	Chang et al. (1997)

HlyA, TDH, TLH and d-VPH correspond to HlyA (El Tor) haemolysin, thermostable direct haemolysin, thermolabile haemolysin and thermostable haemolysin families respectively.
a. Data from (Zhang and Austin, 2005).

2.3.2. *Vibrio parahaemolyticus*

V. parahaemolyticus was first identified as a cause of food-borne illness in Japan in 1950. It results in one of the leading causes of gastroenteritis expressed in diarrhea, headache, vomiting, and abdominal cramps following the consumption of contaminated food or water; in addition, it can cause septicemia and wound infections (Morris and Black, 1985; Iwamoto et al., 2010). *V. parahaemolyticus* infections can be lethal for neonates, the elderly, and immunocompromised individuals. In aquatic ecosystems, organisms like oysters which are filter-feeding mollusks, tend to accumulate different microorganisms from seawater during their filtration (DePaola et al., 1990; Klein et al., 2014). Oysters have the capacity to filter 96–816 L of water per day (Barnes et al., 2007). In this process, they are able to accumulate *V. parahaemolyticus* resulting in a concentration100-fold higher than the water of the surrounding area (Morris, 2003). During the warmer months, *V. parahaemolyticus* contamination in oysters might reach 100% (Morris, 2003). Bacterial illnesses were frequently caused from the consumption of undercooked and raw oysters (Joseph et al., 1982; Ellison et al., 2001; Bisha et al., 2012). Daniels et al. (2000) reported that outbreaks associated with *V. parahaemolyticus* infections in the United States from 1973-1998 occurred during the warmer months, and the median infection rate averaged 56% among people who consumed contaminated seafood. In 2006, a total of 177 *V. parahaemolyticus* infections were reported from New York, Oregon, and Washington states, and the laboratory-confirmed cases were over three-folds higher than the average number in all US states during the same period of 2002-2004 (CDC, 2006). *V. parahaemolyticus* have caused forty outbreaks in 15 U.S. states from 1973 to 1998 (Daniels et al., 2000; Bisha et al., 2012). The economic costs associated with *V. parahaemolyticus* infections are estimated to be US$21 million per year (Ralston et al., 2011). Ralston et al. (2011) proposed

that *Vibrio* spp. are responsible for 9800 infections from seafood consumption or recreational exposure annually. According to the FoodNet of CDC's Emerging Infections Program (CDC, 2005), in comparison with baseline data from 1996 to 1998 and 2005, the incidence of the most common foodborne pathogens in the United States have decreased or were close to the target levels of the national health objectives while infections caused by *Vibrio* spp. have increased by 41% and *V. parahaemolyticus* is responsible for 54% of the increase.

According to its pathogenicity *V. parahaemolyticus* can be classified into two groups: pathogenic and nonpathogenic. It can be also classified into different serotypes according to its capsule (K) and somatic (O) antigens. According to the antigen classification system, there are 13(O) different antigens and 71 (K) different types (Chen et al., 2012). *V. parahaemolyticus* grows between 9 and 44°C with an optimum multiplication temperature of 35 °C. *V. parahaemolyticus* communities disseminate in marine and estuarine environments. (Murray et al., 2012). *V. parahaemolyticus*, lactose and sucrose negative, and can grow in a medium containing 3, 6 and 8% but not 10% of NaCl. The pH range for optimum growth is7.8 to 8.6. *V. parahaemolyticus* are facultative anaerobic bacteria, that are very sensitive to stream heat, dry and freeze conditions (Kaysner and DePaola, 2004).

The global occurrence of *V. parahaemolyticus* infections and outbreaks following the consumption of raw or undercooked oysters has raised the interest on this pathogen. Although molecular methods are widely used to confirm the presence of *V. parahaemolyticus* studies indicate a need for identifying genetic markers to increase the accuracy of detection methods. The following section describes those factors reported in the literature and their effect on the pathogenicity of *V. parahaemolyticus*.

2.3.3. *Vibrio parahaemolyticus* Pathogenicity

V. parahaemolyticus like other *Vibrio* spp. possesses two chromosomes 1 and 2; however, the size of *V. parahaemolyticus* chromosomes are 3.3 and 1.9 Mb, respectively. Chromosome 2 of *V. parahaemolyticus* contains a higher number of unique genes compared to chromosome 1 indicating a greater difference of structure and gene content (Makino et al., 2003). *V. parahaemolyticus* strains possess a *tlh* species specific gene, which codes for the thermolabile direct hemolysin (TLH) (Bej et al., 1999; Luan et al., 2007b). However, the virulence of clinical *V. parahaemolyticus* strains are associated with the *tdh* and/or *trh* genes that code for the thermostable direct hemolysin (TDH) and/or a TDH-related hemolysin (TRH), respectively (Taniguchi et al., 1985; Shirai et al., 1990; Okuda et al., 1997). Both *tdh*/*trh* genes are associated with β hemolysis on Wagatsuma blood agar, which is known as the Kanagawa Phenomenon (KP). The presence of *tdh*/*trh*-positive *V. parahaemolyticus* in seafood pose a serious risk to public health (Zarei et al., 2012). Yamazaki et al. (2010) reported that TDH/TRH proteins are the main pathogenic factors in *V. parahaemolyticus*. Furthermore, TDH/TRH have been used as accepted markers for *V. parahaemolyticus* detection in seafood (Wagley et al., 2009). Honda et al (1988) reported that TDH/TRH have a homolog amino acid sequence consisting of 165 residues and sharing 67% similarity. Clinical isolates that possess both *tdh/trh* genes are known to be KP^+. However, many of clinical isolates that are KP^+ were tdh^- and/or trh^- and vice versa (Honda et al., 1980; Kelly and Stroh, 1989; Shirai et al., 1990; Garcia et al., 2009). Unlike clinical isolates, only a few of the environmental isolates (1-2%) were KP^+ (Miyamoto et al., 1969), and only 6% of bacterial isolates from molluscans in Atlantic and Gulf Coast (Cook et al., 2002). In Mexico, Cabrera-Garcia et al. (2004) found that just 9% of the environmental isolates were both tdh^+ and KP^+. Rojas et al. (2011) observed that only 10.5% of

the environmental *V. parahaemolyticus* isolates possess the *tdh* gene. Also, the presence of the *tdh* gene in *V. parahaemolyticus* has been detected in 12% of the environmental isolates (Wagley et al., 2008). Parveen et al. (2008) reported that real-time PCR method showed that 13 and 20% of Maryland Chesapeake Bay *V. parahaemolyticus* isolates from water and oyster samples possess *tdh* gene, respectively, indicating the lower incidence of *tdh* gene compared to the oyster and water samples of the Gulf Coast. However, the presence of the *trh* gene in Maryland Chesapeake Bay *V. parahaemolyticus* isolates was detected in 40% of the water and oyster samples which agrees well with the levels in Gulf Coast oyster and water enrichment samples (Parveen et al., 2008). Higher percentage of *tdh*/*trh* occurrence in *V. parahaemolyticus* isolated from the environment was reported by Velazquez-Roman et al. (2012) found 52% (75 out of 144) of the isolates were *tdh* and/or *trh* positive. Hongping et al. (2011) reported that (83.5%) of clinical isolates and (22%) of sea fish isolates were *tdh*-positive while all fresh water isolates were *tdh*-negative. These studies suggest that, a low number of *tdh*/*trh*-positive *V. parahaemolyticus* are found in environmental and food samples, while the *tdh*/*trh*-negative *V. parahaemolyticus* among clinical samples emphasize the need of assessing other genetic markers for better surveillance and detection of pathogenic *V. parahaemolyticus*. The assumption of a strong correlation of hemolysin in *V. parahaemolyticus* infections is debatable (Kim et al., 2002); other virulence factors such as vascular permeability, stress tolerance, and colonization correlated with the pathogenicity of this species.

Kim et al. (2002) and Miyoshi et al. (2008) have reported that *V. parahaemolyticus* harbors a metalloprotease gene that expresses extracellular zinc metalloprotease and showed sufficient proteolytic activity toward type I collagen. Metalloprotease pathogenicity has been investigated and found to be significant as a virulence factor among *Vibrio* spp. (Kim et al.,

2002). *Vibrio* metalloproteases fall into two classes depending on the arrangement of three zinc ligand residues; class I (HEXXH-E) which contain two histidine residues of HEXXH motif bound to a glutamate and class II (HEXXH) which does not bind to a glutamate residue. Furthermore, *Vibrio* metalloproteases of class I share 68–72% of sequence similarity while class II enzymes share 30–78% (Table 2.3) (Kim et al., 2002). Although *V. parahaemolyticus* metalloprotease VppC have very low sequence homology with other *Vibrio* metalloproteases, *V. alginolyticus* collagenase share high (77%) sequence similarity with VppC. The multiple alignment of class II enzymes [*V. alginolyticus* collagenase, *V. mimicus* metalloprotease (VMC), V. *parahaemolyticus* strain 93 metalloprotease (PrtV), *V. cholerae* 569B exoprotease (VCC)], and VppC resulted in two distinct groups with regards to the amino acid sequence homology. VMC, PrtV, and VCC belong to one group (class II) and share70–78% sequence similarity; and VppC and *V. alginolyticus* collagenase belong to a new group (class III) and have 88% similarity (Table 2.4). The molecular mass and substrate specificity of the metalloproteases support the three-class classification (Kim et al., 2002). The class I enzymes hydrolyze elastin but not collagen; classes II and III enzymes hydrolyze native collagen; class II enzymes do not hydrolyze casein; class III enzymes hydrolyze casein, gelatin and collagen as substrates (Kim et al., 2002). Luan et al. (2007a) reported the proteolytic activity on type I collagen and the cytotoxicity of the zinc metalloprotease protein (VPM) encoded by the *vpm* gene in *V. parahaemolyticus* fall under class III and is identical to VppC.

Another virulence indicator of pathogenic or potential pathogenic *V. parahaemolyticus* was reported to be related to the presence of *toxR* gene. Lin et al. (1993) suggested that *V. parahaemolyticus* possess a homolog of *V. cholerae toxRS* gene sharing 52 and 62% similarities, respectively. Moreover, Vc-ToxR and Vp-ToxR share a similar function since they are strongly

associated with the upregulation of the gene(s) encoding major virulence toxins CTX and TDH (Lin et al., 1993). The study by Whitaker et al. (2012) indicated that the *toxR* gene is required for stress tolerance and colonization of *V. parahaemolyticus*. Genome sequencing of pathogenic *V. parahaemolyticus* revealed another virulence factor called type III secretion systems (T3SS), T3SS1and T3SS2, by which bacterial proteins (effectors) injected directly into host cells. T3SS2 of *V. parahaemolyticus* is encoded in a pathogenicity island on the chromosome 2 while T3SS1 is encoded on chromosome 1 (Makino et al., 2003). According to Ritchie et al. (2012), infant rabbit model infected by *V. parahaemolyticus* revealed that unlike TDH, T3SS2 is essential for intestinal colonization. Hubbard et al. (2016) identified uncharacterized component of T3SS2 to be, contrary to what have been reported in Gotoh et al. (2010), critically regulated by ToxR. Luan et al. (2007b) have compared the reliability and specificity of the four genetic markers *tdh*, *trh*, *toxR*, and *vpm* for the PCR detection of *V. parahaemolyticus* (Tables 2.5, 2.6, and 2.7). Although *toxR* gene may not be considered as a prime virulence factor, its strong association with *V. parahaemolyticus* pathogenicity cannot be ignored. Unlike *tdh*/*trh* gens, the high prevalence of *toxR* gene among *V. parahaemolyticus* isolated from environmental and food samples (Luan et al., 2007b; Paranjpye et al., 2012) can facilitate the surveillance of pathogenic or potential pathogenic *V. parahaemolyticus*. Furthermore, screening *toxR* along with other virulent genes will better illustrate the coexistence association of these genetic markers among *V. parahaemolyticus* isolates.

Table 2.3. *Vibrio* metalloproteases two classification model and homologation percentage[a].

	Class I (HEVSH-E)				Class II (HEYTH)			
	Vpr	***Vv***	***Van***	***Vch* M**	***Vm***	***Vp (93)***	***Vch* AF**	***Val***
Vpr	100.0							
Vv	69.0	100.0						
Van	72.0	70.0	100.0					
***Vch* M**	69.0	68.0	69.0	100.0				
Vm	23.5	25.6	25.5	23.6	100.0			
Vp (93)	15.7	20.0	22.0	26.5	70.0	100.0		
***Vch* AF**	25.2	29.0	31.4	27.5	77.9	71.2	100.0	
Val	23.0	19.6	23.9	18.9	30.0	37.3	30.5	100.0

V. proteolyticus (*Vpr*), *V. vulnificus* (*Vv*), *V. anguillarum* (*Van*), *V. cholerae* (*Vch M*), *V. mimicus* (*Vm*), *V. parahaemolyticus* 93 (*Vp* 93), *V. cholerae* (*Vch* AF), *V. alginolyticus* (*Val*).

a. Data from Kim et al. (2002).

Table 2.4. *Vibrio* metalloproteases three classification model and homologation percentage[a].

	Class I (HEVSH-E)				Class II (HEYTH)			Class III (HEYVH)	
	Vpr	***Vv***	***Van***	***Vch* M**	***Vm***	***Vp (93)***	***Vch* AF**	***Val***	***Vp (04)***
Vpr	100.0								
Vv	69.0	100.0							
Va	72.0	70.0	100.0						
Vch	69.0	68.0	69.0	100.0					
Vm	23.5	25.6	25.5	23.6	100.0				
Vp (93)	15.7	20.0	22.0	26.5	70.0	100.0			
Vch	25.2	29.0	31.4	27.5	77.9	71.2	100.0		
Va	23.0	19.6	23.9	18.9	30.0	37.3	30.5	100.0	
Vp (04)	23.5	22.7	22.7	19.4	30.0	34.0	30.9	88.0	100.0

The accession numbers of various *Vibrio* metalloproteases are as follows: *V. proteolyticus* (*Vpr*) (M64809), *V. vulnificus* (*Vv*) (U48780), *V. anguillarum* (*Van*) (L02528), *V. cholerae* (*Vch* M) (M59466), *V. mimicus* (*Vm*) (AF004832), *V. parahaemolyticus* 93 (*Vp* 93) (Z46782), *V. cholerae* (*Vch* AF) (AF109145), *V. alginolyticus* (*Val*) (X62635), *V. parahaemolyticus 04* (*Vp* 04) (AF32657).

a. Data from Kim et al. (2002).

Table 2.5. Previous reports of primers performance in *Vibrio parahaemolyticus* (*Vp*) detection[a].

Primer Set	Gene	Expected Size of PCR Product (bp)	Strains Tested Positive/Total		Anneal Temperature (°C)	Primer Sequence
			(*Vp*)	Non-(*Vp*)		
1	*GyrB*	285	117/117	0/150	58	VP-1: CGGCGTGGGTGTTTCGGTAGT VP-2r: TCCGCTTCGCGCTCATCAATA
2	*tl*	450	111/111	0/19	58	L-tl: AAAGCGGATTATGCAGAAGCACTG R-tl: GCTACTTTCTAGCATTTTCTCTGCG
3	*tdh*	269	60/111	3/19		L-tdh: GTAAAGGTCTCTGACTTTTGGAC R-tdh: TGGAATAGAACCTTCATCTTCACC
	trh	500	43/111	0/19		L-trh: TTGGCTTCGATATTTTCAGTATCT R-trh: CATAACAAACATATGCCCATTTCC
	toxR	376	373/373	5/290	52.4	toxR-4: GTCTTCTGACGCAATCGTTG toxR-7: ATACGAGTGGTTGCTGTCATG
4	*vpm*	674				VPM1: CAGCTACCGAAACAGACGCTA VPM2: TCCTATCGAGGACTCTCTCAAC

a. Data from Luan et al. (2007b).

Table 2.6. Bacterial strains used in Luan study (Luan et al., 2007b).

Bacterial strains	Source	Number of strains	Country (year of isolation)
V. parahaemolyticus VIB 458	LMG 12094	1	
V. parahaemolyticus VIB 611	ATCC 33844	1	
V. parahaemolyticus VIB 800	Shrimp	1	Thailand
V. parahaemolyticus FYZ8621.4	Clinical isolate from patients	1	China (1998)
V. parahaemolyticus	Clinical isolates from patients	71	China (1998-2002)
V. parahaemolyticus	Environmental isolates from seawater and seafood	10	China (2004-2006)
V. aestuarianus VIB 281	LMG 7909^T	1	USA
V. alginolyticus VIB 283	LMG 4408^T	1	
V. alginolyticus VIB 284	LMG 4409^T	1	Japan
V. cincinatiensis VIB 287	LMG 7891^T	1	USA
V. furnissi VIB 293	LMG 7910^T	1	Japan
V. mimicus VIB298	LMG 7896^T	1	USA
V. pelagia VIB305	LMG 3897^T	1	USA
V. tubiashii VIB309	LMG 10936^T	1	USA
V. vulnificus VIB310	LMG 13545^T	1	USA
V. anguillarum CW4	Fish	1	China
Edwardsiella tarda CW7	Fish	1	China
Aeromonas caviae 1.1960	CCCCM	1	China
Pseudomonas alcaligenes 1.1805	CCCCM	1	China
Aeromonas hydrophila PR156	Heriot-Watt University	1	UK
Aer. hydrophila 1.2017	CCCCM	1	China

Table 2.7. Specificity and reliability of *tdh*, *trh*, *toxR*, and *vpm* in *Vibrio parahaemolyticus* (*Vp*) detection using the polymerase chain reaction (PCR)[a].

Primer Set	Primer Symbol	No. of positive strains/total (*Vp*) strains tested	No. of positive strains/total non (*Vp*) strains tested
1	VP-1/VP-2r	85/85	0/16
2	L-tl/R-tl	85/85 (tl)	0/16
	L-tdh/R-tdh	64/85 (tdh)	
	L-trh/R-trh	0/85 (trh)	
3	toxR-4/toxR-7	85/85	0/16
4	VPM1/VPM2	85/85	0/16

a. Data from Luan et al. (2007b).

CHAPTER 3: MATERIALS AND METHODS

3.1. Study Location and Sampling

Oysters and seawater samples were collected once a month from June to October 2016 from Bowers Beach (BB) and Lewes, Broadkill (LW) in the Delaware Bay. A third site, Slaughter Beach (SL) was added to this study from August to October 2016 (Figure 3.1). Ten to twelve oysters from each site were harvested, one site per week, into sterile plastic bags, and sub-divided to three groups for biological triplicates (A, B, and C). Also, one liter of seawater was collected from each site at the same time. After harvesting, all bags were placed into an ice chest and chilled with ice packs using a sheet of bubble wrap to ensure no direct contact between the ice packs and sample bags. Smart Button Data Logger (ACR SYSTEM INC) was used to confirm that the temperature during transportation was lower than 10C°.

Physico-chemical water quality parameters were recoded from each site in conjunction with each time samples are collected. These parameters include water and air temperature (°C), salinity (ppt), pH, dissolved oxygen (% and mg/L), turbidity (NTU/FTU), chlorophyll *a*, depth (m), conductivity (uS/cm), weather, wind direction, wind speed (mph), and tide (Refer to Appendix A, Page 68). Among these parameters, water temperature, salinity, turbidity, dissolved oxygen (mg/l), and chlorophyll *a* were used in this study for statistical analysis to assess the relationship between these parameters and the CFUs of total *Vibrio* and *Vibrio parahaemolyticus*. YSI 556 Handheld Multiparameter Instrument was used to monitor the water quality parameters water temperature, salinity, pH, conductivity, and dissolved oxygen.

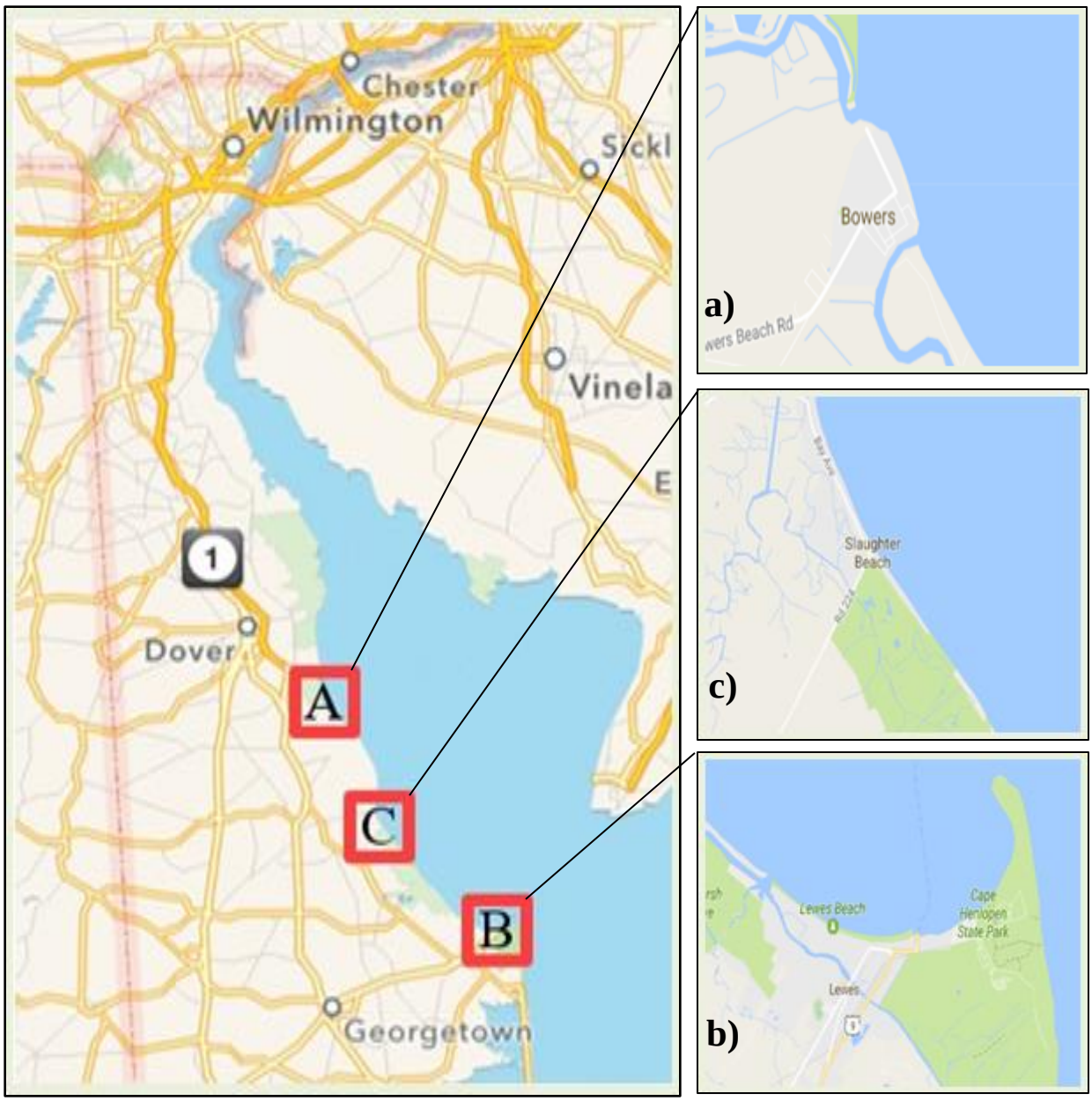

Figure 3.1. Sampling areas in the Delaware Bay: **a)** Bowers Beach (BB), **b)** Lewes, Broadkill (LW), and **c)** Slaughter Beach (SL).

https://www.google.com/maps/place/De/usa,
https://www.google.com/maps/place/Lewes,+DE+USA,
https://www.google.com/maps/place/Bowers,Beach,+DE+USA,
https://www.google.com/maps/place/Slaughter+Beach,+DE+USA

3.2. Processing of Oyster and Seawater Samples.

Culture media, solutions, and agarose used in this study include Tryptic Soya Broth (TSB), CHROMagar medium, (0.1%) Broth Peptone Water (0.1% BPW), TSB + 24% Glycerol, and (1%) Agarose Gel (Refer to Appendix B, Page 72).

Ten to twelve oysters were collected and divided into three groups to be analyzed in triplicates. For each replicate 3-4 oysters were cleaned upon arrival at the laboratory using a scrub brush and tap water before they were shucked with sterile knives. Then, 25 g of tissue was collected into a sterilized blender jar (WARING COMMERCIAL, 7010S), blended for 120 sec at high speed, and 225 mL of (0.1% BPW) was added to prepare the homogenate. This homogenate was labelled as the first (10^{-1}) dilution. The suspension was blended again for 60 sec at high speed. The oyster homogenate and seawater samples from each site were aseptically serial diluted in (0.1% BPW) to a final dilution of (10^{-6}) before 100 µL of each dilution [10^{-1} - 10^{-6}] of both seawater and oyster homogenate samples from each site were aseptically spread plated on CHROMagar plates (CHROMagar™ Vibrio, VB912)**.** Technical duplicates were performed as plate 1 and 2 for each dilution of each sample. The plates were incubated in inverted position for 24h at 37°C.

3.3. Differentiation of *Vibrio* Species and Isolation of *V. parahaemolyticus*

Presumptive colonies of *Vibrio* spp. were observed on the CHROMagar plates, and only 10^{-1} and/or 10^{-2} dilutions were countable. Identification of *Vibrio* spp. on the CHROMagar were interpreted based on color development as shown in Table 3.1 and Figure 3.2. Each plate falls in the colony countable range twenty to two hundred were used for Colony Forming Unit (CFU) calculation, and Log_{10} CFU were obtained.

CFU g^{-1} (or mL^{-1}) = (number of colonies x dilution factor/volume of culture plated)

Table 3.1. The colony color of *Vibrio* spp. on CHROMagar™ Vibrio.

