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Heterogeneity Among Harveyi Clade-Specific Virulence Genes and Attribution of These Genes to Possible Environmental Carriers

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HETEROGENITY AMONG HARVEYI CLADE-SPECIFIC VIRULENCE GENES AND ATTRIBUTION OF THESE GENES TO POSSIBLE ENVIRONMENTAL CARRIERS

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1. ABSTRACT

In the face of global warming, waterborne pathogens are evolving rapidly and expanding geographically. The *Vibrio* Harveyi clade encompasses a number of human and aquatic organism pathogens, and due to their physiology, they may benefit from ongoing changes in the temperature, pH, and salinity of the global Ocean. With this, the Southeastern United States might become an accommodating environment for Harveyi clade species, and it is imperative to monitor for their abundance and distribution in the environment. A qPCR assay was developed for the detection and quantification of clade-specific virulence genes *toxR*$_{vh}$*, hly, toxR, luxR, srp,* *vhh*$_{a,}$ *vhh,* and *vhp*. These genes were detected in waters and sediments at all three study sites in Georgia (USA). This study showed a usual temporal trend with higher gene concentrations in summer months while also revealing a heterogeneity in Harveyi clade virulence genes. These results evidence that the Harveyi clade *Vibrio* are split into two distinct groups in Georgia waters; one group represented by *toxR* and *srp* were less adapted to elevated temperatures, alkali pH, and low salinity compared to the group harboring *luxR, vhp,* *vhh*$_{a,}$ and *vhh*. Moreover, lack of detection of *V. harveyi* and *V. campbellii,* the most known pathogens of the clade, suggests that these *Vibrios* are not the carriers for the aforementioned virulence genes. The positive response to elevated temperature and low pH exhibited by *Vibrio* pathogens in this research demands their future study as prospective agents of future *Vibrio* disease outbreaks.

2. INTRODUCTION

Climate change unequivocally poses the greatest threat to humanity and biodiversity on Earth, and environmental changes due to anthropogenic activities are directly impacting the composition and stability of ecosystems (Hautier et al., 2015). Marine biomes contain the greatest abundance and diversity of life and are one of the most susceptible ecosystems to the effects of climate change. Global warming and the consequential increase in sea surface temperatures (SST) have been notably linked to the extinction and endangerment of aquatic organisms, however, one of the main threats of climate change is the increased occurrence of diseases (Cavicchioli et al., 2019; Brandão et al., 2022). Changes in water temperature and other physicochemical parameters caused by global warming are inducing significant effects on aquatic microbial communities. Moreover, rising SST's have been associated with increased growth and survival of disease-causing viruses and bacteria, as well as have been implicated in their geographical expansion (Hauser et al., 2021; Coates and Norton, 2021; El-Sayed and Kamel, 2020).

One of the largest concerns for increased pathogen survival and expansion is the impact on the global aquaculture industry (World Bank, 2014). During 2020, the global production of aquatic animals was almost 200 million metric tons. To meet the increasing demand for protein driven by population growth, it is expected that this output will double by 2030 (World Fish, 2023). Currently, infectious diseases are the most formidable threat to the industry, and it is estimated to result in losses of more than $6 billion USD annually (FAO, 2022). Over-intensive farming, overcrowding, and antibiotic resistance are significant causes of disease spread in aquaculture, and the effects of global warming on microbial communities are expected to contribute to infection rates (FAO, 2022; Cavicchioli et al., 2019).

Global producers of marine aquaculture, especially Asian countries, are severely impacted by bacterial infections (World Bank, 2014). Most common among these pathogens are members of the *Vibrio* genus, which are well-known agents of disease in both humans and animals (Deng et al., 2020). Vibrio species are ubiquitous in aquaculture environments, and many are notorious pathogens to marine vertebrates and invertebrates. Vibrio infections are becoming more prevalent in tropical and subtropical regions, likely due to their high metabolic versatility and ability to quickly adapt under adverse conditions (Montánchez and Kaberdin, 2020).