SPECIES	COLONY COLOR
V. parahaemolyticus	Mauve
V. vulnificus	Green/Blue
V. cholerae	Green/ Blue
V. alginolyticus	Colorless

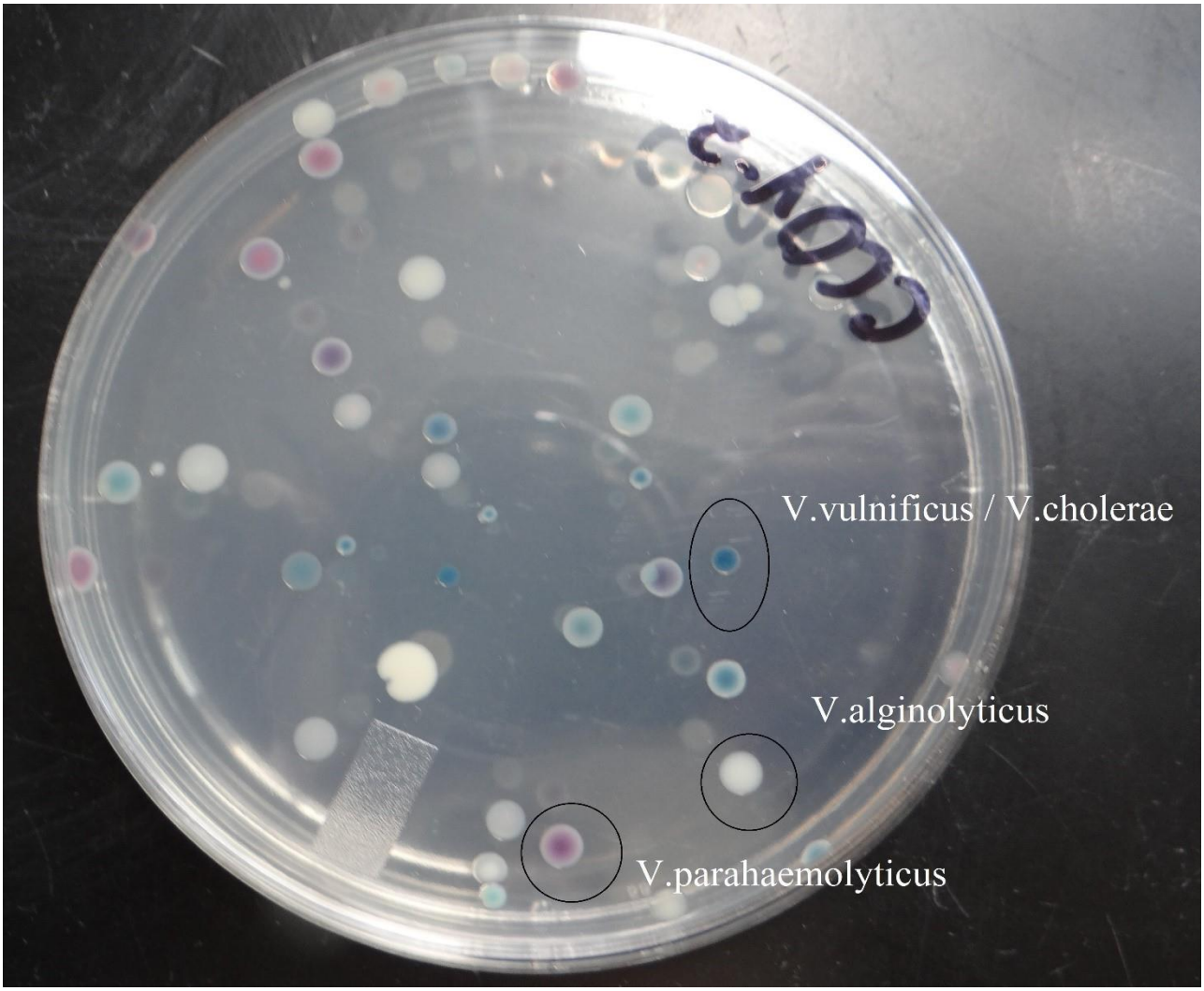

Figure 3.2 The colony color of *Vibrio* spp. on CHROMagar™ Vibrio.

Mauve colonies on the CHROMagar plates confirmed the presence of *V. parahaemolyticus*. Using the sterile loop, 20% of the mauve colonies from each plate were chosen and inoculated into 1.5 mL microcentrifuge tube of TSB, applying aseptic techniques. Bacterial isolates were incubated with overnight shaking (175 rpm) at 37°C (New Brunswick Scientific I 24 Incubator Shaker Series). Bacterial isolates in the microcentrifuge tubes were then centrifuged at 15,000 rpm for 2 min (Eppendorf Centrifuge 5424), and the supernatant was discarded. Equal amounts (600 µL) of (0.1% BWP) and (TSB + [24% G]) were added and the pellet was resuspended and then frozen at -20°C (Figure 3.3).

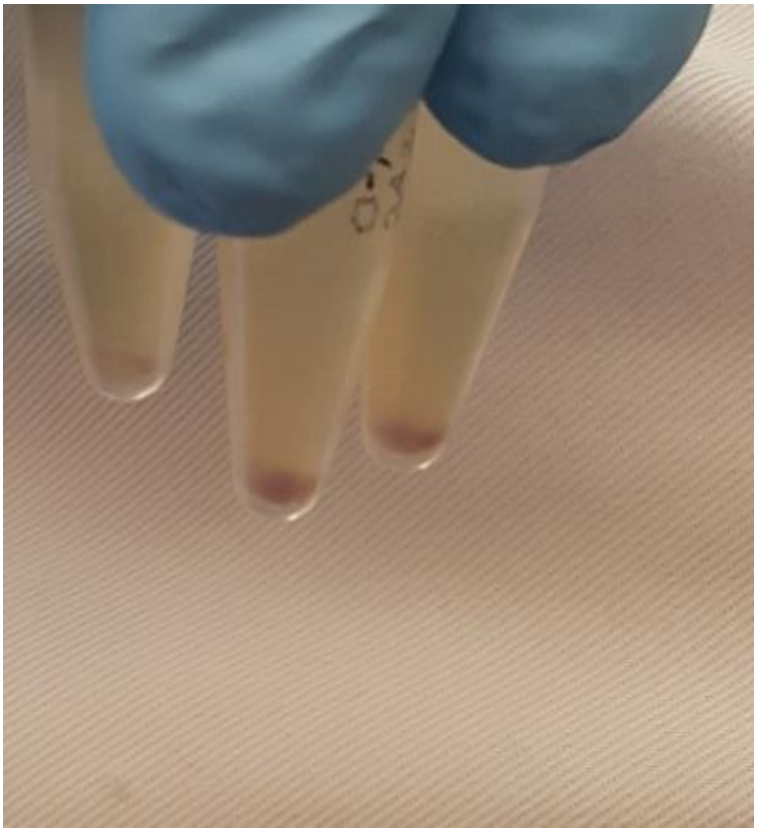

Figure 3.3 Bacterial Pellets of the mauve colonies from CHROMagar™ Vibrio plates stored at -20°C.

3.4. Molecular Characterization of *V. parahaemolyticus* Isolates

3.4.1. PCR Procedures and Conditions

Five sets of primers previously assessed by Luan et al. (2007b) and Nordstrom et al. (2007) were used in this study as shown in Table 3.2. The PCR reaction mixture consisted of 1 µL of the tested DNA template, 2 µL of the 5x green buffer (Taq Buffer; PROMEGA, USA), 0.1 µL of Taq polymerase (Taq; PROMEGA; USA), 0.4 µL of 2.5 mM deoxynucleotide, 0.2 µL of each forward and reverse primers (IDT; USA), and 6.1 µL of nuclease free water. The amplification conditions of *tlh, tdh, trh, toxR,* and *vpm* genes are shown in Table 3.3, and PCR reactions were performed using S1000; Thermal Cycler; Bio-Rad.

Three control samples were used in this study as follow:

1) 1 µL of nuclease free water (No DNA Template- negative control)

2) 1 µL of *V. vulnificus* (Non-target DNA Template-negative control)

3) 1 µL of *V. parahaemolyticus* DNA template-positive control)

Table 3.2. Summary of the properties of the primers used in this study.

Primer set	Template sequence	Size of PCR product	Primer sequence 5'-3'
1	*tlh*	200	F-tlh: ACTCAACACAAGAAGAGATCGACAA R-tlh: GATGAGCGGTTGATGTCCAA
2	*tdh*	269	F-tdh: TCCCTTTTCCTGCCCCC R-tdh: CGCTGCCATTGTATAGTCTTTATC
3	*trh*	290	F-trh: TTGCTTTCAGTTTGCTATTGGCT R-trh: TGTTTACCGTCATATAGGCGCTT
4	*toxR*	367	toxR-4: GTCTTCTGACGCAATCGTTG toxR-7: ATACGAGTGGTTGCTGTCATG
5	*vpm*	675	vpm1: CAGCTACCGAAACAGACGCTA vpm2: TCCTATCGAGGACTCTCTCAAC

Table 3.3. PCR conditions used in this study.

PCR Amplification Conditions		
***tlh* gene (species- specific)**	***tdh*/*trh* genes (toxic factors)**	***toxR*/*vpm* genes (virulent indicators)**
Denaturation Temp	Denaturation Temp	Denaturation Temp
95°C/30sec	95°C/30sec	94°C/1 min
Annealing Temp	Annealing Temp	Annealing Temp
60°C/45sec	60°C/45sec	58°C/1 min
Extension Temp	Extension Temp	Extension Temp
68°C/1 min	68°C/1 min	72°C/1 min

3.4.2. Gel Electrophoresis Procedure

PCR amplicons were visualized using the gel electrophoresis system (FB-SB-1316; Electrophoresis System; Fisher Scientific; USA). A gel casting stand containing a solidified 1% agarose gel with 20 gel wells was placed into the Gel box (electrophoresis unit), and the gel box was filled with 1xTAE running buffer until the gel was covered. PCR samples were loaded into the gel wells in the following order: no DNA template- negative control, non-target DNA template-negative control, bacterial isolates, DNA template-positive control, and molecular weight ladder (100bp). The gel was run at 130 V until the dye line is approximately 80-85% of the way down the gel. DNA bands were pictured using gel documentation system (Syngene, G: BOX EF) where DNA fragments are usually appearing as bands.

3.5. Data Analysis

3.5.1 Multiple Linear Regression Test

Multiple regression analysis was used to understand whether *Vibrio* levels (CFU) in oyster and seawater samples from Delaware Bay could be predicted based on temperature,

salinity, dissolved oxygen, and chlorophyll *a*. Here, the continuous dependent variable would be "Log_{10} CFU g^{-1} (or mL^{-1})", while the continuous independent variables were temperature, salinity, dissolved oxygen, and chlorophyll *a*. Multiple regression can be used to determine how much of the variation in CFUs is explained by the independent variables "as a whole", but also what the "relative contribution" of each of these independent variables was in explaining the variance. Furthermore, multiple regression tests were used to explain if there is an association between *V. parahaemolyticus* (CFU) levels and temperature, turbidity, dissolved oxygen, and chlorophyll *a* collectively or for each parameter. Moreover, the assumptions that are necessary to be met when using multiple regression analysis were checked, and pretreatments of the data were performed as needed.

3.5.2. One-Way ANOVA

One-Way ANOVA test was used to determine whether there were any statistically significant differences between the means of two or more independent groups. In this case the one-way ANOVA test was used to determine whether the mean of *Vibrio* spp. concentration (CFU) among the sample types was different. However, this test focuses on the occurrence of the significant differences, and does not indicate which specific group was significantly different from each other. The independent variable in this case was the sample type consisting of two groups: oyster and seawater samples, while the dependent variable was the Log_{10} CFUs of *Vibrio*. The assumptions that are necessary for one-way ANOVA test was checked.

CHAPTER 4: RESEARCH FINDINGS AND DISCUSSION

4.1. Physico-chemical Water Quality Parameters

Physico-chemical water quality parameters (Refer to Appendix A Page 68) illustrate that water temperature ranged from 14.63 °C (LW, October) to 28 °C (BB, August). Salinity levels were in the range of 5.37ppt (LW, October) to 32ppt (SL, August). The lowest-range and highest-range dissolved oxygen (3.12 to 8.23 mg/L) were recorded during the months of August and October from BB and LW sites, respectively. The minimum pH value of 6.44 (LW) and the maximum of 8.82 (BB) were observed during the month of October. In terms of turbidity and chlorophyll *a*, the minimum and maximum levels ranged from 19 to 55.35 NTU/FTU and 0.134 to 1.174, respectively. Notably, at the LW site and during the month of October, water quality parameters displayed the lowest range of water temperature (14.63°C), minimum level of salinity (5.37ppt), highest range of dissolved oxygen (8.23mg/L), and minimum pH value of (6.44). This shows that temperature and salinity are inversely correlated with dissolved oxygen concentrations (NOAA, 2017).

4.2. Identification and Detection of Presumptive *Vibrio* Species

Using culture-based characterization method, *Vibrio* species were distinguished on the CHROMagar™ Vibrio based on the color development. Colonies were categorized into mauve, green/blue, and colorless indicating the presence of *V. parahaemolyticus*, *V. vulnificus*/*V. cholerae*, and *V. alginolyticus* respectively. The averages of Colony Forming Units CFU g^{-1} (or mL^{-1}) for each month for both oyster and seawater samples are displayed in Tables 4.1, 4.2, and 4.3 from BB, LW, and SL sites, respectively.

Table 4.1. Averages of CFU g^{-1} (or mL^{-1}) in relation to sample type, and sampling time from Bowers Beach (BB) study site.

Month	Oyster Mauve	Water Mauve	Oyster Green/Blue	Water Green/Blue	Oyster Colorless	Water Colorless	Total O/W
June	2017	33	5817	50	3417	17	11251/100
July	**9633**	1100	7983	1867	**10350**	1500	27966/4467
Aug	980	617	3920	1767	3180	1583	8080/3967
Sep	417	<10	250	33	83	100	750/133
Oct	17	33	17	33	50	17	84/83

O/W= Total CFU g^{-1} of Oyster Samples / and Total CFU mL^{-1} of Seawater Samples; <10 (non-detectable).

Table 4.2. Averages of CFU g^{-1} (or mL^{-1}) in relation to sample type, and sampling time from Lewes, Broadkill (LW) study site.

Month	Oyster Mauve	Water Mauve	Oyster Green/Blue	Water Green/Blue	Oyster Colorless	Water Colorless	Total O/W
June	367	83	533	67	450	383	1350/533
July	1850	167	867	<10	1183	433	3900/600
Aug	1133	117	2083	133	**17350**	100	20566/350
Sep	<10	17	<10	50	<10	<10	<10/67
Oct	<10	<10	<10	<10	<10	33	<10/33

O/W= Total CFU g^{-1} of Oyster Samples / and Total CFU mL^{-1} of Seawater Samples; <10 (non-detectable).

Table 4.3. Averages of CFU g^{-1} (or mL^{-1}) in relation to sample type, and sampling time from Slaughter Beach (SL) study site.

Month	Oyster Mauve	Water Mauve	Oyster Green/Blue	Water Green/Blue	Oyster Colorless	Water Colorless	Total O/W
Aug	117	20	17	18	233	47	367/85
Sep	17	33	<10	33	<10	100	17/166
Oct	<10	<10	<10	<10	<10	17	<10/17

O/W= Total CFU g^{-1} of Oyster Samples / and Total CFU mL^{-1} of Seawater Samples; <10 (non-detectable).

Tables 4.1 to 4.3 illustrate that the total mean levels of *Vibrio* in oysters ranged from <10 CFU g^{-1} (non-detectable) to 2.8×10^4 CFU g^{-1}, while the total mean levels of *Vibrio* in seawater samples ranged from 1.7×10 CFU mL^{-1} to 4.47×10^3 CFU mL^{-1}. Mean levels of *V. alginolyticus* (colorless colonies) from LW site during the month of August was the highest (1.74×10^4 CFU g^{-1}) among *Vibrio* spp. from oyster samples. On the other hand, the highest mean levels of *V. parahaemolyticus* (mauve colonies) was 9.63×10^3 CFU g^{-1} in the oyster samples during the

month of July from BB site, and that was higher than *V. parahaemolyticus* (CFU) levels (6.0×10^2 CFU g^{-1}) detected by direct plating-colony hybridization procedure in Maryland Chesapeake Bay oysters (Parveen et al, 2008). According to the U.S. Food and Drug Administration (FDA) safety levels in regulations and guidance, *V. parahaemolyticus* levels (Kanagawa positive or negative) in this study did not exceed the safety limits ($\geq 1\times10^4$ CFU g^{-1}) for ready-to-eat food (FDA, 1986). Clearly, all presumptive *Vibrio* spp (CFU) levels, agrees well with the strong correlations between water temperature and *Vibrio* densities that reported in the literature (Parveen et al., 2008; Takemura et al., 2014; Urquhart et al., 2016), indicating the increase of *Vibrio* levels with the increase of temperature and vice versa. CFU counts of *Vibrio* spp. from seawater samples are notably much lower than oyster samples demonstrating that oysters can concentrate *Vibrio* spp. up to ten-fold compared to the water of the surrounding areas (Morris, 2003). Seawater samples from LW on July demonstrated <10 CFU g^{-1} (non-detectable) of *V. vulnificus/V. cholerae* indicating that there are parameters other than temperature that may affect the growth of this species (Urquhart et al., 2016). Both oyster and seawater samples from LW and SL sites in October showed <10 CFU g^{-1} (or CFU mL^{-1}) (non-detectable) of *V. vulnificus/V. cholerae* and *V. parahaemolyticus*; however, *V. alginolyticus* were detected in the seawater samples from LW and SL showing 3.3×10 and 1.7×10 CFU mL^{-1} respectively. Detection limit of *V. parahaemolyticus* and *V. vulnificus*/*V. cholerae* in oyster was 1.7×10 CFU g^{-1}, while of *V. alginolyticus* was 5.0×10 CFU g^{-1}. However, in the seawater samples, the detection limit of *V. parahaemolyticus* and *V. alginolyticus* was 1.7×10 CFU mL^{-1}, while of *V. vulnificus*/*V. cholerae* was 1.8×10 CFU mL^{-1}. Log_{10} CFU g^{-1} (or mL^{-1}) were obtained and the figures, (4.1 to 4.8) demonstrate the CFU g^{-1} (or mL^{-1}) and Log_{10} CFU g^{-1} (or mL^{-1}) in relation of sample type, site, time of collection, and *Vibrio* spp. (color of colonies).

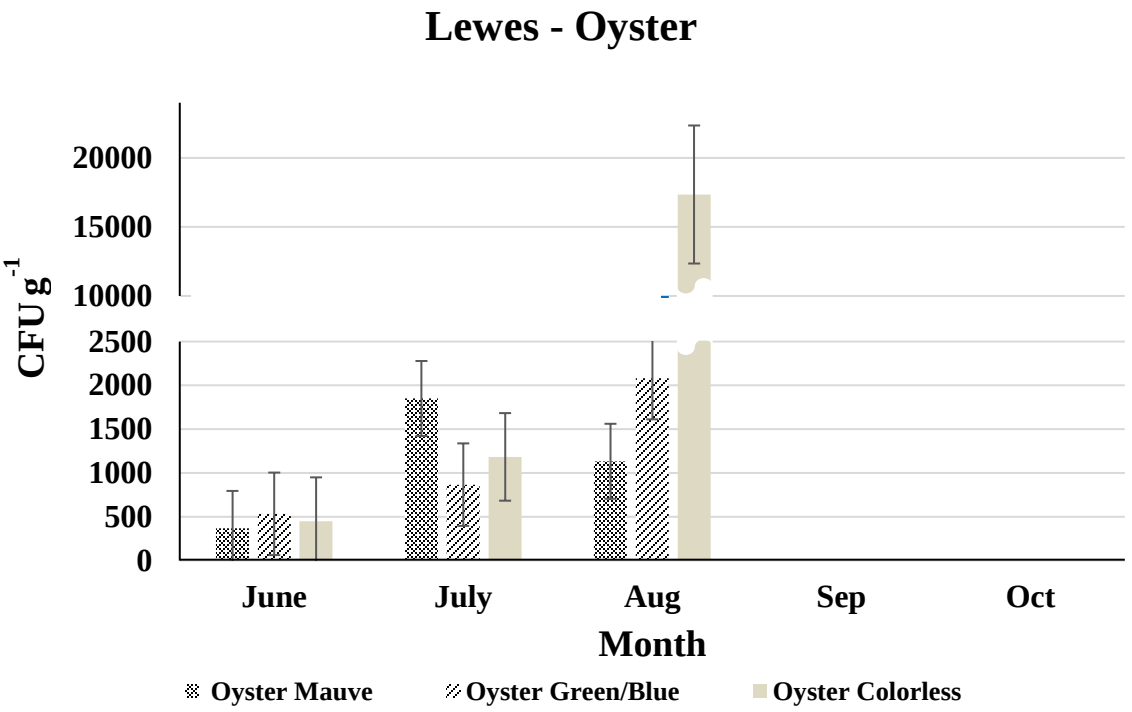

Figure 4.1.a. Average *Vibrio* levels in oyster samples from Lewes, Broadkill in relation to time collection (CFU g^{-1}).

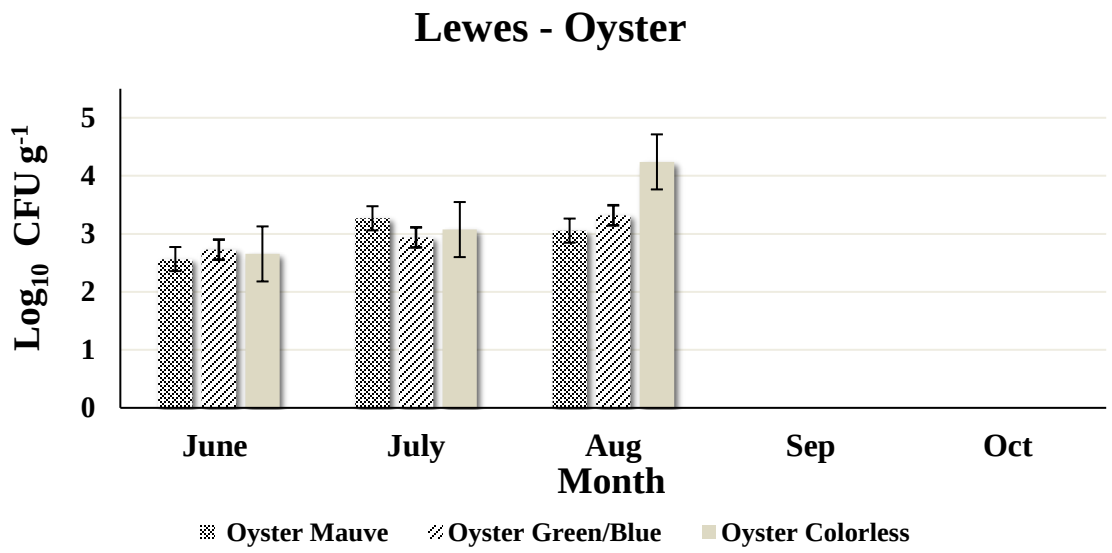

Figure 4.1.b. Average *Vibrio* levels in oyster samples from Lewes, Broadkill in relation to time collection (Log_{10} CFU g^{-1}).

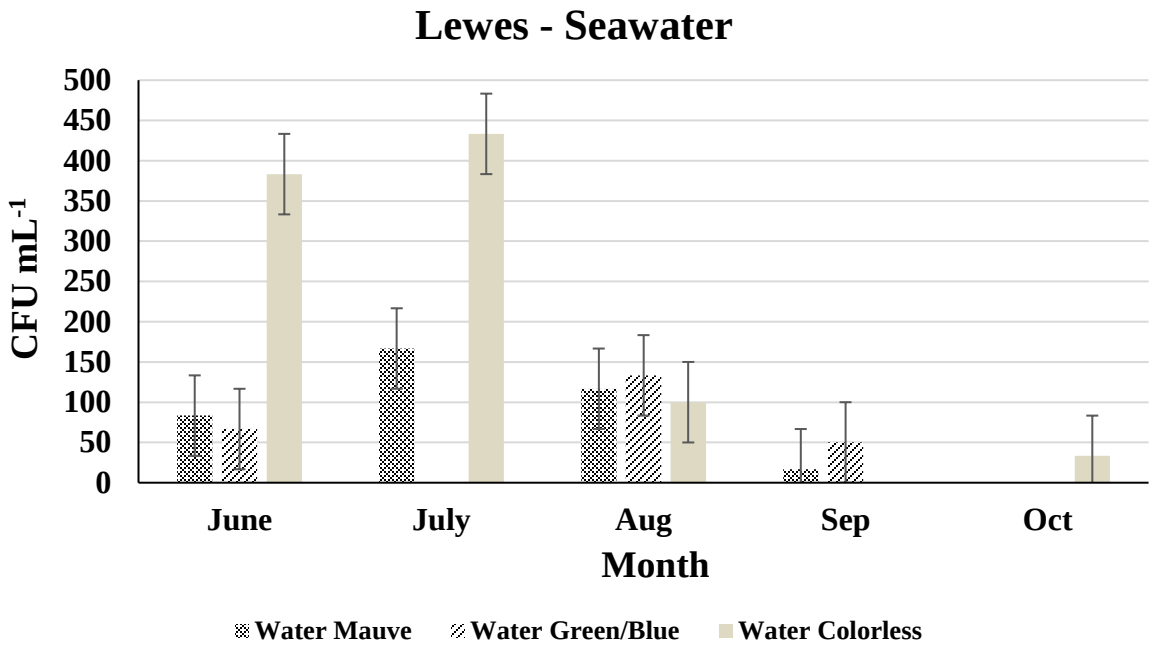

Figure 4.2.a. Average *Vibrio* levels in seawater samples from Lewes, Broadkill in relation to time collection (CFU mL^{-1}).