Due to climate change, the Southeastern United States presents an increasingly accommodating environment for the expansion of microbial species. The vulnerability of the region to new pathogens is particularly worrisome due to the abundance of aquaculture products produced in the southeastern USA (NOAA, 2020). Pathogenic vibrio including *Vibrio parahaemolyticus* and *V. vulnificus* have been well-documented in Coastal Georgia, and there is probable reason to believe that other members of the genus are present. Species belonging to the Harveyi clade are among the most prolific agents of infection in global aquaculture, however, some of the most common members of the clade have yet to be studied in the region. This study sought to detail the seasonal distribution dynamics of the Harveyi clade virulence genes in water and sediments of Coastal Georgia, to elucidate environmental drivers for their distribution, and to determine the presence of clade-members *Vibrio harveyi* and *Vibrio campbellii* in this environment.

3. LITERATURE REVIEW

3.1 Vibrio

The *Vibrio* genus encompasses more than 100 species of gram-negative, halophilic bacteria, many of which are ubiquitous in estuarine and marine environments (Baker-Austin et al., 2018). *Vibrio* species have highly plastic genomes and can survive in a large range of environmental conditions; furthermore, as seen by their perseverance and geographical expansion over the past two decades, *Vibrio* spp. are readily adapting to climate changes (Baker-Austin et al., 2012). The emergence of *Vibrio* species in new regions and hosts is largely attributed to rising sea surface temperatures (SST), suggesting that vibrio are microbial barometers of climate change (Baker-Austin et al., 2017). In the North Atlantic and North Sea, climate-dependent sea surface warming has been directly linked to an increased presence of vibrio (Vezzulli et al., 2016). Additionally, heatwaves in Northern Europe over the last three decades positively correlate with vibrio wound infections (Fleischmann et al., 2022). There have also been reports of commensal and mutualistic *Vibrio* spp. converting to opportunistic pathogens in the presence of elevated temperatures (Luna et al., 2010).

Many vibrio are agents of disease in both humans and animals, and with their continuous expansion, instances of Vibrio-related illness are expected to rise in the following years (Vezzulli et al., 2013). Vibriosis, the disease caused by pathogenic vibrios, is characterized as a gastrointestinal illness in humans and presents as a conglomerate of symptoms in animals (Bell and Bott, 2021). The CDC reports that 95% of foodborne illnesses in the United States are due to *Vibrio* (CDC, 2023). Though vibrios are of relevance to human health, the most vulnerable entity to Vibrio-related diseases is the aquaculture industry. Asia is the largest global supplier of

aquaculture products, and it is estimated that vibrios are responsible for 70% of all diseases in Asian-reared marine animals. In a 2020 study, vibriosis reportedly made up two-thirds of all diseases found in farmed *Epinephelus* spp. in China (Deng et al., 2020). Some *Vibrio* species have been found to have up to a 100% mortality rate in infected mariculture (Kumar et al., 2021). Moreover, the economic impact of vibriosis is devastating, and it is essential to monitor the expansion of pathogenic *Vibrio* species to mitigate effects on the global aquaculture industry.

3.2 The Vibrio Harveyi clade

The *Vibrio* Harveyi clade has particularly become of recent ecological and economical importance due to the number of disease outbreaks caused by its members. The clade is comprised of 12 species including *Vibrio harvyei, Vibrio campbellii, Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio diabolicus, Vibrio rotiferianus, Vibrio jasicida, Vibrio owensii, Vibrio natriegens, Vibrio azureus, Vibrio sagamiensis,* and *Vibrio mytili,* which infect a range of hosts (Culot et al., 2021).

At the core of the clade, *Vibrio harvyei* is currently regarded as the most prolific agent of disease and death in mariculture. Like many vibrios, *V. harvyei* can be found free living in the water column or in biofilms, but it most commonly exists as a parasitic microbe in reared fish, crustaceans, and mollusks (Allen and Finkel, 2022). A number of symptoms and diseases are associated with *V. harveyi* infections, including eye disease, gastroenteritis, necrotizing enteritis, muscle necrosis, scale drop, skin ulceritis, tail rot disease, vasculitis, acute hepatopancreatic necrosis disease (AHND), bacterial white tail disease, black shell disease, early mortality syndrome (EMS), and luminous vibriosis. Efforts to combat disease with vaccines and antibiotics

have shown some promise, though *V. harveyi* continues to cause multi-million dollar losses annually in the aquaculture industry (Zhang et al., 2020).

Recently, Harveyi clade member *Vibrio campbellii* has garnered attention as an emerging opportunistic pathogen. Like *V. harveyi, V. campbellii* causes luminescent vibriosis, ANHD, and EMS in marine vertebrates and invertebrates (Zhang et al., 2020). *V. campbellii* is most commonly associated with shrimp diseases and some hatcheries have reported mortality rates as high as 100% (Kumar et al., 2021). The pathogen shares more than 97% 16S rRNA gene similarity with *V. harveyi* and has faced extensive misidentification; thus, for this reason, it is thought that cases and diseases caused by *V. campbellii* are severely underreported (Srisangthong et al., 2023).

3.3 Virulence and Climate Change

While not all species within the Harveyi clade are agents of vibriosis, a number of virulence genes and factors have been identified in pathogenic species like *V. harveyi* and *V. campbellii*. Typical virulence factors in Harveyi clade vibrios such as haemolysins, phospholipases, metalloproteases, serine proteases, and chitinases are linked to pathogenicity (Ruwandeepika et al., 2011). Whereas other than Harveyi clade *Vibrio* species carry functionally similar virulence genes, these have distinctively different sequence from those carried by the members of the clade. This fact allowed developing primers for virulence genes specific only for bacterias of Harveyi clade (Ruwandeepika et al., 2011). The presence of virulence genes alone does not directly dictate disease progression and outcome for all species but the concentrations of these genes in an environment indicates the abundance of pathogenic Harvey clade bacteria. The

expression level of these factors are indicators of virulence. Regulation and production of virulence factors in pathogenic bacteria is largely controlled through quorum sensing (QS). Though several QS systems have been identified in *Vibrio* spp., most contain the autoinducer-2 (AI-2) system. The AI-2 based QS system is responsible for the production of *luxR*, a QS master regulator in *Vibrio*, which is known to play an important role in biofilm formation, stress response, and virulence gene expression (Brackman et al., 2008). *LuxR* expression in virulent strains of *V. harveyi* and *V. campbellii* has been positively correlated with expression levels of metalloprotease, serine protease, and the regulatory *toxR* gene, however, regulation of virulence factors by *luxR* varies considerably among other vibrios (Chaparian et al., 2020).

The transmembrane transcriptional regulator *toxR* is conserved among vibrios and is considered one of the most important regulators of virulence in pathogenic species. *ToxR* was first discovered in *V. cholerae* as a positive transcriptional regulator of the cholera toxin, but it has since been found to regulate over 20 genes associated with quorum sensing, bile resistance, and the expression of outer membrane proteins (Ruwandeepika et al., 2011; Almagro-Moreno et al., 2015; Gubensak et al., 2021). The *toxR* regulon has not been well studied outside of *V. cholerae,* however, recent work with *V. parahaemolyticus* and *V. harveyi* suggests that *toxR* is an essential regulator of biofilm formation, type-3 secretion system expression, and other virulence factors in Harveyi clade vibrios (Zhang et al., 2018). Though more research is needed to conclusively elude all the roles of *toxR*, it has been shown that higher *toxR* expression in pathogenic Harveyi clade vibrios is significantly correlated with brine shrimp larvae survival. In an *in vivo* study, *toxR* expression was 122-fold higher in virulent strains of *V. harveyi, V. campbellii,* and *V. parahaemolyticus* than in their avirulent counterparts (Ruwandeepika et al., 2011).

Unfortunately, little research has been done regarding the effects of climate change on virulence gene production or expression in the Harveyi clade. Comprehensive studies assessing the geographical spread of pathogenic *Vibrio* spp. indicate that rising sea surface temperatures (SST) is the primary driver, but the mechanisms by which *toxR*, *luxR*, and other critical virulence genes facilitate expansion are somewhat elusive. A recent analysis of *V. harveyi* revealed that major adaptive mechanisms were poorly coordinated under elevated temperatures and carbon limitation, ultimately affecting cell survival. Surprisingly, however, gene expression analysis showed that lytic enzymes, siderophores, type-3 secretion systems, motility, and numerous other virulence factors had enhanced expression under stress conditions. The results of this study suggest that temperature-dependent expression of virulence factors is important for the spread of *Vibrio* spp. and *Vibrio*-associated diseases (Montánchez et al., 2020).