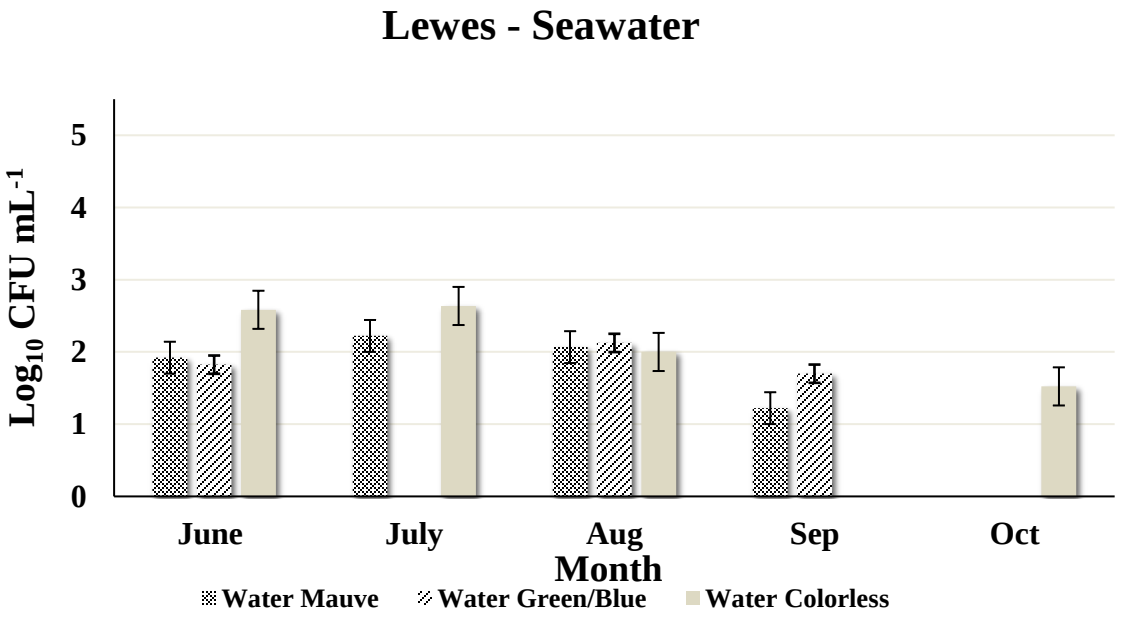

Figure 4.2.b. Average *Vibrio* levels in seawater samples from Lewes, Broadkill in relation to time collection (Log_{10} CFU mL^{-1}).

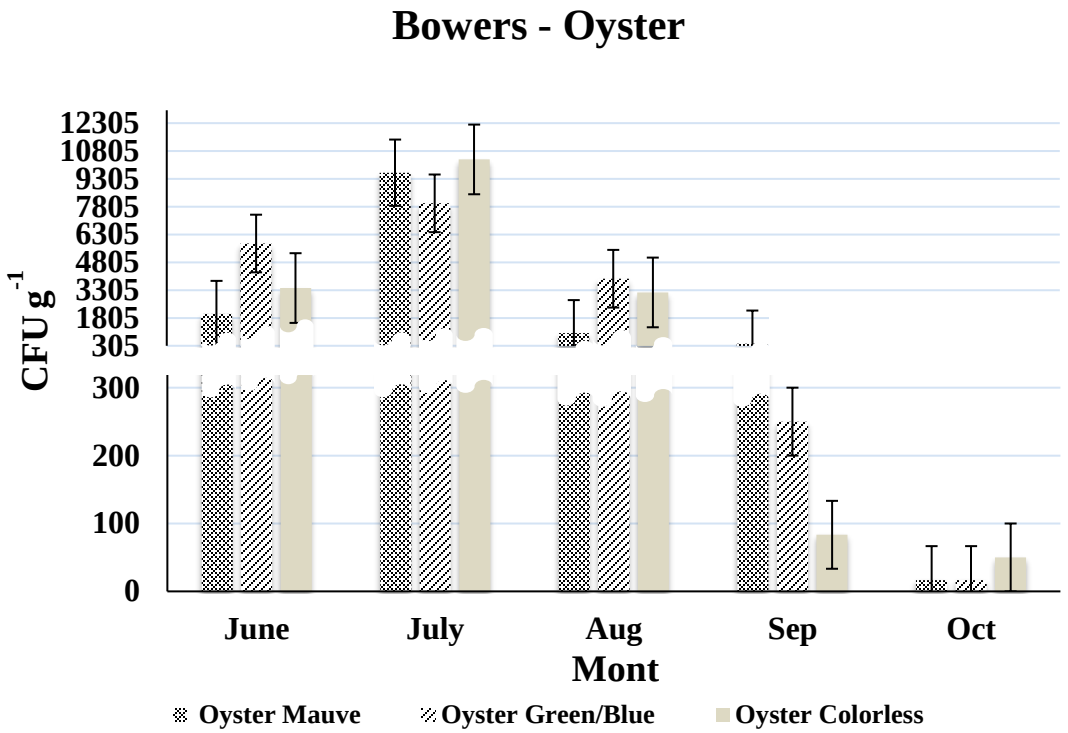

Figure 4.3.a. Average *Vibrio* levels in oyster samples from Bowers Beach in relation to time collection (CFU g^{-1}).

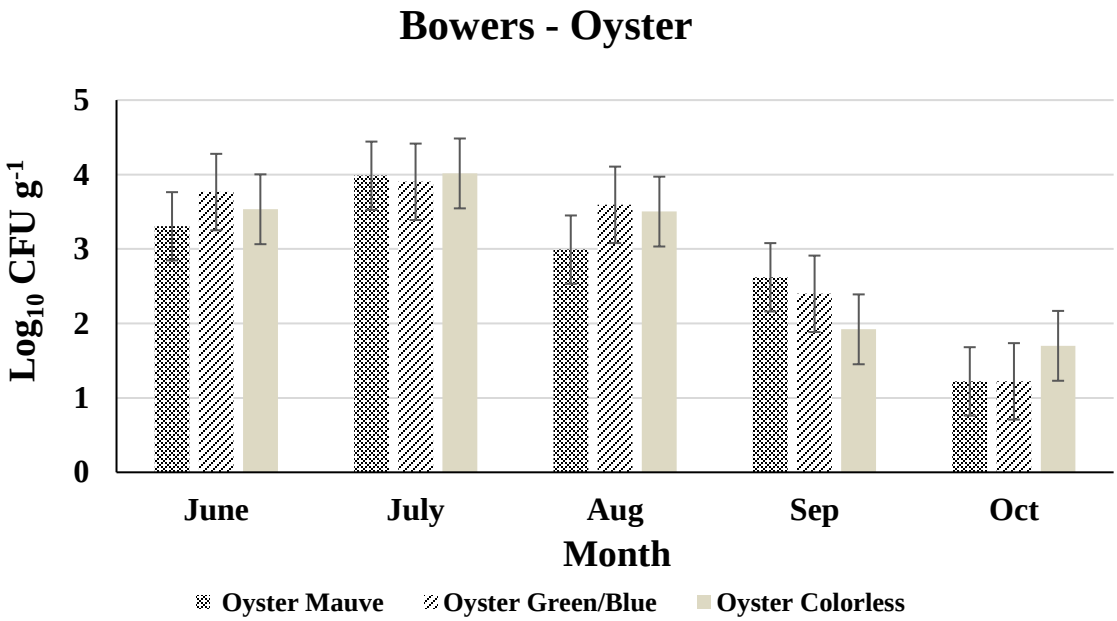

Figure 4.3.b. Average *Vibrio* levels in oyster samples from Bowers Beach in relation to time collection (Log_{10} CFU g^{-1}).

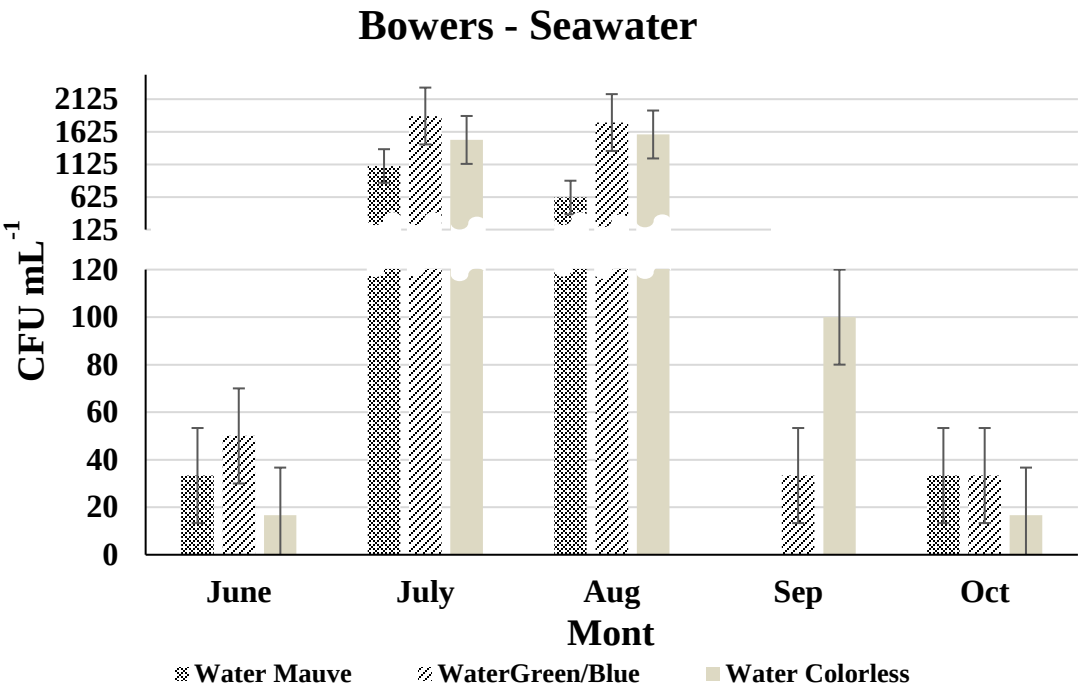

Figure 4.4.a. Average *Vibrio* levels in seawater samples from Bowers Beach in relation to time collection (CFU mL^{-1}).

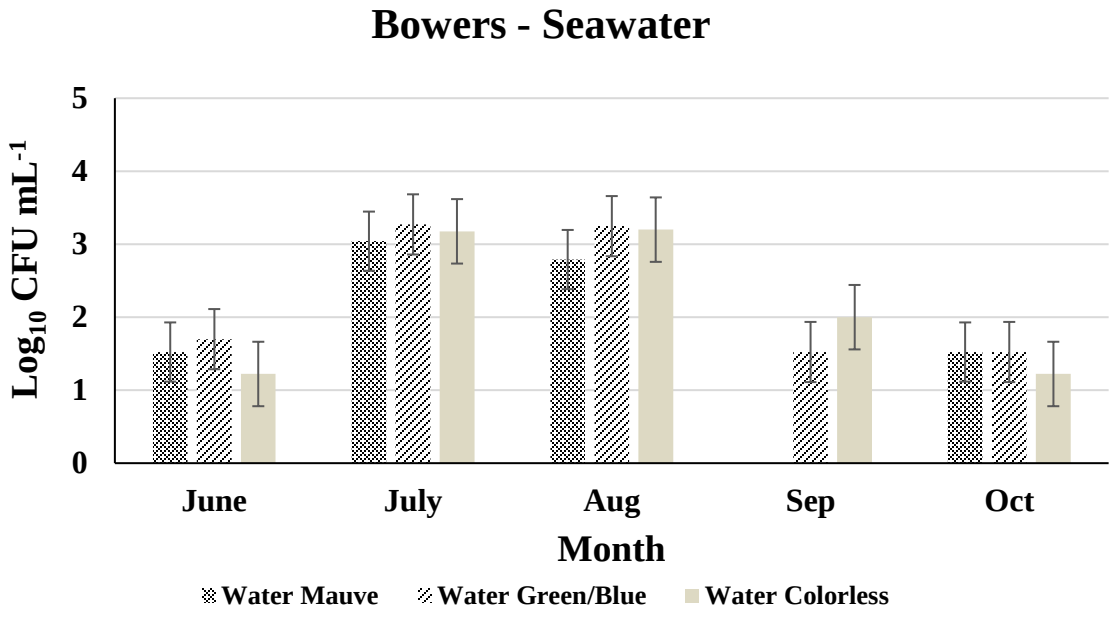

Figure 4.4.b. Average *Vibrio* levels in seawater samples from Bowers Beach in relation to time collection (Log_{10} CFU mL^{-1}).

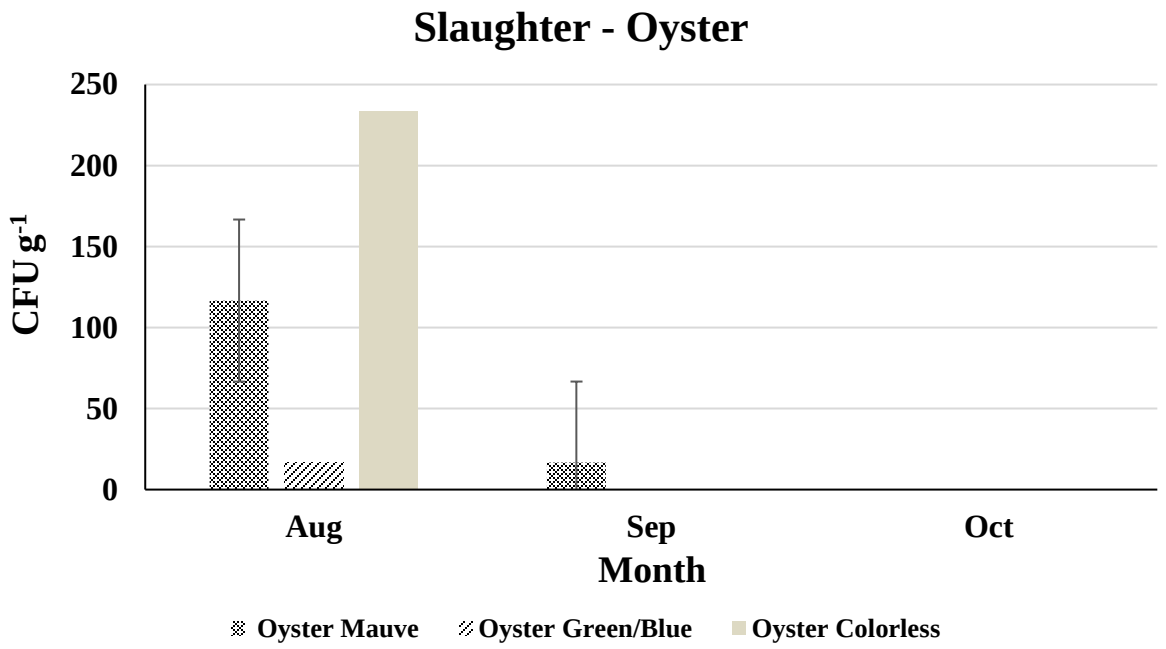

Figure 4.5.a. Average *Vibrio* levels in oyster samples from Slaughter Beach in relation to time collection (CFU g^{-1}).

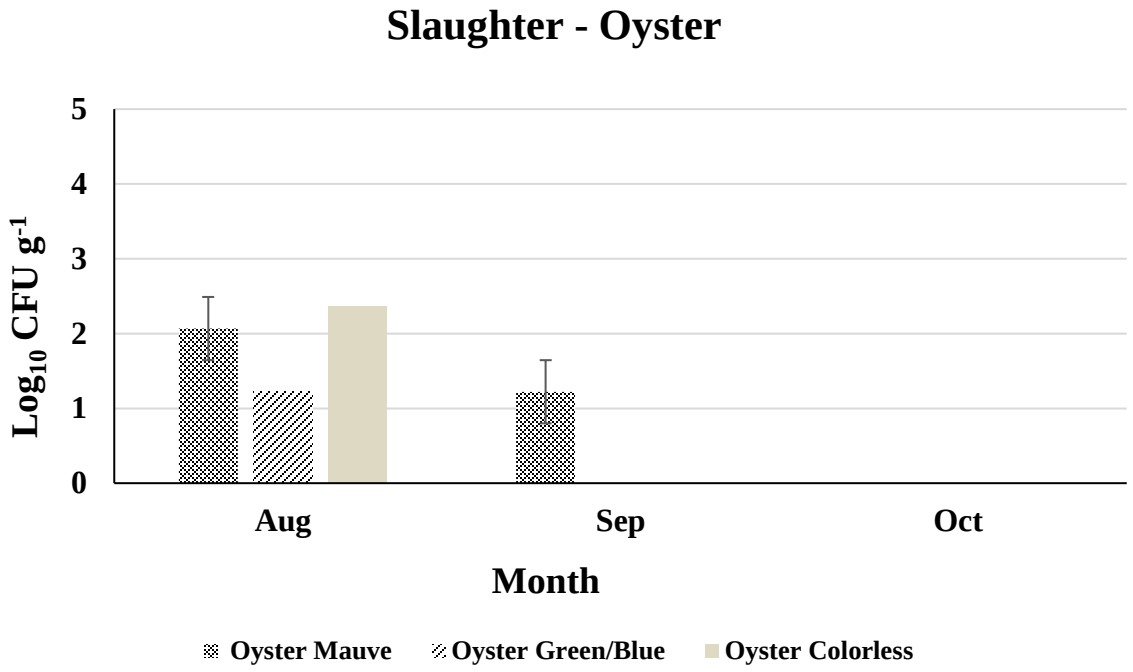

Figure 4.5.b. Average *Vibrio* levels in oyster samples from Slaughter Beach in relation to time collection (Log_{10} CFU g^{-1}).

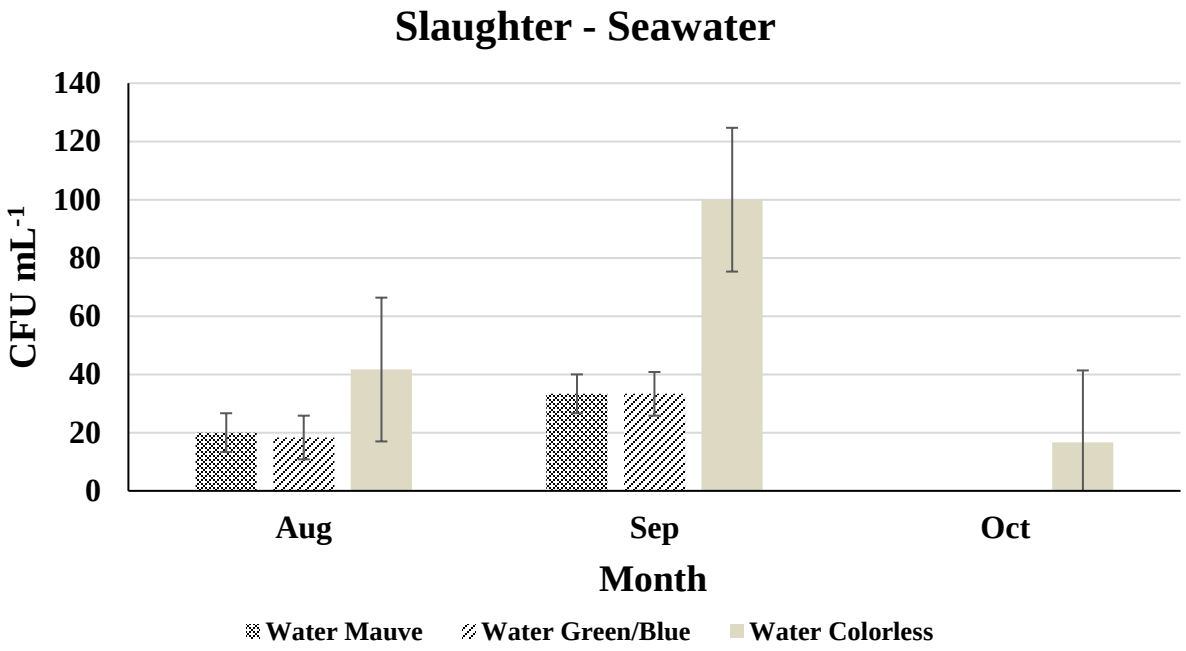

Figure 4.6.a. Average *Vibrio* levels in seawater samples from Slaughter Beach in relation to time collection (CFU mL^{-1}).

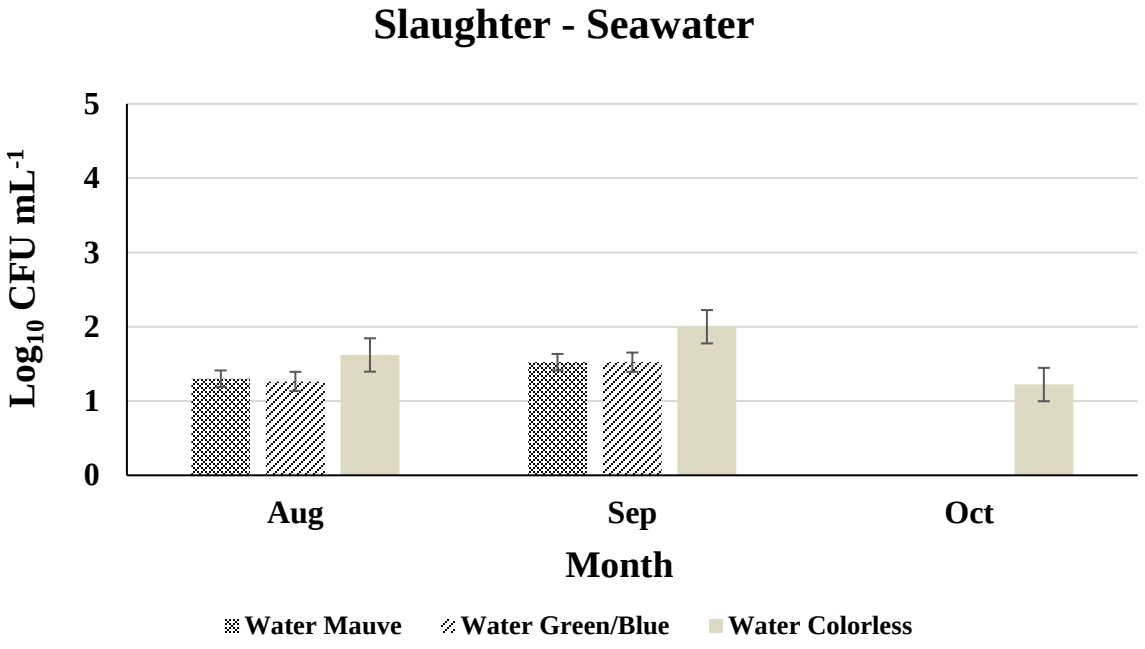

Figure 4.6.b. Average *Vibrio* levels in seawater samples from Slaughter Beach in relation to time collection (Log_{10} CFU mL^{-1}).

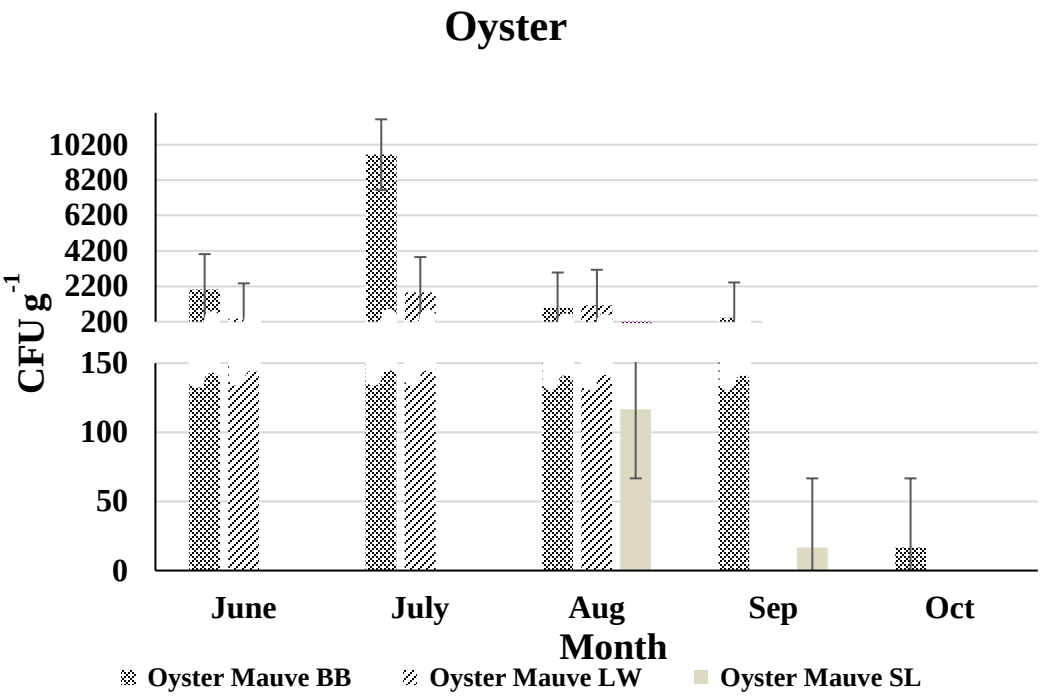

Figure 4.7.a. Average *Vibrio parahaemolyticus* levels in oyster samples in relation to time collection, and sites. Bowers Beach (BB) - Lewes, Broadkill (LW) - Slaughter Beach(SL). (CFU g^{-1}).

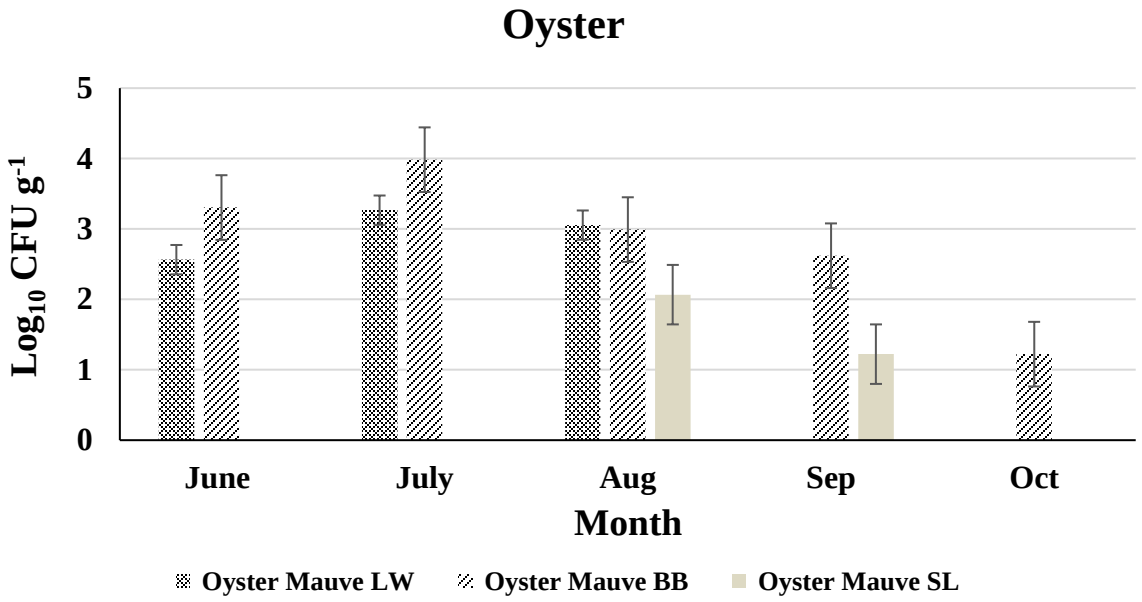

Figure 4.7.b. Average *Vibrio parahaemolyticus* levels in oyster samples in relation to time collection, and sites. Bowers Beach (BB) - Lewes, Broadkill (LW) - Slaughter Beach(SL) (Log_{10} CFU g^{-1}).

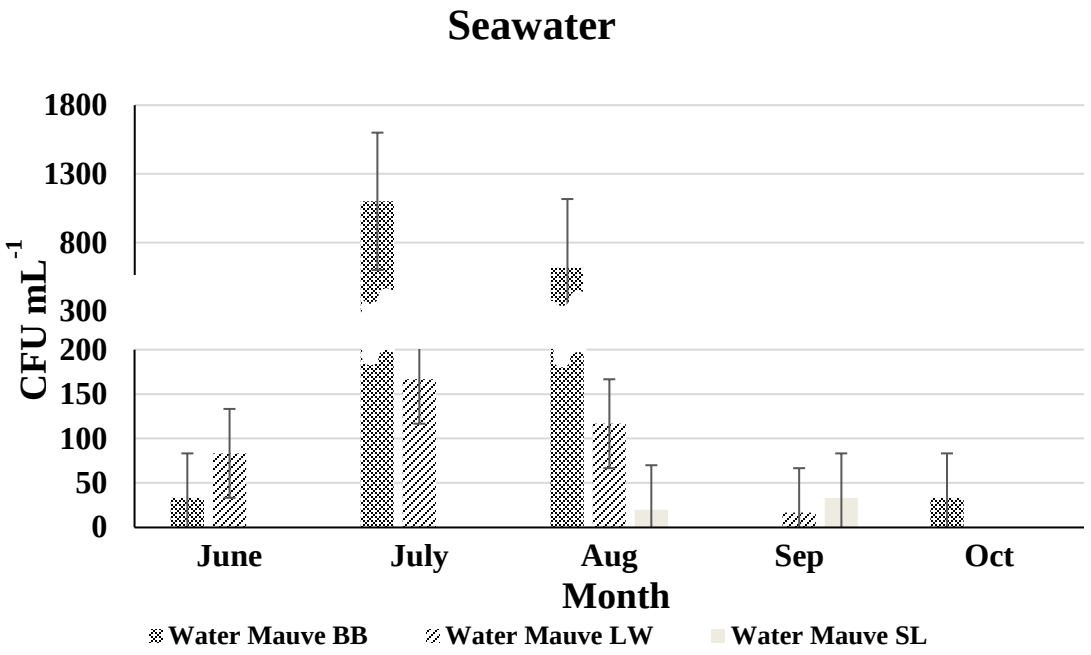

Figure 4.8.a. Average *Vibrio parahaemolyticus* levels in seawater samples in relation to time collection, and sites. Bowers Beach (BB) - Lewes, Broadkill (LW) - Slaughter Beach(SL). (CFU mL^{-1}).

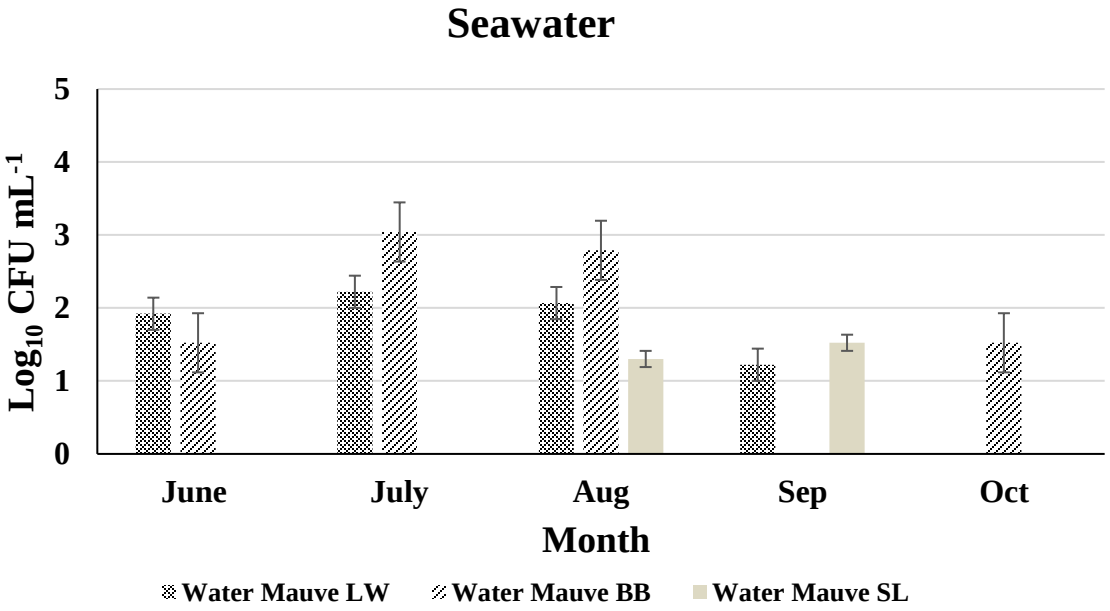

Figure 4.8.b. Average *Vibrio parahaemolyticus* levels in seawater samples in relation to time collection, and sites. Bowers Beach (BB) - Lewes, Broadkill (LW) - Slaughter Beach (SL) (Log_{10} CFU mL^{-1}).

4.2. Molecular Identification and Characterization of *V. parahaemolyticus*

A total of 165 *V. parahaemolyticus* isolates (mauve colonies) were examined for the presence of *tlh*, *tdh*, *trh*, *toxR*, and *vpm* genes. The representative gel photos for the PCR targeting *tdh*, *trh*, *toxR*, *vpm*, and *tlh* genes are shown in figures 4.9 to 4.13, respectively.

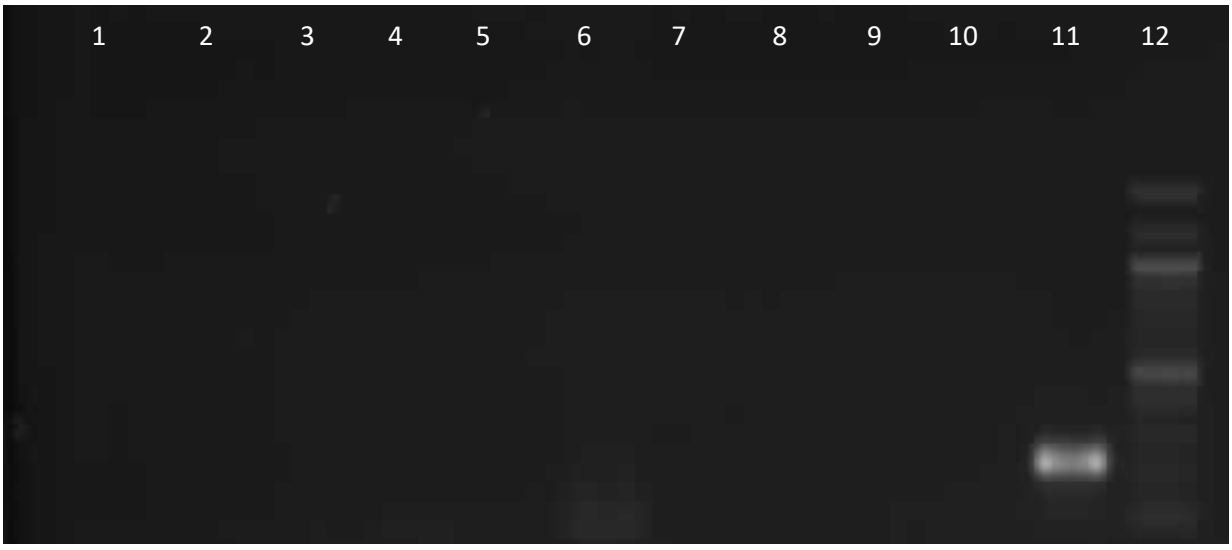

Figure 4.9. PCR amplicons targeting *tdh* gene. Lane 1, no DNA negative control; lane 2, non-target DNA negative control; lanes 3-10 bacterial isolates (269 bp); lane 11, *V. parahaemolyticus* positive control (269 bp); lane 12, 100 bp DNA marker.

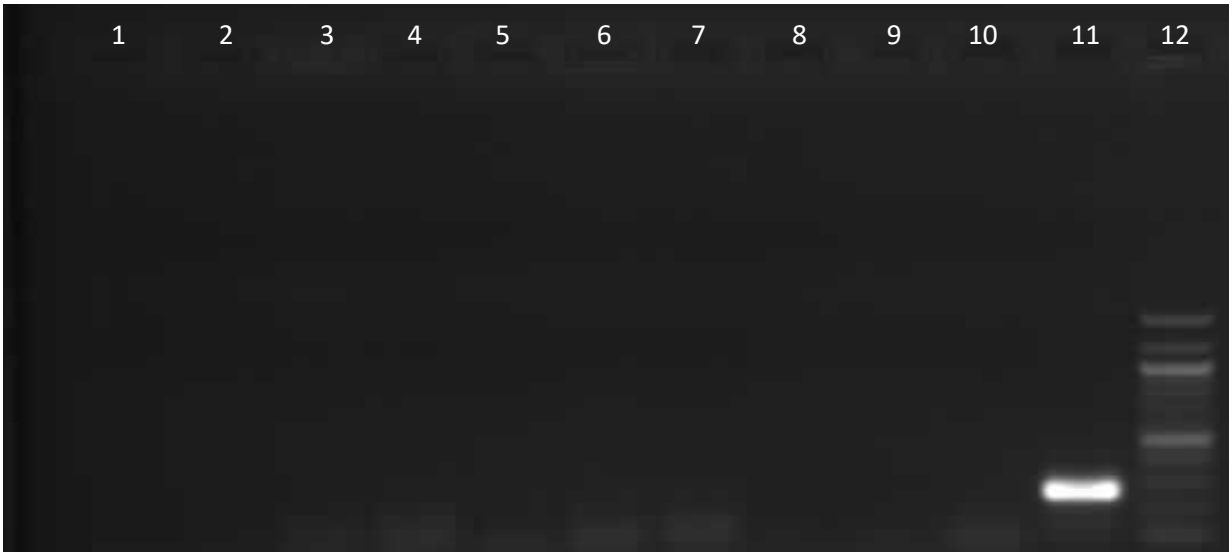

Figure 4.10. PCR amplicons targeting *trh* gene. Lane 1, no DNA negative control; lane 2, non-target DNA negative control; lanes 3-10 bacterial isolates (290 bp); lane 11, *V. parahaemolyticus* positive control (290 bp); lane 12, 100 bp DNA marker.

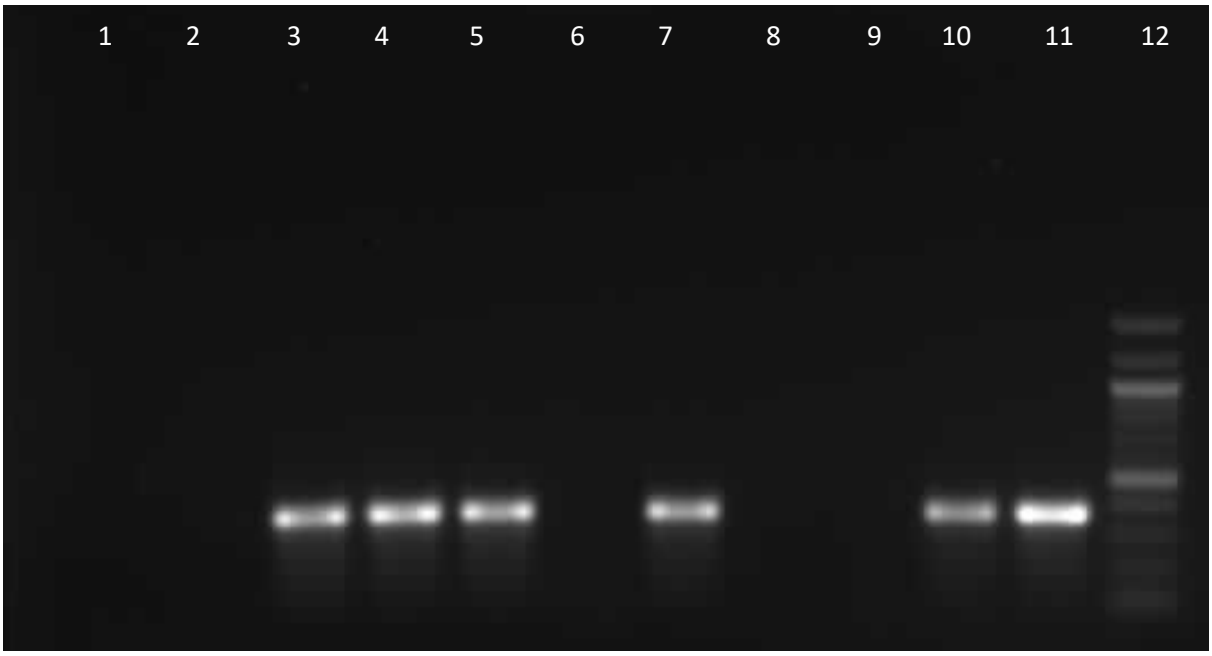

Figure 4.11. PCR amplicons targeting *toxR* gene. Lane 1, no DNA negative control; lane 2, non-target DNA negative control; lanes 3-10 bacterial isolates (367 bp); lane 11, *V. parahaemolyticus* positive control (367 bp); lane 12, 100 bp DNA marker.

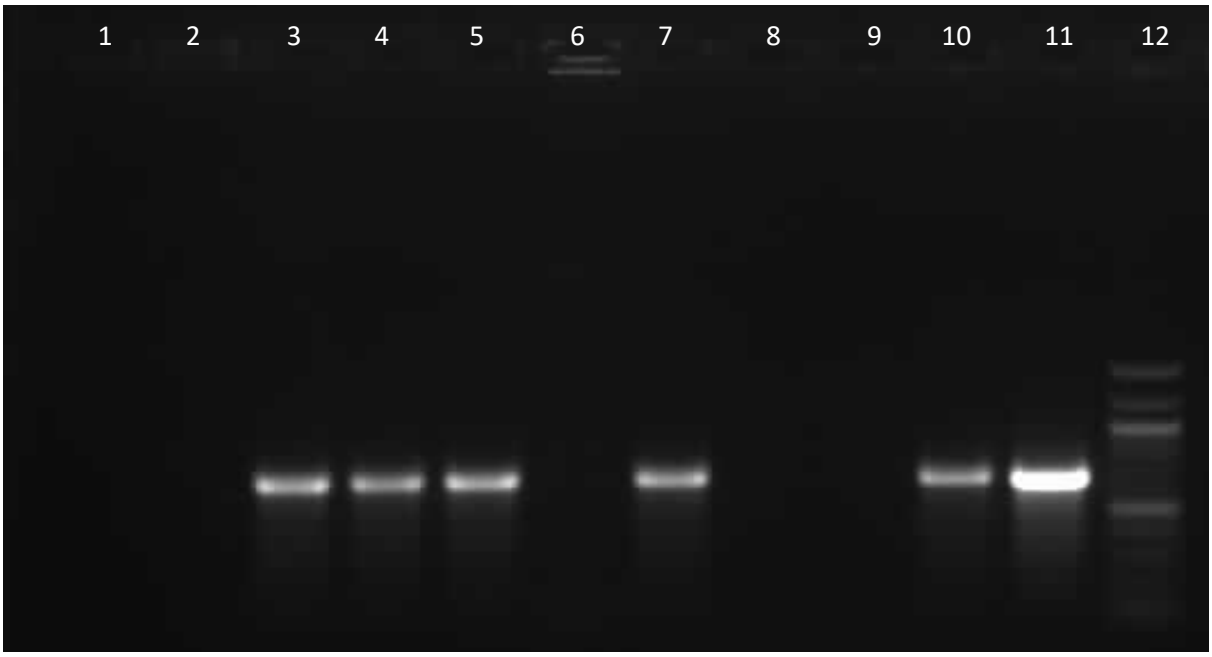

Figure 4.12. PCR amplicons targeting *vpm* gene. Lane 1, no DNA negative control; lane 2, non-target DNA negative control; lanes 3-10 bacterial isolates (675 bp); lane 11, *V. parahaemolyticus* positive control (675 bp); lane 12, 100 bp DNA marker.

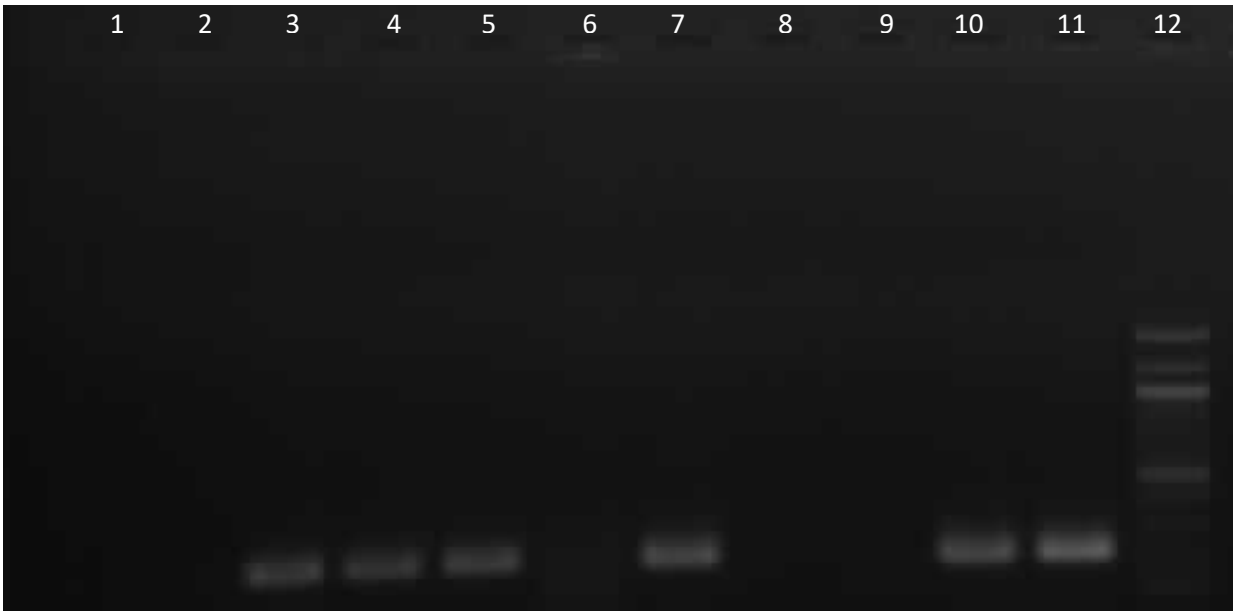

Figure 4.13. PCR amplicons targeting *tlh* gene. Lane 1, no DNA negative control; lane 2, non-target DNA negative control; lanes 3-10 bacterial isolates (190 bp); lane 11, *V. parahaemolyticus* positive control (190 bp); lane 12, 100 bp DNA marker.

The number of samples according to their collection site, time of collection, and sample type are shown in Tables 4.4 to 4.7.

Table 4.4. Gene Occurrence Among the Total of 165 *V. parahaemolyticus* Isolates.

	# of Isolates (165)	
Gene	**Presence**	**%**
tdh	31	19
trh	39	24
toxR	108	65.5
vpm	110	67
tlh	137	83

Table 4.5. Distribution of Gene Presence According to The Sampling Time.

a) Gene Presence Among *V. parahaemolyticus* Isolates Collected in June.

Gene	# of Isolates (76) Presence	%
tdh	4	15.4
trh	8	30.8
toxR	20	76.9
vpm	18	69.2
tlh	23	88.5

# of Isolates are the number of mauve colonies from the CHROMagar plates.

b) Gene Presence Among *V. parahaemolyticus* Isolates Collected in July.

Gene	# of Isolates (76) Presence	%
tdh	27	35.5
trh	30	39.5
toxR	50	66
vpm	61	80
tlh	70	92

# of Isolates are the number of mauve colonies from the CHROMagar plates.

c) Gene Presence Among *V. parahaemolyticus* Isolates Collected in August.

Gene	# of Isolates (50) Presence	%
tdh	0	0
trh	1	2
toxR	30	60
vpm	25	50
tlh	35	70

# of Isolates are the number of mauve colonies from the CHROMagar plates.

d) Gene Presence Among *V. parahaemolyticus* Isolates Collected in September.

Gene	# of Samples (10) Presence	%
tdh	0	0
trh	0	0
toxR	7	70
vpm	6	60
tlh	8	80

# of Isolates are the number of mauve colonies from the CHROMagar plates.

e) Gene Presence Among *V. parahaemolyticus* Isolates Collected in October.

Gene	# of Samples (3) Presence	%
tdh	0	0
trh	0	0
toxR	1	33
vpm	0	0
tlh	1	33

# of Isolates are the number of mauve colonies from the CHROMagar plates.

Table 4.6. Distribution of Gene Presence According to the Study Sites.

a) Gene Presence Among *V. parahaemolyticus* Isolates Collected from Bowers Beach.

Gene	# of Isolates (84) Presence	%
tdh	8	9.5
trh	14	17
toxR	52	62
vpm	56	67
tlh	75	89

# of Isolates are the number of mauve colonies from the CHROMagar plates.

b) Gene Presence Among *V. parahaemolyticus* Isolates Collected from Lewes, Broadkill.

Gene	# of Isolates (67) Presence	%
tdh	23	34
trh	25	37
toxR	49	73
vpm	50	75
tlh	54	81

# of Isolates are the number of mauve colonies from the CHROMagar plates.

c) Gene Presence Among *V. parahaemolyticus* Isolates Collected from Slaughter Beach.

Gene	# of Isolates (14) Presence	%
tdh	0	0
trh	0	0
toxR	7	50
vpm	4	29
tlh	7	50

# of Isolates are the number of mauve colonies from the CHROMagar plates.

Table 4.7. Distribution of Gene Presence According to Sample Sources and Collection Time.

Collection Time	Sample Location	Number of Samples	*tlh*$^+$ *trh*$^+$ *tdh*$^+$ *toxR*$^+$ *vpm*$^+$	*tlh*$^+$ *trh*$^+$ ***tdh***$^-$ *toxR*$^+$ *vpm*$^+$	*tlh*$^+$ ***trh***$^-$ *tdh*$^+$ *toxR*$^+$ *vpm*$^+$	*tlh*$^+$ *trh*$^+$ *tdh*$^+$ ***toxR***$^-$ *vpm*$^+$	*tlh*$^+$ ***trh***$^-$ ***tdh***$^-$ *toxR*$^+$ *vpm*$^+$	*tlh*$^+$ *trh*$^+$ ***tdh***$^-$ ***toxR***$^-$ *vpm*$^+$	*tlh*$^+$ *trh*$^+$ ***tdh***$^-$ *toxR*$^+$ ***vpm***$^-$	*tlh*$^+$ *trh*$^+$ ***tdh***$^-$ ***toxR***$^-$ ***vpm***$^-$	*tlh*$^+$ ***trh***$^-$ ***tdh***$^-$ *toxR*$^+$ ***vpm***$^-$	*tlh*$^+$ ***trh***$^-$ ***tdh***$^-$ ***toxR***$^-$ *vpm*$^+$	*tlh*$^+$ ***trh***$^-$ ***tdh***$^-$ ***toxR***$^-$ ***vpm***$^-$	***tlh***$^-$ ***trh***$^-$ ***tdh***$^-$ ***toxR***$^-$ ***vpm***$^-$
June	**BB**	16	2	4	1	-	4	-	1	1	-	-	2	1
	LW	10	-	-	1	-	6	-	-	-	1	-	-	2
July	**BB**	36	1	2	4	-	11	1	-	1	-	8	6	2
	LW	40	19	4	1	2	8	-	-	-	-	-	2	4
August	**BB**	22	-	1	-	-	11	-	-	-	3	-	3	4
	LW	16	-	-	-	-	9	-	-	-	-	-	2	5
	SL	12	-	-	-	-	4	-	-	-	2	-	-	6
September	**BB**	7	-	-	-	-	6	-	-	-	-	-	1	-
	LW	1	-	-	-	-	-	-	-	-	-	-	-	1
	SL	2	-	-	-	-	-	-	-	-	1	-	-	1
October	**BB**	3	-	-	-	-	-	-	-	-	1	-	-	2
	LW	-	-	-	-	-	-	-	-	-	-	-	-	-
	SL	-	-	-	-	-	-	-	-	-	-	-	-	-

Bowers Beach (BB) - Lewes, Broadkill (LW) - Slaughter Beach(SL)

About 19% of the samples tested were positive for *tdh* and 24% were positive for *trh* while 83%, 65.5%, and 67% of the samples were positive for *tlh*, *toxR*, and *vpm* respectively (Figure 4.14). The low density of *tdh*/*trh* is in agreement with the low level of these genes among environmental and food samples reported in the literature (Wagley et al., 2008; Parveen et al., 2008; Rojas et al., 2011). This also demonstrates the specificity of the *tlh* gene for *V. parahaemolyticus* species, and reliability of *toxR* /*vpm* genes for pathogenic *V. parahaemolyticus* as reported in Luan et al. (2007b). The prevalence of all genes among oyster and seawater samples resemble the same pattern of its prevalence among total isolates (Figure 4.15). At both sites (Bowers Beach and Lewes, Broadkill), the occurrence of *vpm* gene was the highest and followed by *toxR*. However, in Slaughter Beach the presence of *toxR* gene was almost twice the presence of the *vpm* gene, and that probably due to the late addition of Slaughter Beach as a sampling site (Figure 4.16).

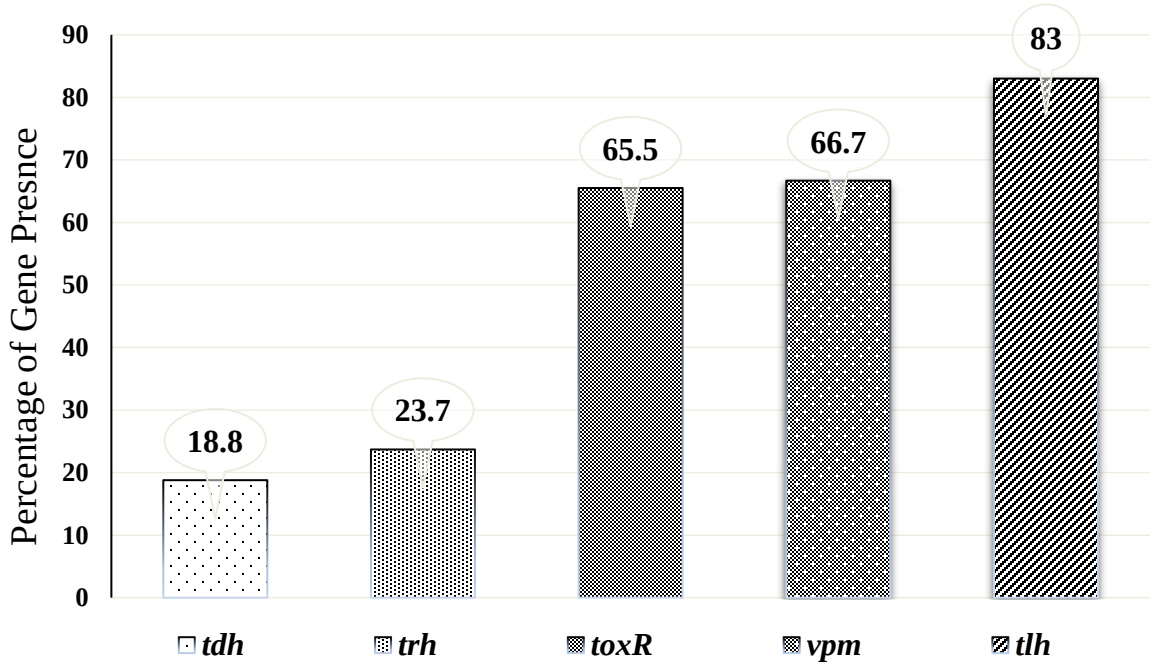

Figure 4.14. Occurrence Percentage of Genes Among Total *V. parahaemolyticus* Isolates.

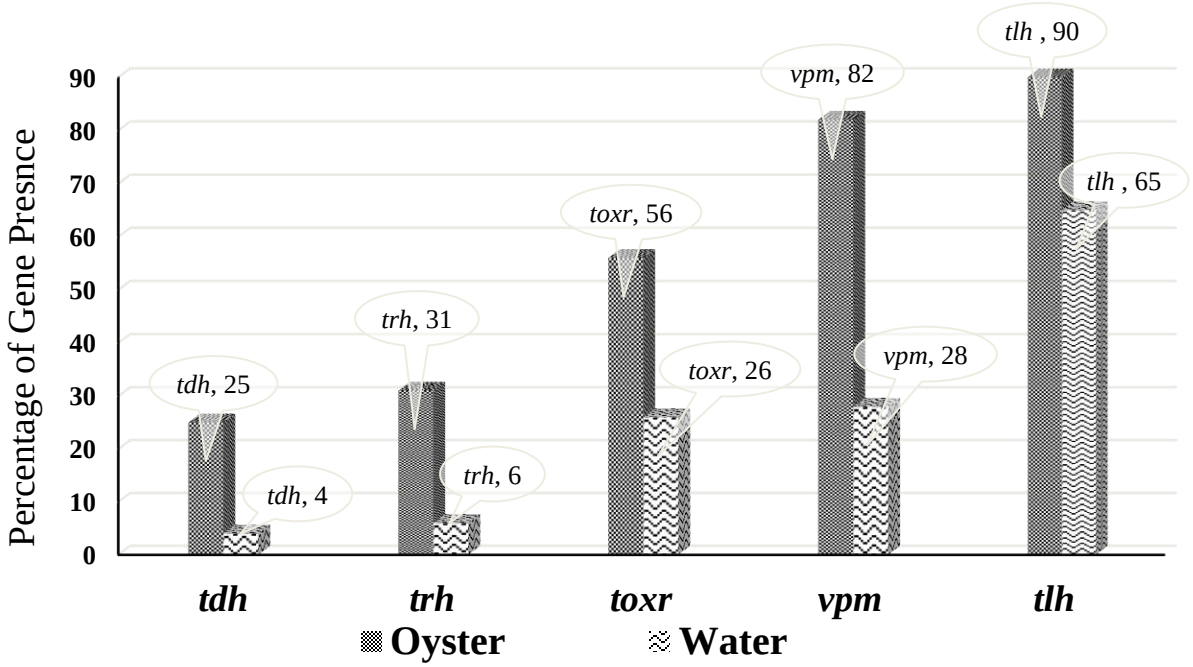

Figure 4.15. Occurrence Percentage of Genes Among *V. parahaemolyticus* Isolates from Oyster and Seawater samples.

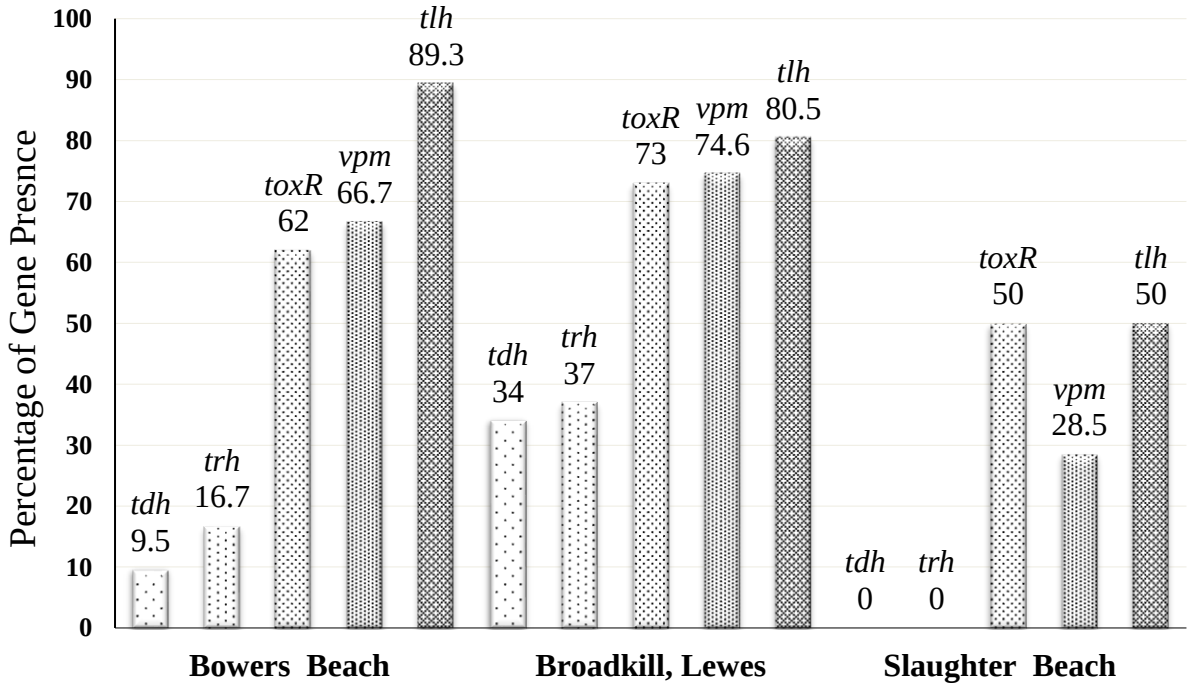

Figure 4.16. Occurrence Percentage of Genes Among *V. parahaemolyticus* Isolates from the Three Study sites.

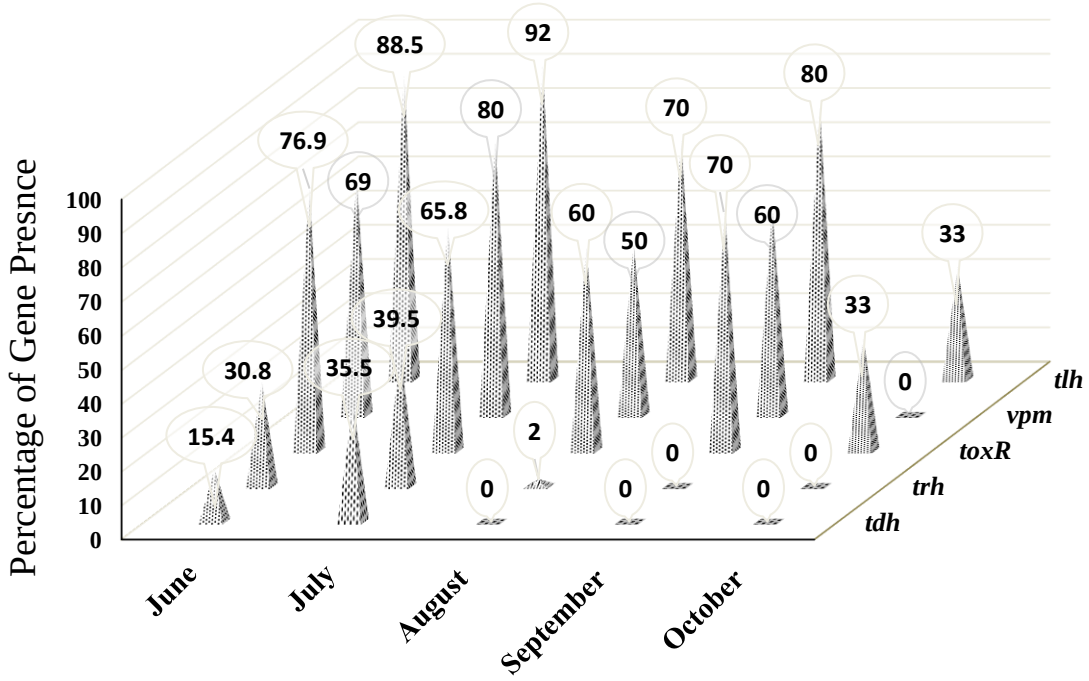

Figure 4.17. Occurrence Percentage of Genes Among *V. parahaemolyticus* Isolates During Study Months.

In contrast to the gene occurrence according to the study site and sample type, presence of *vpm* gene was higher than *toxR* only in the month of July (Figure 4.17). Furthermore, during the month of October where the water temperature ranged from 14.63 to 17.91°C, *V. parahaemolyticus* possess only *tlh*/*toxR* genes. That supports the importance of *toxR* gene for the stress tolerance of *V. parahaemolyticus* (Whitaker et al., 2012). The coexistence of *tlh, toxR, and vpm* was the dominant pattern in 36% of the total samples, and followed by the coexistence of all genes in 13% of the total samples. Furthermore, *tlh*, *toxR*, and *vpm* was the most prevalent pattern regarding the sites, time, and origin of samples (Table 4.5; Figures 4.18, 4.19, and 4.20). As a result, the third null hypothesis was rejected, and the alternative hypothesis was accepted.

Pathogenicity of strains was observed in 73% of the samples which possessed one or more of the virulence genes. However, non-pathogenic strains, possessing only *tlh* gene, made up only 10% of the total samples. Of the non-pathogenic strains, 87.5% belonged to the seawater samples (Table 4.5). The results demonstrated that virulent strains were much more likely in oysters rather than seawater samples. Furthermore, isolates obtained during the month of July possessed the highest number of virulent genes (Figure 4.17). Lewes, Broadkill and Bowers Beaches provided the greatest amount of pathogenic *V. parahaemolyticus*, respectively (Table 4.6 a and b). The high frequency of *trh* gene compared to the *tdh* gene agrees well with its occurrence in Gulf Coast and Chesapeake Bay oysters (Parveen et al., 2008). The absence of *tdh*/*trh* positive *V. parahaemolyticus* during the months of August, September, and October highlight the importance of understanding *V. parahaemolyticus* dynamics in relation with water quality parameters. Variation of gene patterns among the examined samples suggest a variation of *V. parahaemolyticus* virulent serotypes that inhabit Delaware Bay; particularly Bowers, Lewes Broadkill, and Slaughter Beaches.

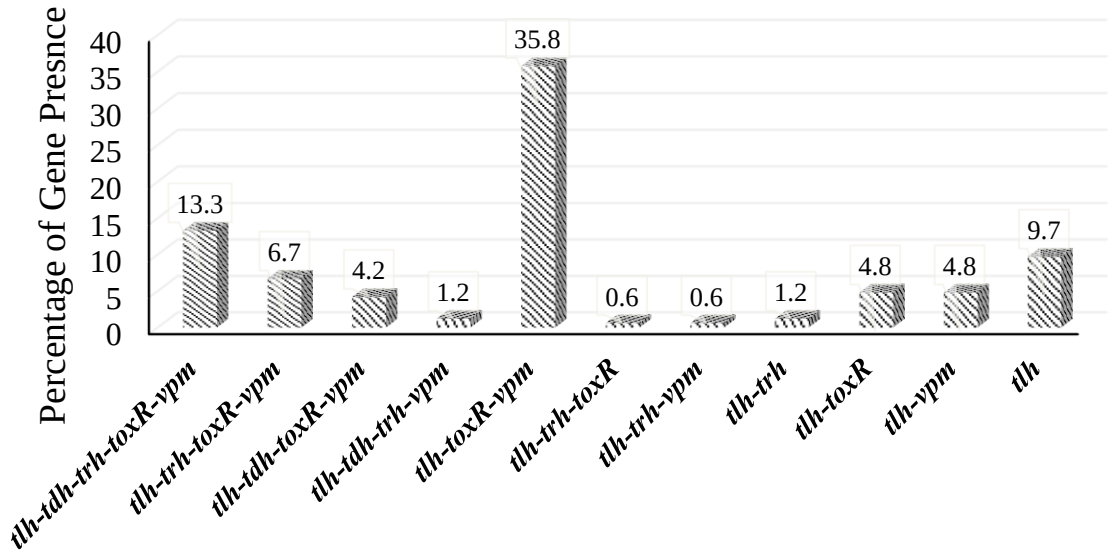

Figure 4.18. Genes Coexistence Among Total *V. parahaemolyticus* Isolates.

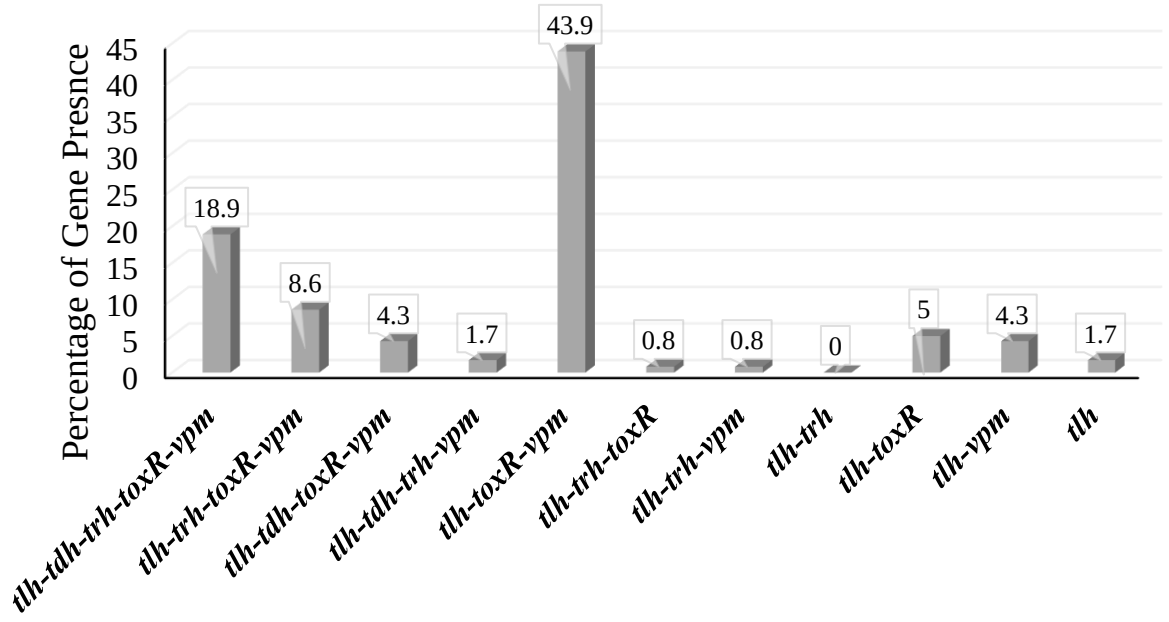

Figure 4.19. Gene Coexistence Among *V. parahaemolyticus* Isolates from Oyster.

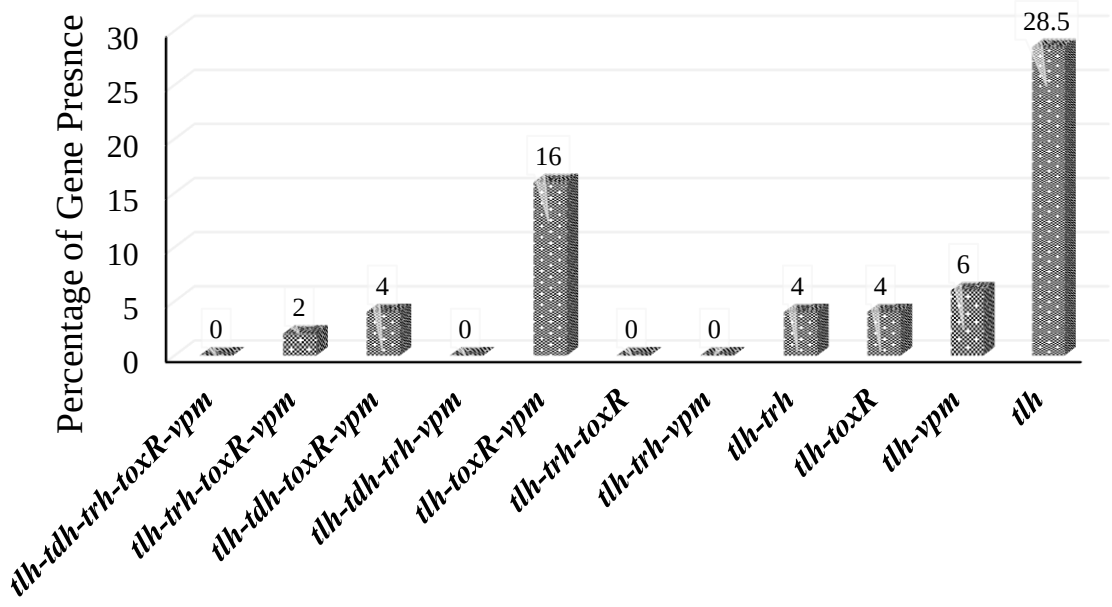

Figure 4.20. Gene Coexistence Among *V. parahaemolyticus* Isolates from Seawater.

4.3 Multiple Linear Regression Test

4.3.1. Analysis of *Vibrio* spp. (CFU) levels in Relation to Water Quality Variables

A multiple regression analysis was run to explain the proportion of the variation of *Vibrio* spp. (Log_{10} CFU) levels by the independent variables temperature, salinity, dissolved oxygen (DO), turbidity, and chlorophyll *a*. In addition, the test was used to determine how much *Vibrio* spp. Log_{10} CFUs changes for a one-unit change in the temperature, salinity, dissolved oxygen (DO), turbidity, and chlorophyll *a*. Data interpretation will go through three stages: (a) determine whether the multiple regression model is a good fit for the data; (b) understand the coefficients of the regression model; and (c) explain and predict the dependent variable based on values of the independent variables.

The assumption of linearity was met as assessed by the plot of studentized residuals (difference between actual and predicted values) against the predicted value "as whole variables", and by partial regression plots of each independent variables against Log_{10} CFUs. With regards to the time series of samples collection (LW 1st/BB 2nd/SL 3rd), and thus the possibility of autocorrelation, independence of residuals was checked as assessed by a Durbin-Watson statistic of 1.620 indicating no autocorrelation (Table 4.9). There was homogeneity (pretty symmetrically distributed), as assessed by visual inspection of a plot of studentized residuals versus predicted values (Refer to Appendix C, Page 75). There was no evidence of multicollinearity (high intercorrelations between the independent variables), as assessed by tolerance values greater than 0.1 (Table 4.8). There were no studentized deleted residuals (outlier) greater than ±3 standard deviations, no leverage (extreme x value) greater than 0.2, and

values for Cook's distance above 1 (data not shown). The assumption of normality was met, as assessed by the Histogram Plot, and confirmed by P-P Plot (Refer to Appendix C, Page 75).

The multiple correlation coefficient (*R*), shows value equal to 0.784, indicating a moderate to strong linear association between the variables (Table 4.9). The coefficient of determination "adjusted R^2" is another common measure used to assess goodness of overall model fit, and adjusted R^2, as shown in table 4.9, is 0.588 explaining (58.8%) of the variability of *Vibrio* Log_{10} CFUs by the addition of the temperature, salinity, dissolved oxygen (DO), chlorophyll *a*, and turbidity. All variables "overall model" added statistically significant difference ($p< 0.05$) to the prediction (Table 4.10). Temperature, salinity, dissolved oxygen, chlorophyll *a*, and turbidity "as a whole" were statistically significant in predicting *Vibrio* spp. (Log_{10} CFU) levels, $F (5, 72) = 22.983$, $p < 0.05$. The slope coefficients, which represents the change in the dependent variable for a one-unit change in each independent variable, (Table 4.8) shows that the *p*-value of the temperature, turbidity, and dissolved oxygen deemed significant ($p < 0.05$); however, there was no significant differences added by salinity and chlorophyll *a* (Laerd Statistics., 2017). These observations agree with the correlation of temperature and turbidity with *Vibrio* (CFU) levels reported in Ozbay (2016). Hence, the first alternative hypothesis with regards to total *Vibrio* levels was accepted.

4.3.2. Analysis of *V. parahaemolyticus* (CFU) Levels in Relation to Water Quality Variables

A multiple regression analysis was run to predict *Vibrio parahaemolyticus* (Log_{10} CFU) levels in relation to the independent variables temperature, turbidity, dissolved oxygen (DO), and chlorophyll *a*. There was linearity as assessed by the plots of each independent variables against

Table 4.8. Multicollinearity, and the Slope Coefficients of Each Independent Variable Statistics (Log_{10} CFUs of *Vibrio*).[a]

Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B		Correlations			Collinearity Statistics
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound	Zero-order	Partial	Part	Tolerance
1 (Constant)	-3.703	1.176		-3.149	0.002	-6.047	-1.359				
Temp	0.279	0.041	1.158	6.814	**0.000**	0.197	0.360	0.704	0.626	0.498	**0.185**
Salinity	-0.025	0.017	-0.157	-1.482	0.143	-0.058	0.009	0.363	-0.172	-0.108	**0.479**
Turbidity	-0.020	0.006	-0.243	-3.261	**0.002**	-0.032	-0.008	-0.345	-0.359	-0.239	**0.961**
DO mg/L	0.217	0.082	0.380	2.639	**0.010**	0.053	0.380	-0.487	0.297	0.193	**0.257**
Chlorophyll*a*	-0.510	0.278	-0.167	-1.836	0.071	-1.063	0.044	0.253	-0.211	-0.134	**0.648**

a. Dependent Variable: Log_{10} CFUs of *Vibrio* spp.

Table 4.9. **Model Summary[b]**-Linearity Association and Goodness of Overall Model Fit.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics					Durbin-Watson
					R Square Change	F Change	df 1	df 2	Sig. F Change	
1	**0.784a**	0.615	**0.588**	0.67392	0.615	**22.983**	**5**	**72**	0.000	**1.620**

a. Predictors: (Constant), Chlorophyll *a*, Salinity, DO mg/L, Temp.

b. Dependent Variable: Log_{10} CFUs of *Vibrio* spp.

Table 4.10. ANOVA[a] - Statistical Significance of the Addition of All Independent Variables "Overall Model".

Model		Sum of Squares	df	Mean Square	F	Sig.
1	**Regression**	52.191	5	10.438	22.983	**0.000b**
	Residual	32.700	72	0.454		
	Total	84.891	77			

a. Dependent Variable: Log_{10} CFUs of *Vibrio* spp.

b. Predictors: (Constant), Chlorophyll *a*, Salinity, DO mg/L, Temp.

CFUs, and by the plot of studentized residuals (difference between actual and predicted values) against the predicted values (Refer to Appendix D, Page 85). Independence of residuals was checked as assessed by a Durbin-Watson statistic of 1.762 indicating no autocorrelation. The assumption of homogeneity was met according to the plot of studentized residuals versus predicted values (Refer to Appendix D, Page 85). Tolerance values were greater than 0.1, indicating that there is no high intercorrelations between the independent variables (multicollinearity) (Table 4.11). Outlier (studentized deleted residuals) were less than ±3 standard deviations, leverage (extreme x value) were not greater than 0.2, and values for Cook's distance above 1 (data not shown). Normality test was assessed by the Histogram Plot, and confirmed by P-P Plot (Refer to Appendix D, Page 85).

The multiple regression model "as a whole" statistically significantly predicted *Vibrio parahaemolyticus* Log_{10} CFUs, F (4, 95) = 32.393, ($p < 0.05$). Adjusted $R^2 = 0.56$ explaining (56%) of the variability of *Vibrio parahaemolyticus* Log_{10} CFUs by the addition of the temperature, salinity, dissolved oxygen (DO), and chlorophyll *a*. As shown in table 4.11, only the temperature variable added statistically significantly to the prediction, ($p < 0.05$) (Laerd

Statistics., 2017). As a result, the first null hypothesis with regards to total *V. parahaemolyticus* level was rejected, and the alternative hypothesis was accepted.

Table 4.11. Multicollinearity, and the Slope Coefficients[a] of Each Independent Variable Statistics of *Vibrio parahaemolyticus* (Log_{10} CFU) levels.

Variable	Unstandardized Coefficients		Standardized Coefficients Beta	Sig.	Tolerance
	B	Std. Error			
(Constant)	-5.816	2.332		**0.021**	
Temperature	0.324	0.074	1.173	**0.000**	**0.221**
Chlorophyll *a*	-0.727	0.532	-0.208	0.186	**0.693**
DO mg/L	0.285	0.162	0.437	0.093	**0.260**
Turbidity	-0.018	0.012	-0.194	0.144	**0.982**

a Dependent Variable: Log_{10} CFUs of *Vibrio parahaemolyticus.*

4.4. One-Way ANOVA Test

One-Way ANOVA was conducted to determine if the Log_{10} CFUs of *Vibrio* spp. is significantly different between sample types. Samples were classified into two groups: oyster (n = 13) and seawater (n = 13) (Table 4.12). There were no outliers in the data, as assessed by inspection of the boxplot (Figure 4.21). *Vibrio* Log_{10} CFUs data were normally distributed for the oyster and seawater groups as assessed by Shapiro-Wilk's test ($p > 0.05$) (Table 4.13). There was heterogeneity of variance, as assessed by Levene's test of homogeneity of variances ($p = 0.003$) (Table 4.14). There were no statistically significant differences in *Vibrio* (Log_{10} CFU) levels between the different sample groups as shown in table 4.15 with Welch's F (1, 16.303) = 0.107 ($p = 0.747$). The group means were not significantly different ($p > 0.05$) so that the second null hypothesis cannot be rejected and the second alternative hypothesis cannot be accepted, (Laerd Statistics., 2017).

Table 4.12. Descriptive data for *Vibrio* (Log_{10} CFU) levels among oyster and seawater samples.

					95% Confidence Interval for Mean			
Log_{10} CFU	**N**	**Mean**	**Std. Deviation**	**Std. Error**	**Lower Bound**	**Upper Bound**	**Minimum**	**Maximum**
Oyster	13	2.4784	1.69569	0.47030	1.4537	3.5031	0.00	4.45
Seawater	13	2.3105	0.73020	0.20252	1.8693	2.7518	1.22	3.65
Total	26	2.3945	1.28197	0.25141	1.8767	2.9123	0.00	4.45

Table 4.13. Normality Distribution.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Sample	**Statistic**	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
Log10 CFU	**Oyster**	0.159	13	0.200*	0.878	13	**0.067**
	Seawater	0.164	13	0.200*	0.933	13	**0.367**

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction.

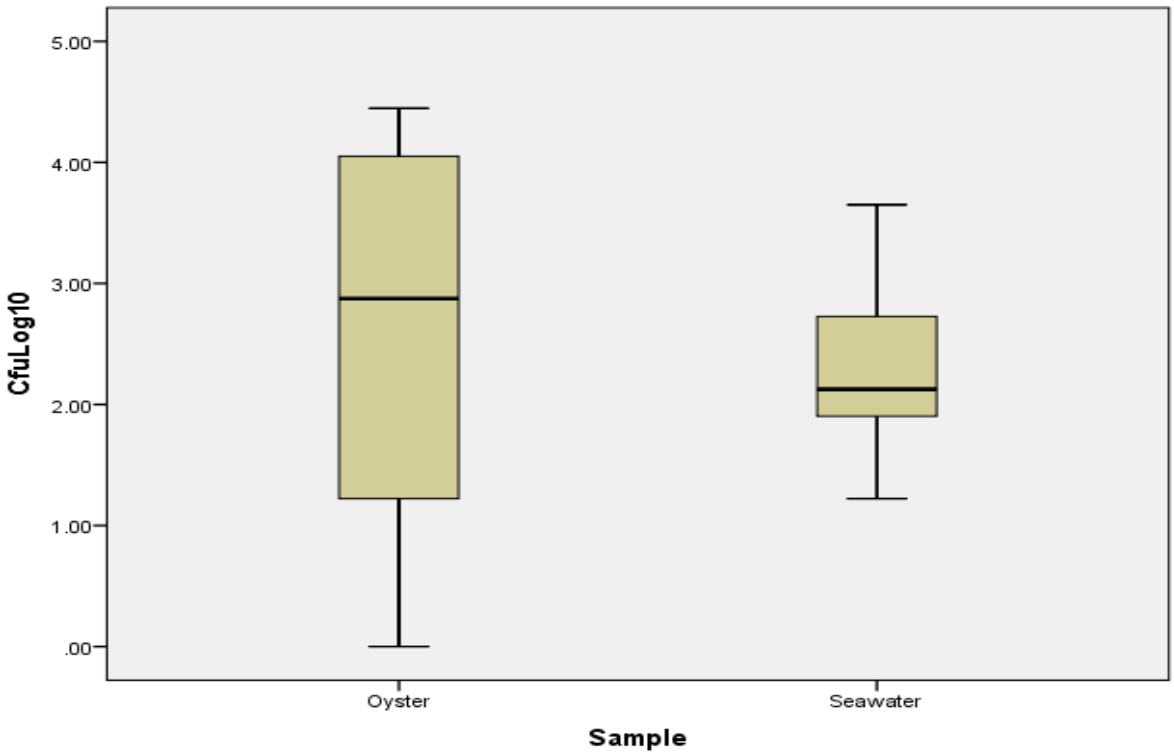

Figure 4.21. Boxplot to Confirm Outlier Test.

Table 4.14. Homogeneity of Log10 CFU Variances.

Levene Statistic	df1	df2	Sig.
10.899	1	24	**0.003**

Table 4.15. Robust Tests of Equality of Log_{10} CFU Means.

	Statistic[a]	df1	df2	Sig.
Welch	0.107	**1**	**16.303**	**0.747**

a. Asymptotically F distributed.

CHAPTER 5: CONCLUSION AND FUTURE RECOMMENDATIONS

The purpose of this study was to identify and differentiate *Vibrio* species and characterize pathogenic *Vibrio parahaemolyticus* in oyster and seawater samples from Delaware Bay. Four presumptive *Vibrio* species: *Vibrio parahaemolyticus, Vibrio cholerae/Vibrio vulnificus,* and *Vibrio alginolyticus* were identified and differentiated on CHROMagar™ Vibrio media based on the color of their colonies which were mauve, green/blue, and colorless, respectively. Colony Forming Units (CFU) were calculated and the Log_{10} CFU g^{-1} (or mL^{-1}) were obtained. There was a significant association between the increase of *Vibrio* spp. levels and water temperature, dissolved oxygen, and turbidity. The correlation of temperature and turbidity with total *Vibrio* (CFU) levels is in agreement with the previous study of Delaware Inland Bays Eastern Oysters in which increased temperature and decreased turbidity increased the level of total *Vibrio* (Ozbay, 2016). On the other hand, *V. parahaemolyticus* (CFU) levels are significantly associated with only the temperature. First alternative hypothesis of the correlation of *Vibrio* spp. and *V. parahaemolyticus* levels with temperature was accepted. There was no significant association, however, between *Vibrio* spp. level and the type of samples, and thus the second alternative hypothesis was rejected. The highest values of CFU were observed from *Vibrio alginolyticus* in the months of July and August, followed by *Vibrio parahaemolyticus* in July. CFUs value from oyster samples were much higher than from seawater samples indicating that *Vibrio* concentrations in oyster are higher than the water of the surrounding areas.

Of the bacterial isolates 83% were *tlh*-positive, confirming the specificity of *tlh* gene for *V. parahaemolyticus* species. The presence of *toxR* (66.7%) and *vpm* (65.5%) genes demonstrated the highest occurrence compared to *tdh* (18.8%) and *trh* (23.7%) genes. Thus, the reliability of *toxR* and *vpm* as gene markers for pathogenic or potential pathogenic *Vibrio*

parahaemolyticus is notably higher, and the third hypothesis was accepted. Occurrence of virulent genes in oyster samples was notably significant. Most of the seawater isolates (87.5%) possessed only *tlh* gene. Coexistence analysis of the virulent genes showed that the *tlh-toxR-vpm* pattern was the highest (35.8%, 43.9%, and 16%) among total bacterial, oyster, and seawater isolates, respectively. The gene pattern *tlh-tdh-trh-toxR-vpm* was the second pattern (18.9% and 13.3%) among oyster and total isolates respectively. Gene coexistence indicate gene occurrence correlation, and our results showed that *toxR* is conceivably correlated to the presence of *tdh* which confirms the strong association of *toxR* and *tdh* gene regulation reported in the literature (Lin et al., 1993). Future studies may focus on conducting different classification tests such as hemolytic activity on Wagatsuma blood agar and anti-O/ anti-K sera on one bacterial isolate from each coexistence gene pattern group. Furthermore, 16S rDNA gene sequencing for *V. parahaemolyticus* isolates characterization will illustrate the correlation of species divergence and presence of virulent genes. Also, research additional genetic markers to validate the virulence of *V. parahaemolyticus.* To the best of our knowledge, this is the only study that confirmed the occurrence of total and pathogenic *V. parahaemolyticus*, using CHROMagar and five genetic markers, in the oysters (*Crassostrea virginica*) of Delaware Bay. This study provided informative data to better understand the infections dynamics associated with oyster consumption and recreational water activities caused by pathogenic *Vibrio* species. Moreover, research directed at coastal environmental issues and public health received important data for crucial management decisions.

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Appendix A

Physico-Chemical Water Quality Parameters Data

Physico-Chemical Water Quality Parameters Data in Relation to Bowers Beach Site.

Bowers Beach	OTV[a] CFU g^{-1}	WTV[b] CFU mL^{-1}	OTV[a] Log_{10} CFU	WTV[b] Log_{10} CFU	H_2O[c] °C	Salinity ppt	Turbidity NTU/FTU	DO[d] %	pH	Chlorophyll a
June	11251	100	4.05	2.00	24.18	20	29	101.7	8.18	1.174
July	27966	4467	4.45	3.65	27.74	27	19	69.6	7.88	–
August	8080	3967	3.91	3.60	28	25	43.5	58	7.55	0.238
September	750	133	2.88	2.12	23.67	26	45.1	67.2	8.04	1.14
October	84	83	1.92	1.92	17.91	25.78	55.1	99.9	8.82	0.218

OTV[a] = Oyster Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
WTV[b] **=** Water Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
H_2O[c] = Water Temperature
DO[d] = Dissolved Oxygen

Physico-Chemical Water Quality Parameters Data in Relation to Bowers Beach Site.

Bowers Beach	OTV[a] CFU g^{-1}	WTV[b] CFU mL^{-1}	OTV[a] Log_{10} CFU	WTV[b] Log_{10} CFU	Air[c] °C	Depth m	Cond[d] uS/cm	DO[e] mg/l	WX[f]	WD[g]	Wind mph	Tide
June	11251	100	4.05	2.00	27.78	–	32200	6.28	1	7	11	4
July	27966	4467	4.45	3.65	27.22	5.14	42100	3.86	0	8	8	3
August	8080	3967	3.91	3.60	25	1.4	39300	3.35	1	0	0	1
September	750	133	2.88	2.12	21.67	3.85	40,700	4.02	1	6	2	1
October	84	83	1.92	1.92	16.11	3.5	36700	8.09	0	6	4	3

OTV[a] = Oyster Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
WTV[b] **=** Water Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
Air[c] = Air Temperature
Cond[d] **=** Conductivity
DO[e] = Dissolved Oxygen
WX[f] = Weather: (**0** = Clear); (**1** = Partly cloudy); (**2** = continuous clouds); (**3** = blowing snow/sand); (**4** = fog/haze); (**5** = drizzle); (**6** = rain); (**7** = snow/snow with rain); (**8** = showers); (**9** = thunder storms)
WD[g] = Wind Direction **(**0 = No Wind); (1 = N); (2 = NE); (3 = E); (4 = SE); (5 = S); (6 = SW); (7 = W); (8 = NW)
Tide Codes: (1 = Ebb); (2 = Slack after ebb); (3 = flood); (4 = slack after flood)
Ebb Current: The movement of a tidal current away from the coast or down an estuary

Physico-Chemical Water Quality Parameters Data in Relation to Lewes, Broadkill Site.

Lewes, Broadkill	OTV[a] CFU g^{-1}	WTV[b] CFU mL^{-1}	OTV[a] Log_{10} CFU	WTV[b] Log_{10} CFU	H_2O[c] °C	Salinity ppt	Turbidity NTU/FTU	DO[d] %	pH	Chlorophyll a
June	1350	533	3.13	2.73	22.7	23	29	57.8	7.2	0.292
July	3900	600	3.59	2.78	22.98	32	33	76.8	7.84	0.366
August	20566	350	4.31	2.54	26.43	25	40.8	56.9	7.88	0.824
September	0	67	0.00	1.83	21.32	24	39.04	58.5	7.75	0.134
October	0	33	0.00	1.52	14.63	5.37	54.8	83.6	6.44	0.52

OTV[a] = Oyster Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
WTV[b] = Water Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
H_2O[c] = Water Temperature
DO[d] = Dissolved Oxygen

Physico-Chemical Water Quality Parameters Data in Relation to Lewes, Broadkill Site.

Lewes, Broadkill	OTV[a] CFU g^{-1}	WTV[b] CFU mL^{-1}	OTV[a] Log_{10}CFU	WTV[b] Log_{10}CFU	Air[c] °C	Depth m	Cond[d] uS/cm	DO[e] mg/l	WX[f]	WD[g]	Wind mph	Tide
June	1350	533	3.13	2.73	22.22	1.22	36500	3.71	2	6	9	2
July	3900	600	3.59	2.78	26.67	2.96	49000	4.33	1	1	1	3
August	20566	350	4.31	2.54	25	5.4	43200	3.12	1	1	7	3
September	0	67	0.00	1.83	22.78	5.3	36700	3.42	0	8	11	3
October	0	33	0.00	1.52	11.11	3.2	7500	8.23	0	8	25	1

OTV[a] = Oyster Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
WTV[b] = Water Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
Air[c] = Air Temperature
Cond[d] = Conductivity
DO[e] = Dissolved Oxygen
WX[f] = Weather: (**0** = Clear); (**1** = Partly cloudy); (**2** = continuous clouds); (**3** = blowing snow/sand); (**4** = fog/haze); (**5** = drizzle); (**6** = rain); (**7** = snow/snow with rain); (**8** = showers); (**9** = thunder storms)
WD[g] = Wind Direction **(**0 = No Wind); (1 = N); (2 = NE); (3 = E); (4 = SE); (5 = S); (6 = SW); (7 = W); (8 = NW)
Tide Codes: (1 = Ebb); (2 = Slack after ebb); (3 = flood); (4 = slack after flood)
Ebb Current: The movement of a tidal current away from the coast or down an estuary

Physico-Chemical Water Quality Parameters Data in Relation to Slaughter Beach Site.

Slaughter Beach	OTV[a] CFU g^{-1}	WTV[b] CFU mL^{-1}	OTV[a] Log_{10} CFU	WTV[b] Log_{10} CFU	H_2O[c] °C	Salinity ppt	Turbidity NTU/FTU	DO[d] %	pH	Chlorophyll a
August	367	85	2.56	1.93	26.74	32	55.35	75.1	8.06	0.833
September	17	166	1.23	2.22	20.82	26.48	55.05	60.5	7.31	0.314
October	0	17	0.00	1.23	14.68	16.64	20	88	7.44	0.276

OTV[a] = Oyster Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
WTV[b] = Water Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
H_2O[c] = Water Temperature
DO[d] = Dissolved Oxygen

Physico-Chemical Water Quality Parameters Data in Relation to Slaughter Beach Site.

Slaughter Beach	OTV[a] CFU g^{-1}	WTV[b] CFU mL^{-1}	OTV[a] Log_{10} CFU	WTV[b] Log_{10} CFU	Air[c] °C	Depth m	Cond[d] uS/cm	DO[e] mg/l	WX[f]	WD[g]	Wind mph	Tide
August	367	85	2.56	1.93	23.89	4.03	49000	4	0	2	6	3
September	17	166	1.23	2.22	11.11	2.93	40,700	4.7	1	4	16	1
October	0	17	0.00	1.23	12.78	3.32	25000	7.79	0	6	9	1

OTV[a] = Oyster Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
WTV[b] = Water Total *Vibrio* (*V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*/*V. vulnificus*)
Air[c] = Air Temperature
Cond[d] = Conductivity
DO[e] = Dissolved Oxygen
WX[f] = Weather: (**0** = Clear); (**1** = Partly cloudy); (**2** = continuous clouds); (**3** = blowing snow/sand); (**4** = fog/haze); (**5** = drizzle); (**6** = rain); (**7** = snow/snow with rain); (**8** = showers); (**9** = thunder storms)
WD[g] = Wind Direction (0 = No Wind); (1 = N); (2 = NE); (3 = E); (4 = SE); (5 = S); (6 = SW); (7 = W); (8 = NW)
Tide Codes: (1 = Ebb); (2 = Slack after ebb); (3 = flood); (4 = slack after flood)
Ebb Current: The movement of a tidal current away from the coast or down an estuary

Appendix B

Protocols for Preparing Culture Media and Reagents Used in this Study

Preparation of Broth Peptone Water (0.1% BPW) to Be Used for The Serial Dilution

Peptone (BD, Bacto™ Peptone, 211677) 1g

NaCl 20 g

Deionized (DI) water 1000 mL

Peptone and NaCl were added to the DI water while swirling and stirring regularly until peptone was completely dissolved, and it was then autoclaved at 121ºC for 15 min.

Preparation of Tryptic Soy Broth (TSB) Medium

Tryptic Soy Broth (Thermo Fisher Scientific Inc, OXOID, CM0129) 30 g

Deionized (DI) water 1000 mL

Tryptic Soy Broth were dissolved in the DI water while swirling and stirring regularly until complete fusion of the media prior to autoclaving at 121ºC for 15 min.

Preparation of TSB + 24 % Glycerol (TSB+[24%G]) for the Stock Solution

Tryptic Soy Broth (Thermo Fisher Scientific Inc, OXOID, CM0129) 6 g

Glycerol (Glycerol, BP229-1, Fisher BioReagents™) 48 mL

Deionized (DI) water 152 mL

TSB was suspended in the DI water while swirling and stirring regularly until it dissolved completely. Then glycerol was added to the solution before it was autoclaved at 121ºC for 15 min.

Preparing (1%) Agarose Gel for the Gel Electrophoresis procedure

Agarose (Agarose RA™, VWR) 1g

(1x) TAE, 100 mL

1% Ethidium Bromide (Fisher BioReagents) 10 µL

Agarose was suspended in 100 milliliters of (1x) TAE, then heated in the microwave until complete fusion of the agarose was achieved. It was then cooled down until lukewarm before it was poured into the gel casting stand with the well comb in place. Then it remained at room temperature until it completely solidified before the comb was removed carefully, and gel wells were ready to be loaded with the PCR amplicon.

Selective Media Preparation: CHROMagar™ Vibrio (CHROMagar, VB912)

The agar was suspended in the proportion of 74.7 g/L in deionized (DI) water. The media was heated to reach 100ºC while swirling and stirring regularly until complete fusion of the agar occurred. After cooling it down to 45-50ºC it was poured into sterile petri plates using aseptic cabinet (Labconco - 302410000 - 4" Purifier Logic+ Class II A2 Biological Safety Cabinet).

Appendix C

Multiple Regression Analysis of *Vibrio* Log_{10} CFUs Using SPSS

Descriptive Statistics.

	Mean	Std. Deviation	N
Log_{10} CFU	1.9769	1.04999	78
Temp	22.4462	4.35973	78
Salinity	23.7131	6.66155	78
Turbidity	39.9031	12.73366	78
DO mg/L	4.9923	1.84361	78
Chlorophyll *a*	0.5399	0.34371	78

Variables Entered/Removed[a].

Model	Variables Entered	Variables Removed	Method
1	Chlorophyll *a*, Salinity, Turbidity, DO mg/L, Temp[b]	.	Enter

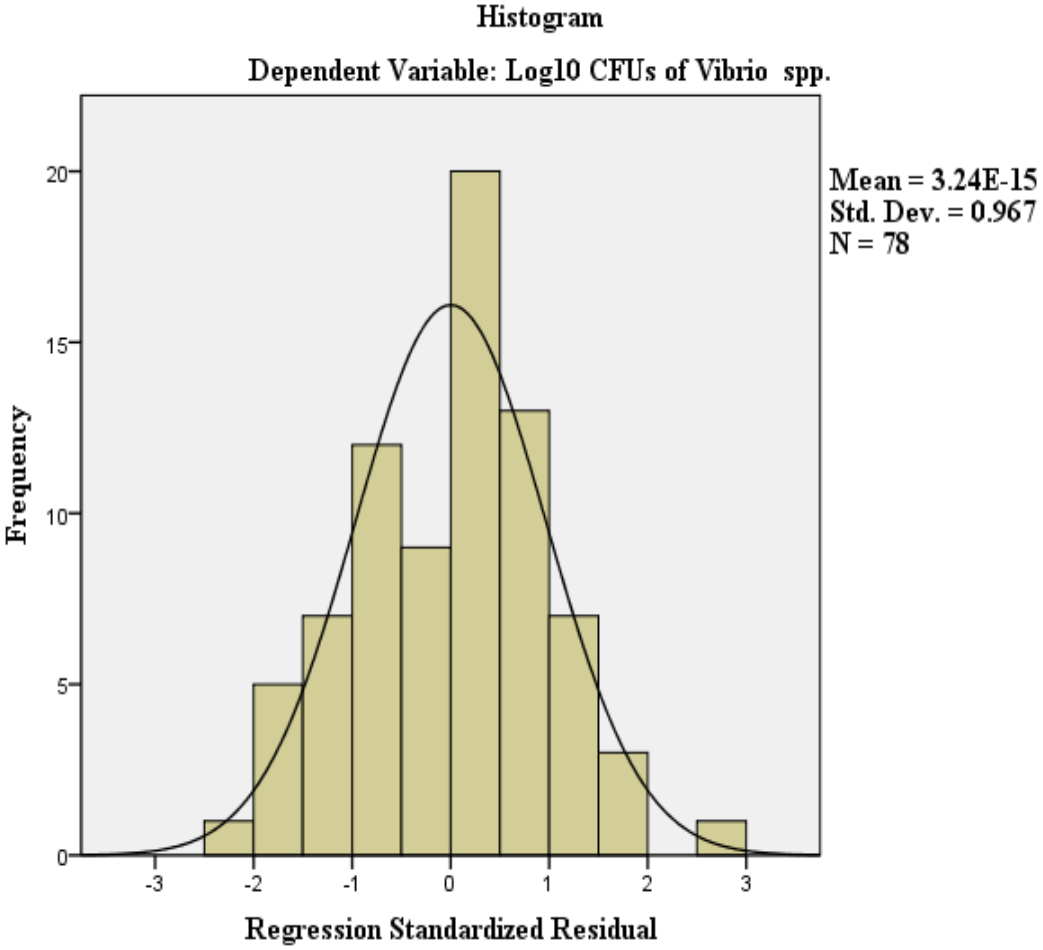
a. Dependent Variable: Log_{10} CFUs of *Vibrio* spp.

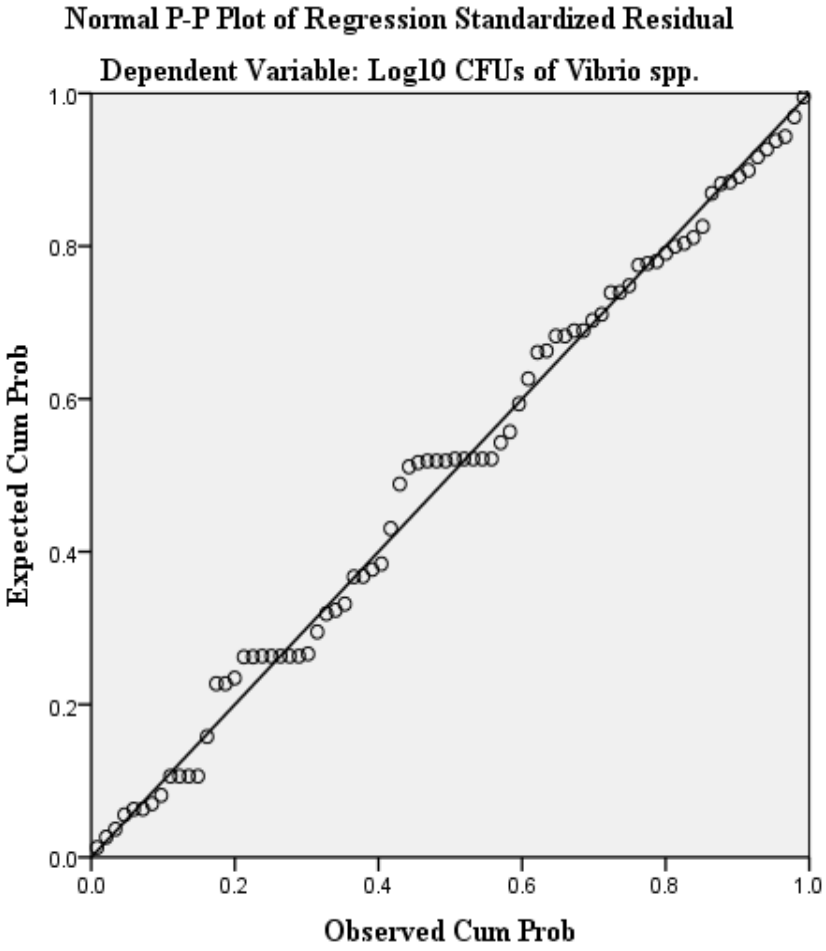
b. All requested variables entered.

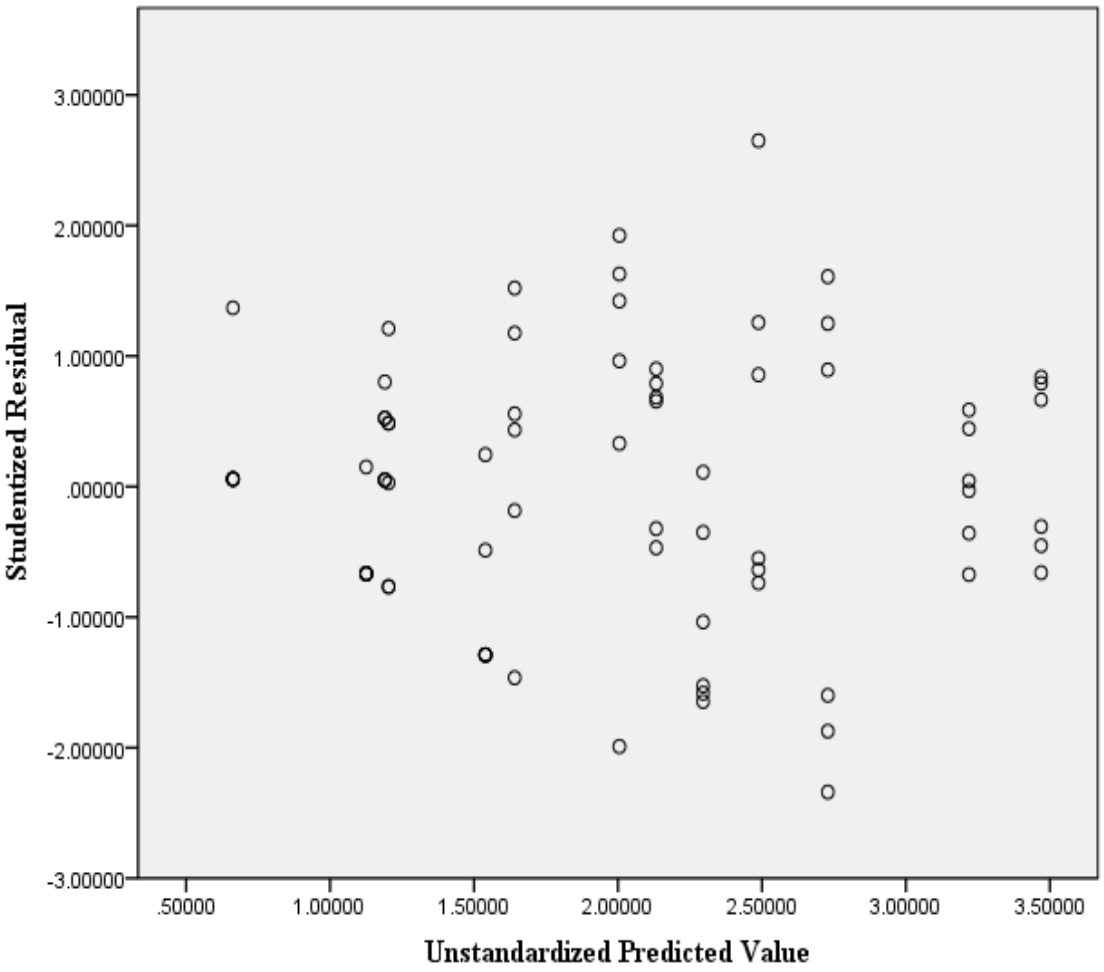
Collinearity Diagnostics[a].

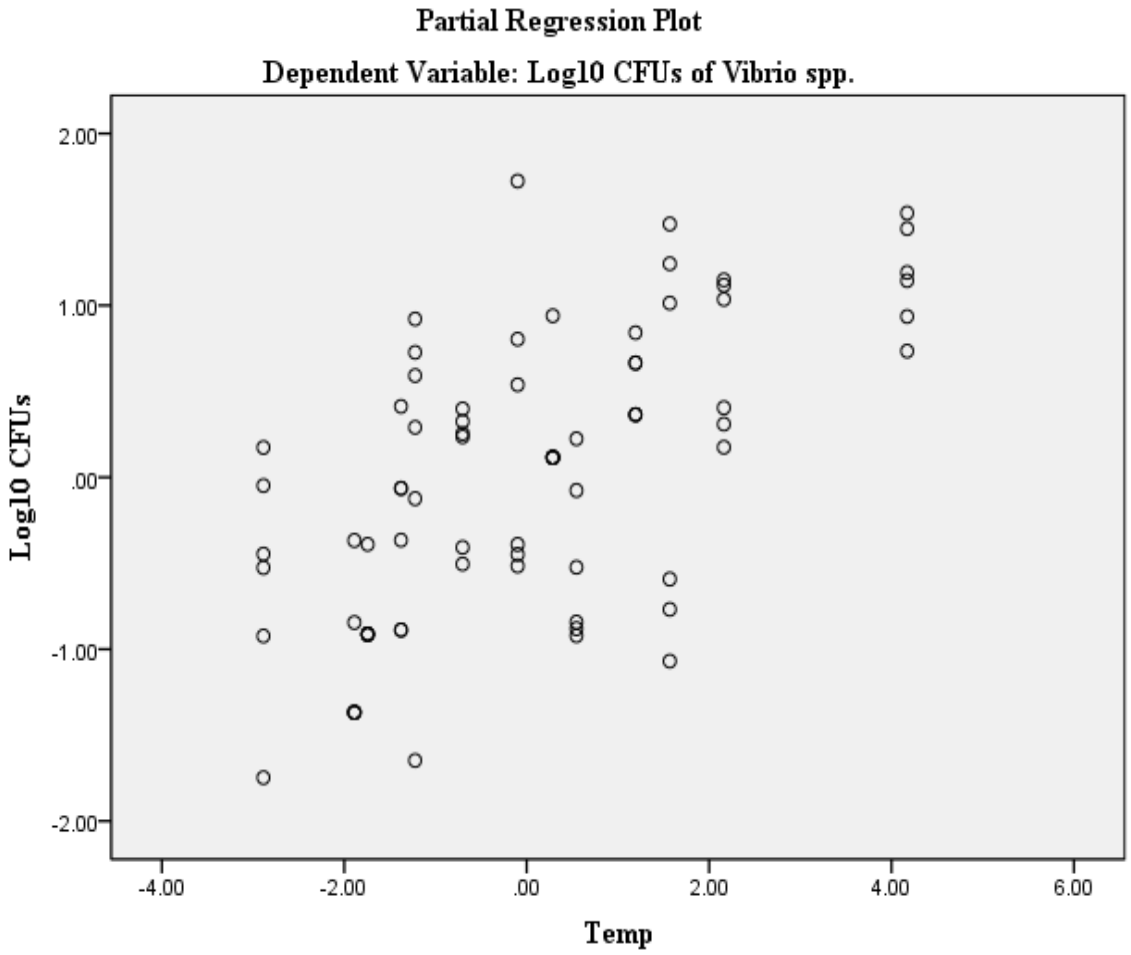
					Variance Proportions				
Model	**Dimension**	**Eigenvalue**	**Condition Index**	**(Constant)**	**Temp**	**Salinity**	**Turbidity**	**DO mg/L**	**Chlorophyll *a***
1	**1**	5.471	1.000	0.00	0.00	0.00	0.00	0.00	0.00
	2	0.263	4.558	0.00	0.00	0.00	0.03	0.02	0.50
	3	0.175	5.596	0.00	0.00	0.06	0.00	0.08	0.13
	4	0.070	8.840	0.00	0.00	0.02	0.94	0.05	0.02
	5	0.018	17.343	0.04	0.12	0.90	0.00	0.07	0.11
	6	0.003	46.032	0.96	0.88	0.01	0.03	0.78	0.23

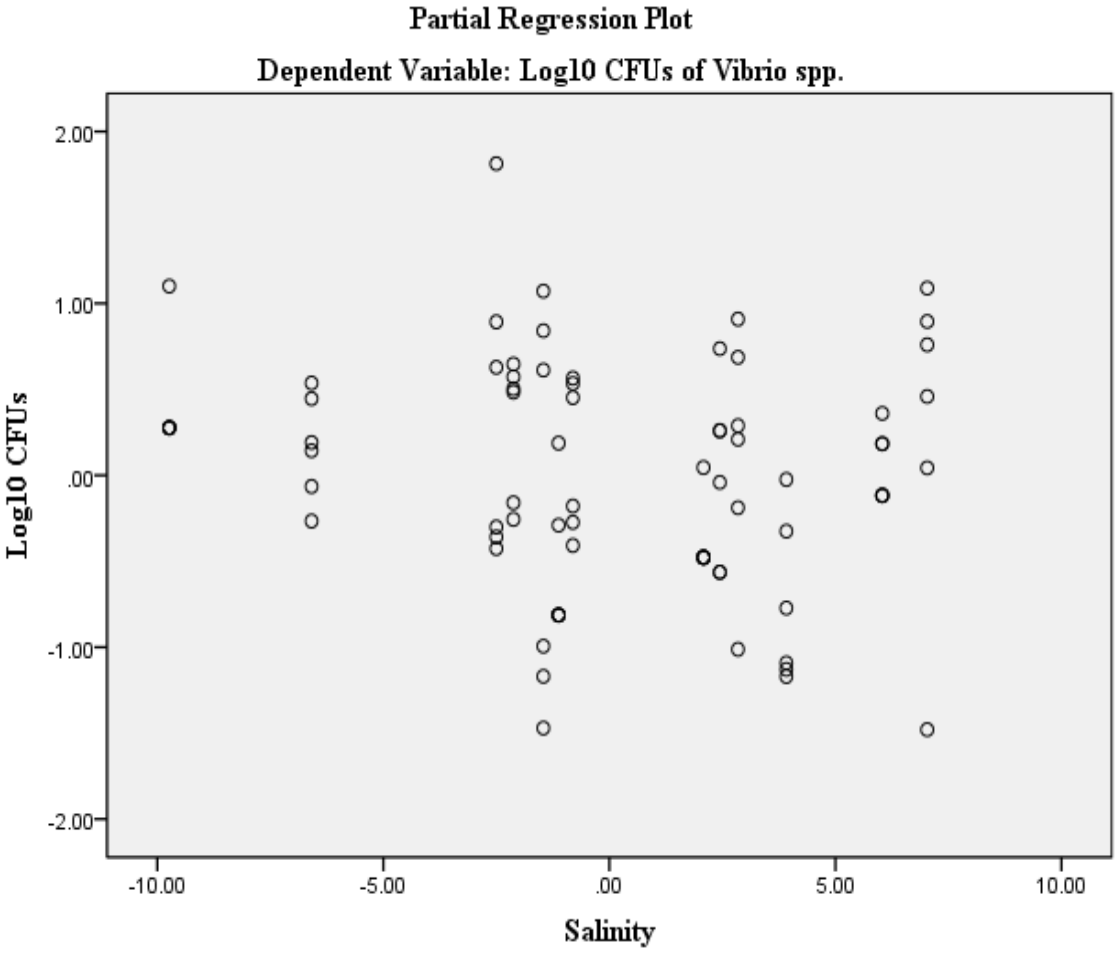
a. Dependent Variable: Log10 CFUs of *Vibrio* spp.

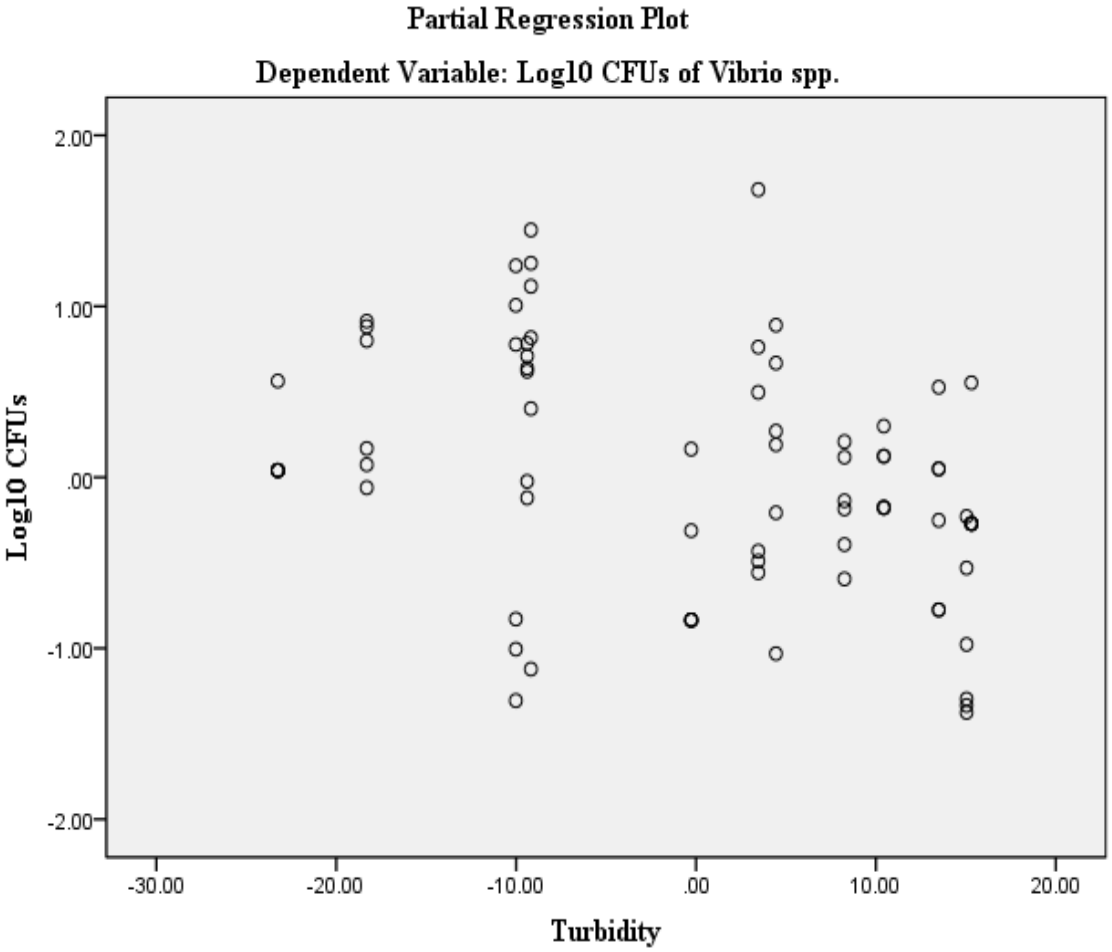

Histogram
Dependent Variable: Log10 CFUs of Vibrio spp.
Mean = 3.24E-15
Std. Dev. = 0.967
N = 78
Frequency
Regression Standardized Residual
0
5
10
15
20
-3
-2
-1
0
1
2
3

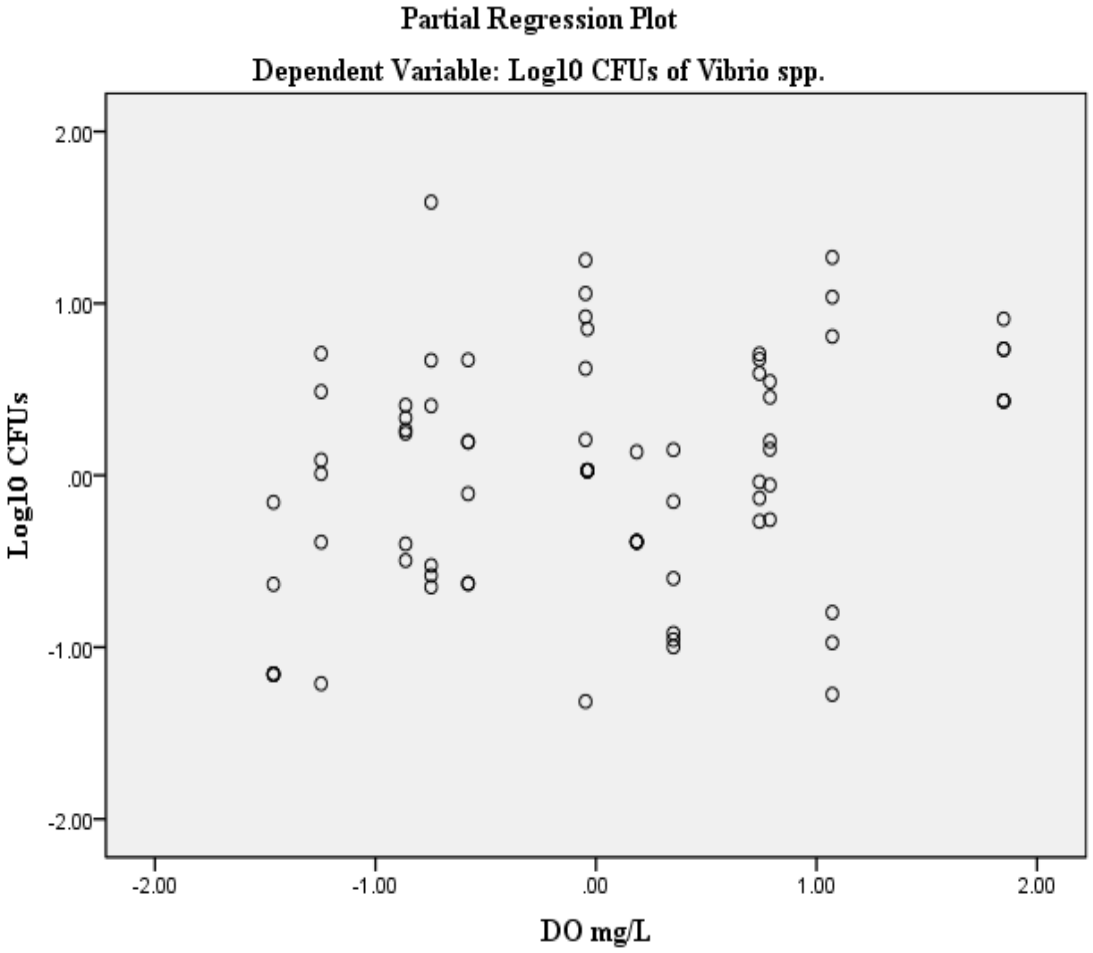

Normal P-P Plot of Regression Standardized Residual
Dependent Variable: Log10 CFUs of Vibrio spp.
Expected Cum Prob
Observed Cum Prob
0.0
0.2
0.4
0.6
0.8
1.0

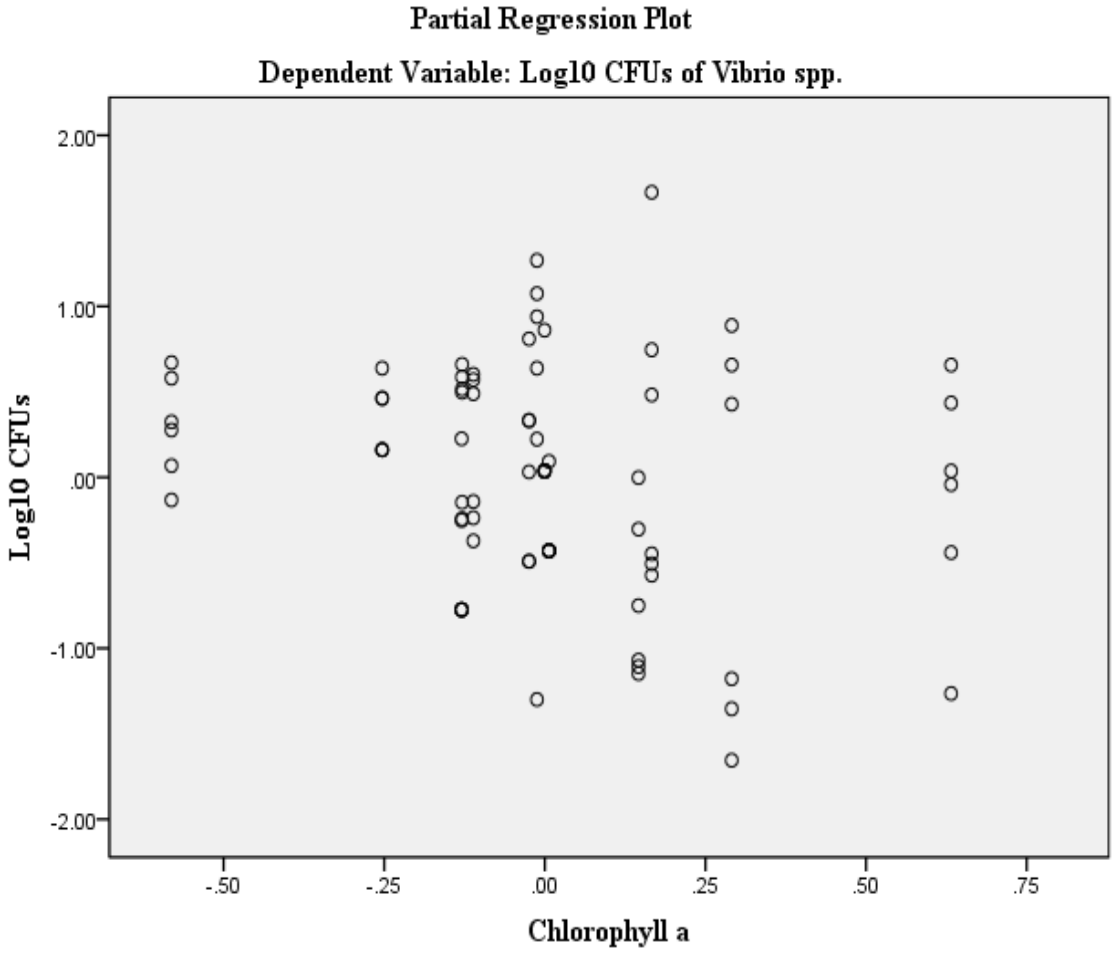

Studentized Residual
3.00000
2.00000
1.00000
.00000
-1.00000
-2.00000
-3.00000
.50000
1.00000
1.50000
2.00000
2.50000
3.00000
3.50000
Unstandardized Predicted Value


Partial Regression Plot
Dependent Variable: Log10 CFUs of Vibrio spp.
Log10 CFUs
2.00
1.00
.00
-1.00
-2.00
-4.00
-2.00
.00
2.00
4.00
6.00
Temp


Partial Regression Plot
Dependent Variable: Log10 CFUs of Vibrio spp.
Log10 CFUs
2.00
1.00
.00
-1.00
-2.00
-10.00
-5.00
.00
5.00
10.00
Salinity


Partial Regression Plot
Dependent Variable: Log10 CFUs of Vibrio spp.
Log10 CFUs
2.00
1.00
.00
-1.00
-2.00
-30.00
-20.00
-10.00
.00
10.00
20.00
Turbidity


Partial Regression Plot
Dependent Variable: Log10 CFUs of Vibrio spp.
Log10 CFUs
2.00
1.00
.00
-1.00
-2.00
-2.00
-1.00
.00
1.00
2.00
DO mg/L


Partial Regression Plot
Dependent Variable: Log10 CFUs of Vibrio spp.
Log10 CFUs
2.00
1.00
.00
-1.00
-2.00
-.50
-.25
.00
.25
.50
.75
Chlorophyll a

Appendix D

Multiple Regression Analysis of *V. parahaemolyticus* Log_{10} CFUs Using SPSS

Descriptive Statistics.

	Mean	Std. Deviation	N
Log_{10} CFU	1.7478	1.21857	26
Temp	22.4462	4.41748	26
Chlorophyll *a*	0.5402	0.34923	26
DO mg/L	4.9923	1.86803	26
Turbidity	39.9031	12.90232	26

Correlations.

		log_{10} CFU	Temp	Chlorophyll *a*	DO mg/L	Turbidity
Pearson Correlation	**Log_{10} CFU**	1.000	0.754	0.218	-0.533	-0.287
	Temp	0.754	1.000	0.399	-0.827	-0.132
	Chlorophyll *a*	0.218	0.399	1.000	-0.114	-0.043
	DO mg/L	-0.533	-0.827	-0.114	1.000	0.122
	Turbidity	-0.287	-0.132	-0.043	0.122	1.000
Sig. (1-tailed)	**Log_{10} CFU**	.	0.000	0.142	0.003	0.078
	Temp	0.000	.	0.022	0.000	0.260
	Chlorophyll *a*	0.142	0.022	.	0.290	0.418
	DO mg/L	0.003	0.000	0.290	.	0.276
	Turbidity	0.078	0.260	0.418	0.276	.
N	**Log_{10} CFU**	26	26	26	26	26
	Temp	26	26	26	26	26
	Chlorophyll *a*	26	26	26	26	26
	DO mg/L	26	26	26	26	26
	Turbidity	26	26	26	26	26

Variables Entered/Removed[a].

Model	Variables Entered	Variables Removed	Method
1	Turbidity, Chlorophyll *a*, DO mg/L, Temp[b]	.	Enter

a. Dependent Variable: *Vibrio parahaemolyticus* Log_{10} CFU.

b. All requested variables entered.

Model Summary[b].

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics					Durbin-Watson
					R Square Change	F Change	df1	df2	Sig. F Change	
1	0.814[a]	0.662	**0.598**	0.77303	0.662	**10.281**	**4**	**21**	**0.000**	**1.762**

a. Predictors: (Constant), Turbidity, Chlorophyll *a*, DO mg/L, Temp., b. Dependent Variable: *Vibrio parahaemolyticus* Log_{10} CFU.

Coefficients[a].

Model	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B		Correlations			Collinearity Statistics
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound	Zero-order	Partial	Part	Tolerance
1 (Constant)	-5.816	2.332		-2.494	0.021	-10.666	-0.967				
Temp	0.324	0.074	1.173	4.352	**0.000**	0.169	0.478	0.754	0.689	0.552	**0.221**
Chlorophyll *a*	-0.727	0.532	-0.208	-1.367	0.186	-1.832	0.379	0.218	-0.286	-0.173	**0.693**
DO mg/L	0.285	0.162	0.437	1.759	0.093	-0.052	0.622	-0.533	0.358	0.223	**0.260**
Turbidity	-0.018	0.012	-0.194	-1.516	0.144	-0.043	0.007	-0.287	-0.314	-0.192	**0.982**

a. Dependent Variable: *Vibrio parahaemolyticus* Log_{10} CFU.

Collinearity Diagnostics[a].

Model	Dimension	Eigenvalue	Condition Index	Variance Proportions				
				(Constant)	Temp	Chlorophyll *a*	DO mg/L	Turbidity
1	**1**	4.550	1.000	0.00	0.00	0.01	0.00	0.00
	2	0.264	4.150	0.00	0.00	0.52	0.02	0.03
	3	0.119	6.191	0.00	0.02	0.16	0.14	0.06
	4	0.064	8.405	0.01	0.02	0.09	0.01	0.88
	5	0.003	41.741	0.99	0.96	0.22	0.83	0.03

a. Dependent Variable: *Vibrio parahaemolyticus* Log_{10} CFU.

ANOVA[a].

Model		Sum of Squares	df	Mean Square	F	Sig.
1	**Regression**	24.574	4	6.143	10.281	**0.000[b]**
	Residual	12.549	21	0.598		
	Total	37.123	25			

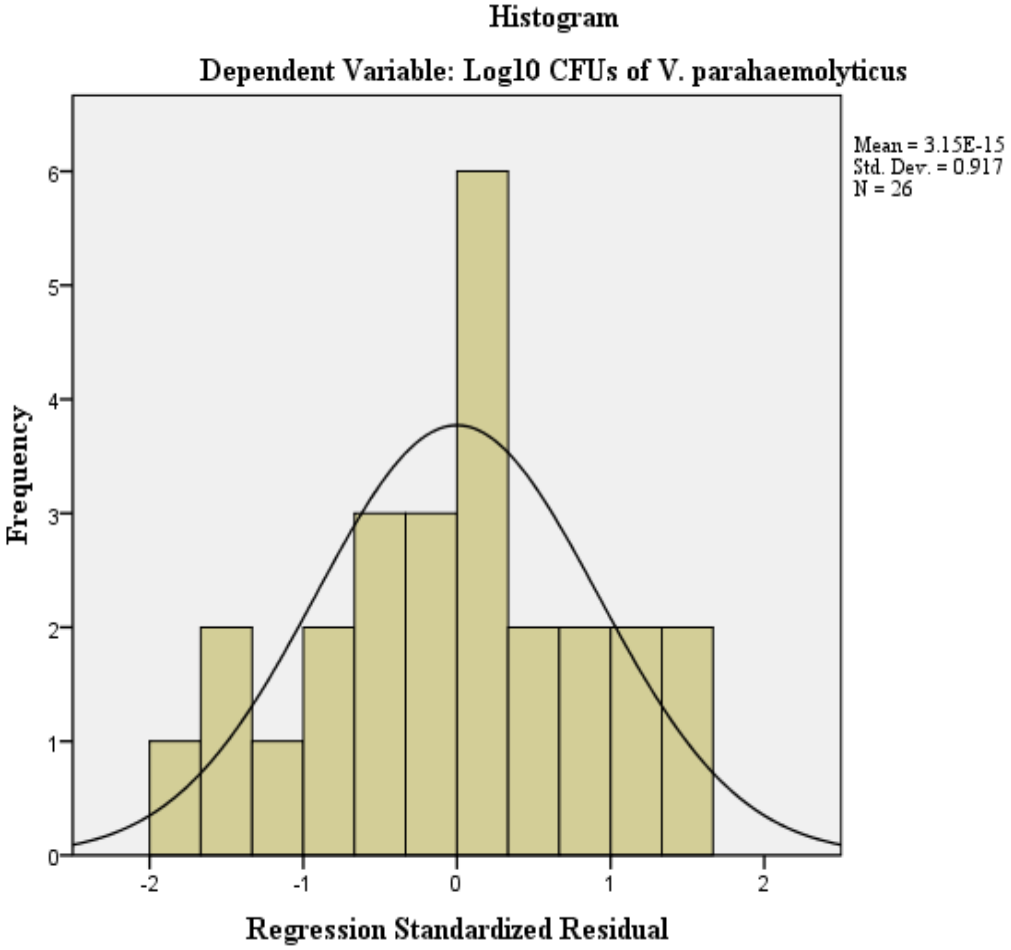
a. Dependent Variable: *Vibrio parahaemolyticus* Log_{10} CFU.

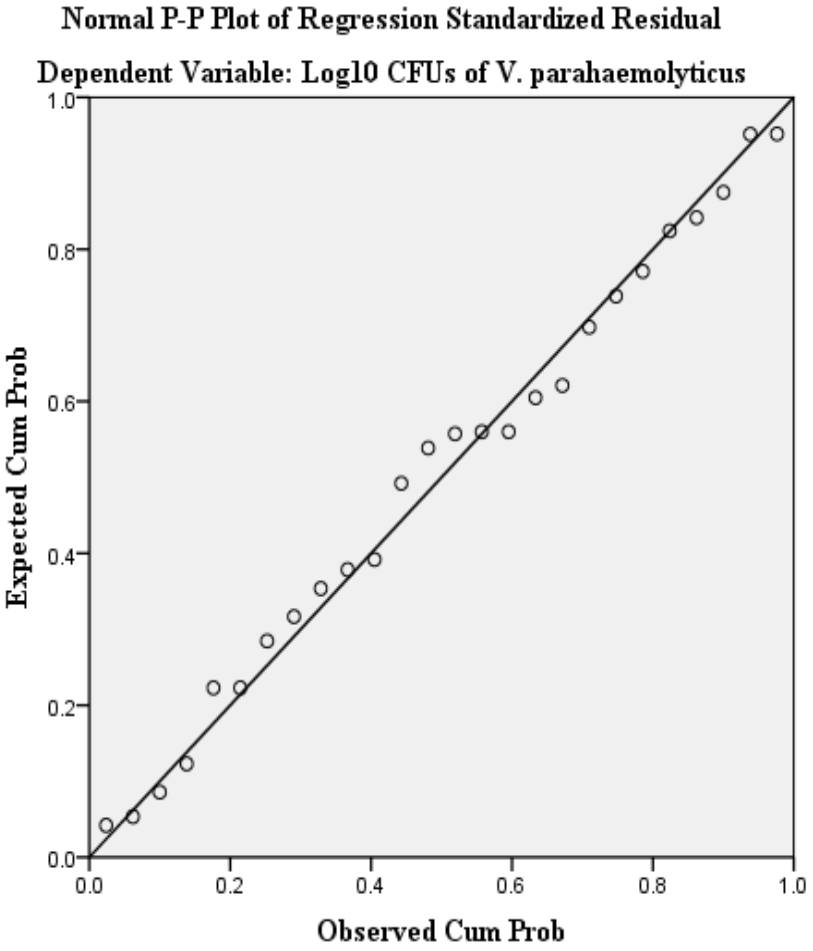
b. Predictors: (Constant), Turbidity, Chlorophyll *a*, DO mg/L, Temp.

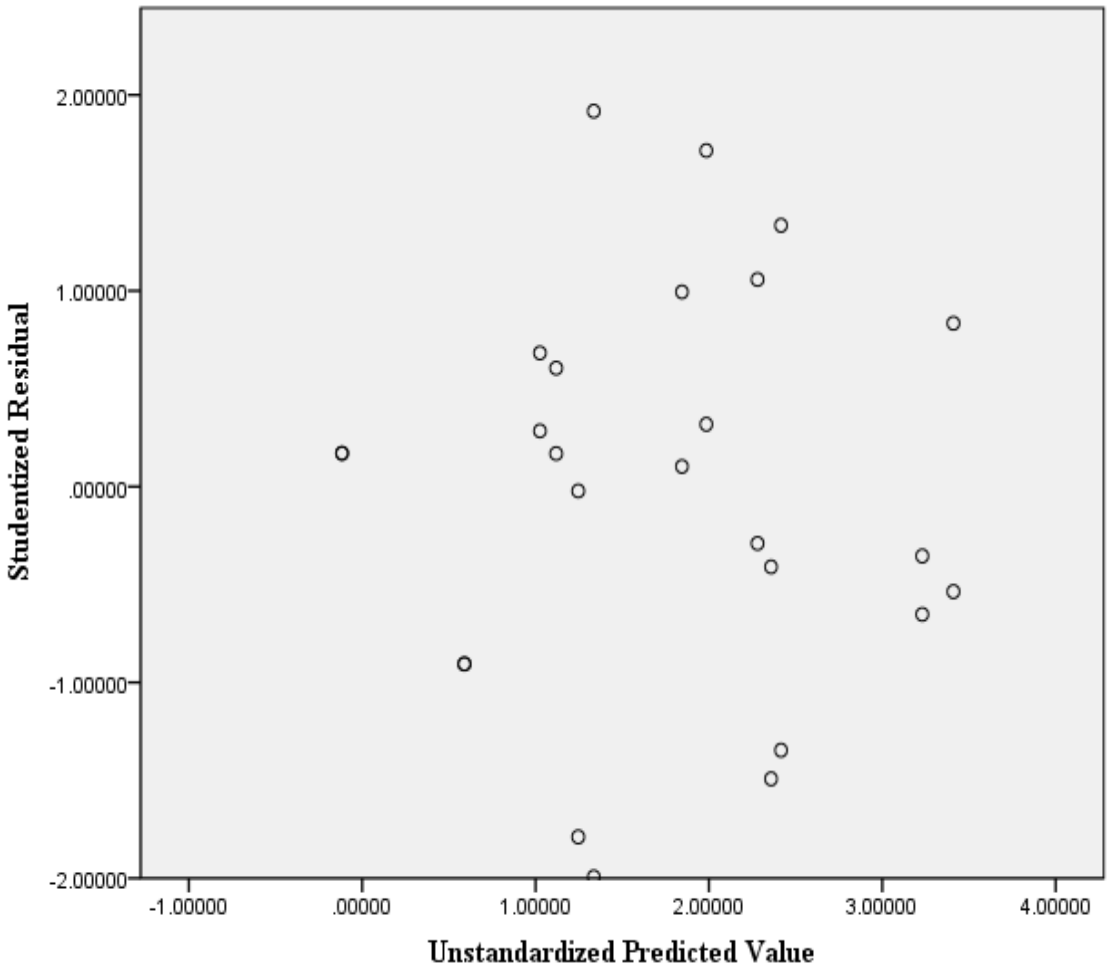
Residuals Statistics[a].

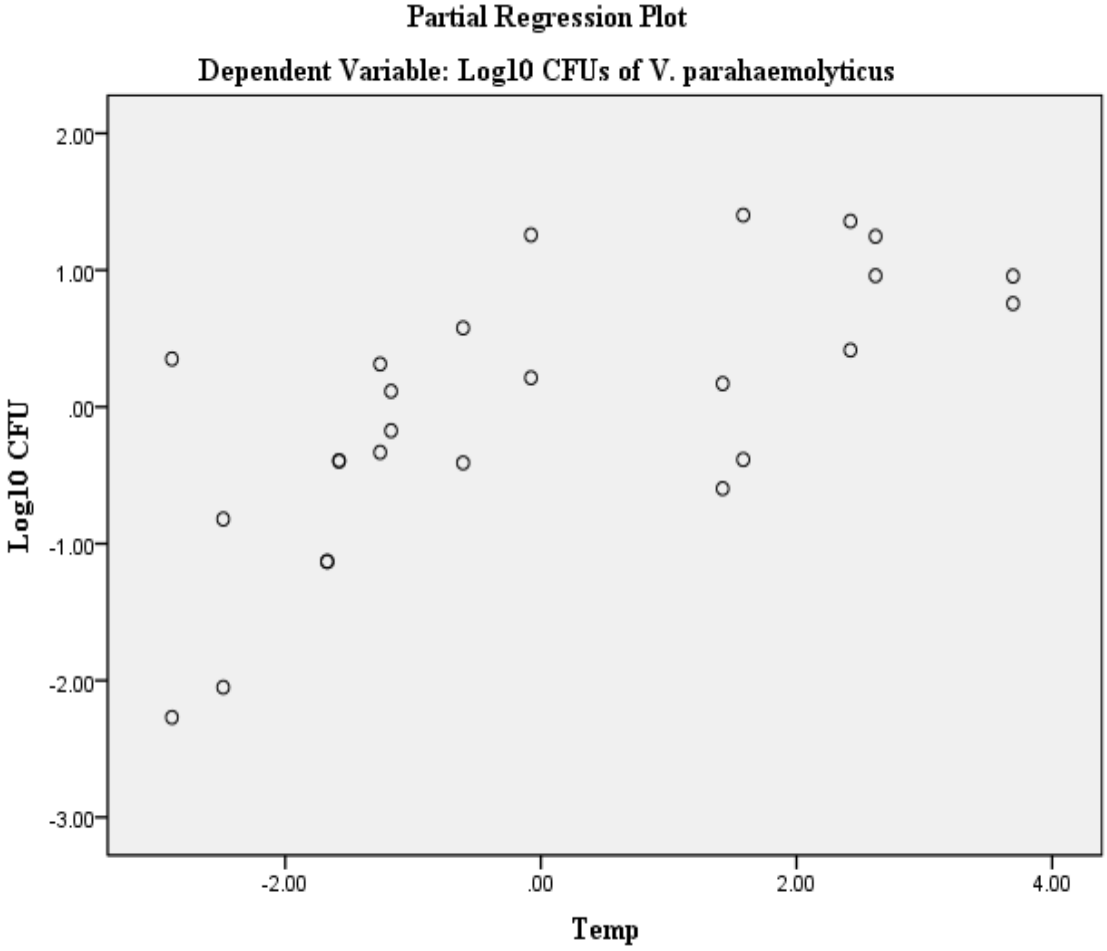
	Minimum	Maximum	Mean	Std. Deviation	N
Predicted Value	-0.1165	3.4102	1.7478	0.99143	26
Std. Predicted Value	-1.880	1.677	0.000	1.000	26
Standard Error of Predicted Value	0.198	0.417	0.333	0.066	26
Adjusted Predicted Value	-0.1492	3.5074	1.7699	1.00625	26
Residual	-1.33542	1.28471	0.00000	0.70849	26
Std. Residual	-1.728	1.662	0.000	0.917	26
Stud. Residual	-1.993	1.917	-0.013	1.025	26
Deleted Residual	-1.77726	1.70977	-0.02206	0.88885	26
Stud. Deleted Residual	-2.160	2.060	-0.016	1.066	26
Mahal. Distance	0.683	6.322	3.846	1.742	26
Cook's Distance	0.000	0.263	0.052	0.073	26
Centered Leverage Value	0.027	0.253	0.154	0.070	26

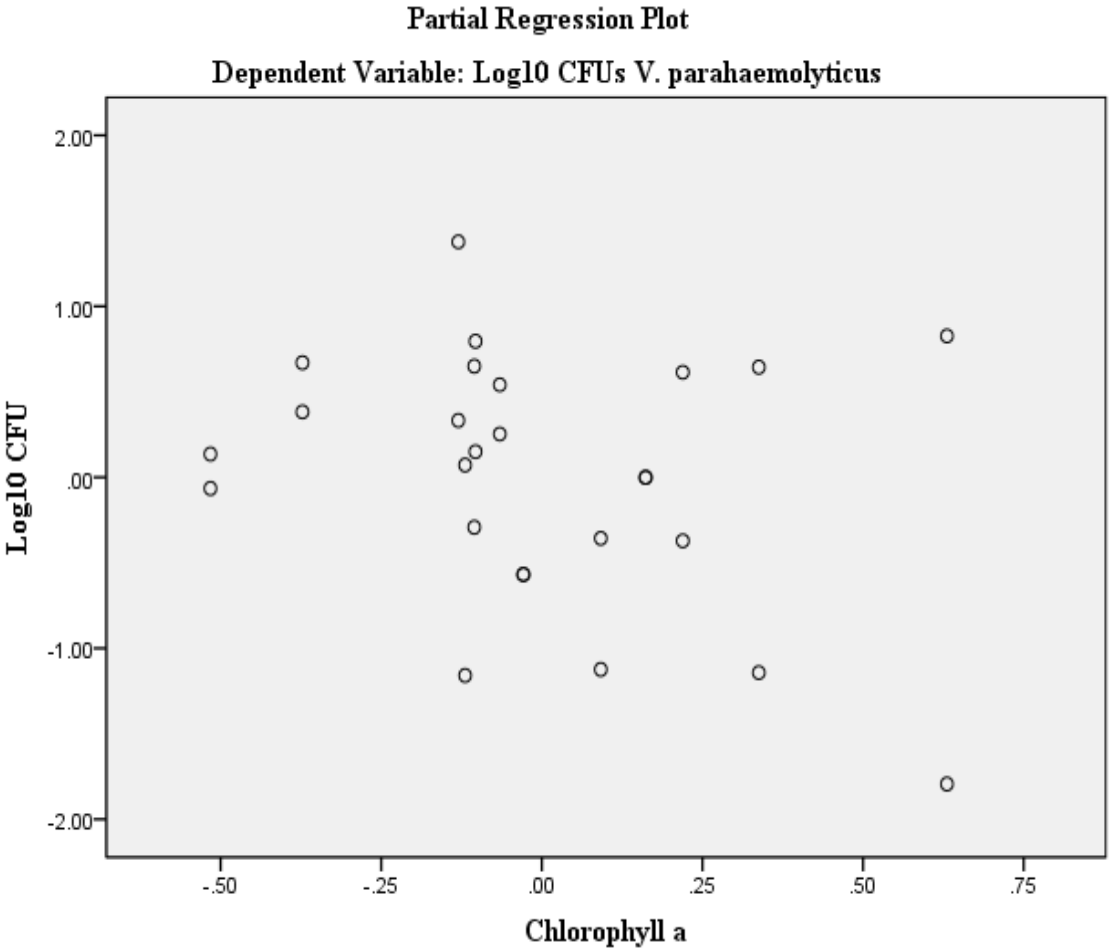
a. Dependent Variable: *Vibrio parahaemolyticus* Log_{10} CFU.

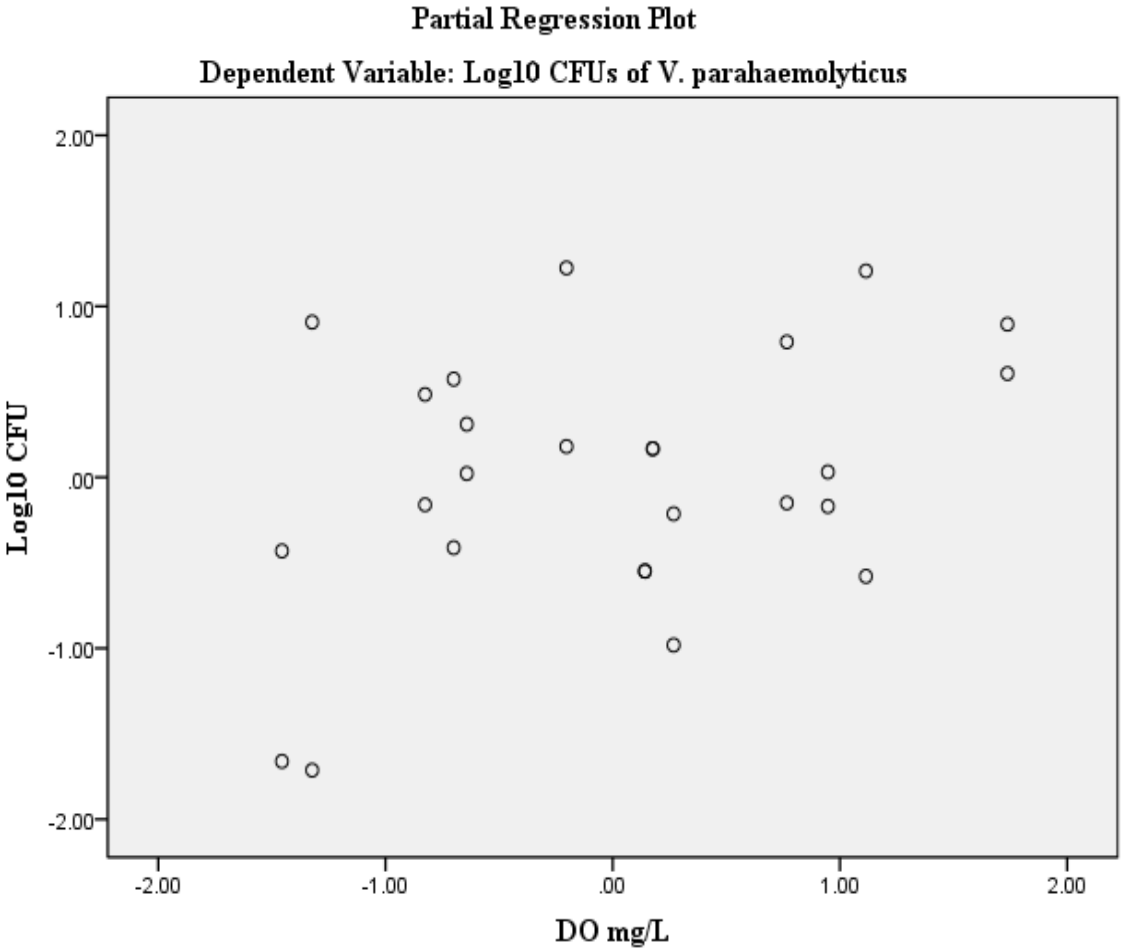

Histogram
Dependent Variable: Log10 CFUs of V. parahaemolyticus
Mean = 3.15E-15
Std. Dev. = 0.917
N = 26
Frequency
0
1
2
3
4
5
6
-2
-1
0
1
2
Regression Standardized Residual

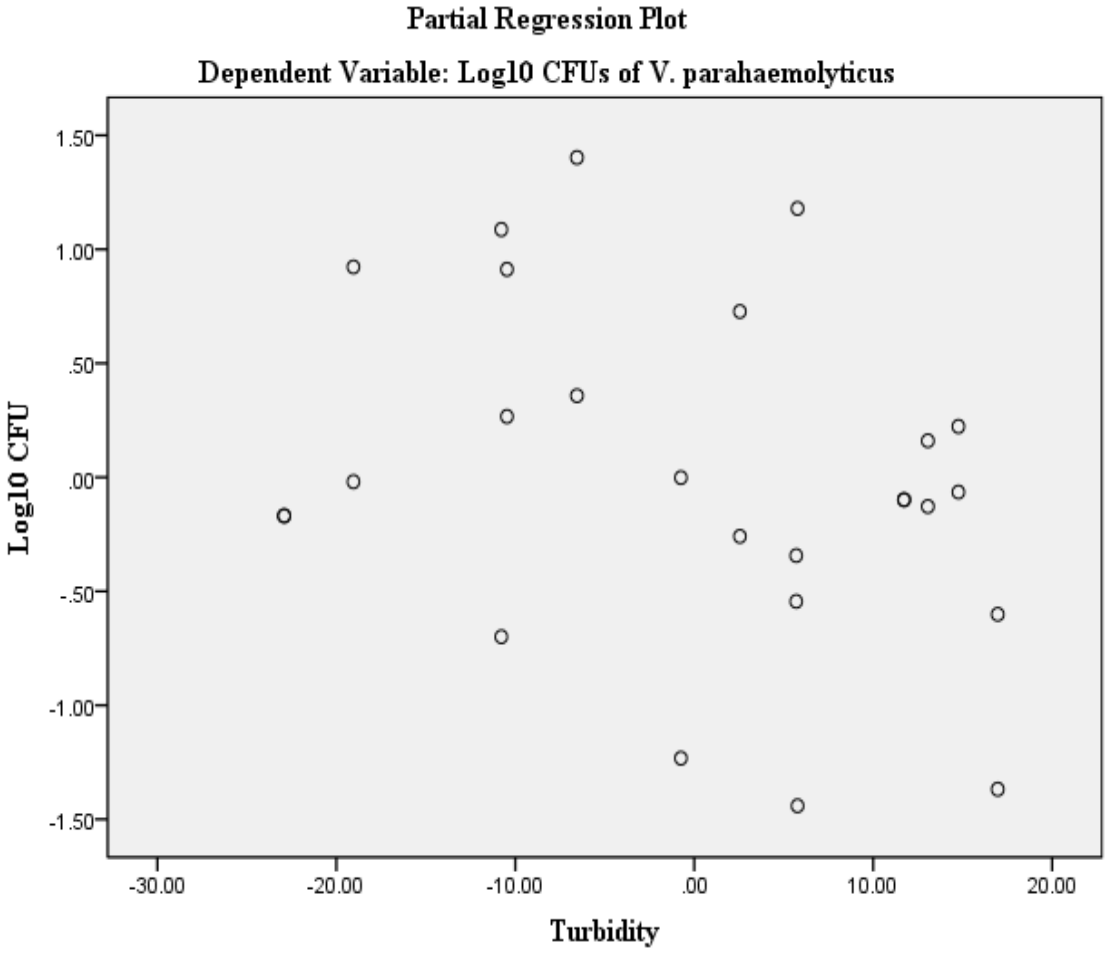

Normal P-P Plot of Regression Standardized Residual
Dependent Variable: Log10 CFUs of V. parahaemolyticus
Expected Cum Prob
Observed Cum Prob
0.0
0.2
0.4
0.6
0.8
1.0


Studentized Residual
2.00000
1.00000
.00000
-1.00000
-2.00000
-1.00000
.00000
1.00000
2.00000
3.00000
4.00000
Unstandardized Predicted Value


Partial Regression Plot
Dependent Variable: Log10 CFUs of V. parahaemolyticus
Log10 CFU
2.00
1.00
.00
-1.00
-2.00
-3.00
-2.00
.00
2.00
4.00
Temp


Partial Regression Plot
Dependent Variable: Log10 CFUs V. parahaemolyticus
Log10 CFU
2.00
1.00
.00
-1.00
-2.00
-.50
-.25
.00
.25
.50
.75
Chlorophyll a


Partial Regression Plot
Dependent Variable: Log10 CFUs of V. parahaemolyticus
Log10 CFU
DO mg/L
2.00
1.00
.00
-1.00
-2.00
-2.00
-1.00
.00
1.00
2.00


Partial Regression Plot
Dependent Variable: Log10 CFUs of V. parahaemolyticus
Log10 CFU
1.50
1.00
.50
.00
-.50
-1.00
-1.50
-30.00
-20.00
-10.00
.00
10.00
20.00
Turbidity