In addition to rising SST's, other effects of climate change are also expected to impact virulence and expansion of *Vibrio* spp. Anthropogenic activities since the industrial revolution have significantly increased the concentration of CO_2 in the atmosphere, ultimately contributing to ocean acidification. *Vibrio* can survive in a range of pH levels but are preferential to alkaline environments. Many bacteria have decreased enzymatic activity and lose culturability at low pH, however, a study assessing *V. harveyi* survival at varying pH indicates that it plays a less important role in survival and virulence than temperature does (Gundogdu et al., 2023). Research with *V. cholerae* has shown that acidity regulates virulence factor expression of the cholerae toxin, but more work is needed to understand the effects of pH on the pathogenicity of other *Vibrio* spp. (Nhu et al., 2021). Although pH-dependent virulence expression is not well-studied, ocean acidification is thought to favor vibrio infections by significantly weakening the immune

systems of marine animals like bivalves, crustaceans, and echinoderms (Baker-Austin et al., 2018).

Glacier shrinkage and changes in rainfall patterns are also expected to impact microbial communities by decreasing the salinity of the world's oceans. *Vibrio* spp. are halophiles that grow optimally between 0.5 and 2.5% salinity, although several species can survive outside this range. Low salinity (1.0-1.5%) has reportedly been linked to increased growth rate and virulence of *V. harveyi* but evidence from other studies with *V. harveyi, V. parahaemolyticus, V. cholerae,* and *V. vulnificus* suggest that presence, growth, and virulence correlate with higher salinity (Prayitno and Latchford, 1995; Wang et al., 2020). Selven et al. found that *Fenneropenaeus indicus* challenged with *V. harveyi* infection had lower survival rates at high salinity (3.5%), and this was largely attributed to increased expression of haemolysin proteins in *V. harveyi* (Selven and Philip, 2013). In another study, however, *V. harveyi* growth and infection in shrimp were found to decrease when salinity was greater than 2% (Matderis et al., 2022) Though these results are contradictory, they can likely be reconciled given that infection susceptibility varies extensively among organisms.

4. MATERIALS AND METHODS

4.1 Field Sites and Sampling Events

Three distinct sampling sites were established within the coastal salt marshes offshore of Townsend, Georgia (Figure 1). Directly north of Fourmile Island, Site 1 was located along the Julienton River at 31°33’34° N, 81°17’16 W. Site 2 was located at the mouth of Sapelo River at 31°32’33 N, 81°16’53 W. Site 3, the southernmost site, was located along the Mud River at 31°30’21 N, 81°16’44 W. Julienton River, Sapelo River, and Mud River are primarily tidal rivers that feed into the Sapelo Sound bay. Each site was in direct proximity to wild clam and oyster beds owned and operated by Sapelo Sea Farms. A total of five sampling events were conducted during low tide in June 2022, August 2022, October 2022, December 2022, and February 2022.

Figure 1. Map of selected study sites

4.2 Water Collection and Processing

For each sampling event, 1 L of water was collected in triplicates at each of the three sites. Water was collected at a depth of approximately 4 feet. Triplicates were collected within 25 yards of each other to provide variation within individual sites. This was completed using sterile 1 L glass bottles and a 4000 Subsurface Grab Bottle Sampler. Bottles were rinsed twice with environmental water prior to collection and samples were immediately placed on ice. Once transported to the laboratory, the 1 L water samples were filtered using a custom water filtration system with 0.22 μm Millipore nitrocellulose filters. Total DNA was extracted using the Qiagen DNEasy® PowerWater kit, and DNA was quantified using a NanoDrop ND-1000 Spectrophotometer. Isolated DNA was stored at -20°C.

4.3 Sediment Collection and Processing

Sediments were collected in triplicates at each site. To provide variation within sites, the triplicates were collected from different clam or oyster beds within 25 yards of each other. This was done by shoveling sediments directly into sterile 1.5-ml microcentrifuge tubes. The tubes were immediately transferred to sterile Whirl-Pak bags and placed over ice. In the laboratory, 250 mg of sediment from each site was used for DNA extraction via the Qiagen DNEasy® PowerSoil Kit. DNA was quantified using a NanoDrop ND-1000 Spectrophotometer, and isolated DNA was stored at -20°C.

4.4 Measurement of Environmental Parameters

A Horiba U-52G Multiparameter Water Quality Meter (Horiba) was used to monitor temperature (°C), pH, salinity (ppt), turbidity (NTU), dissolved oxygen (mg/L), total dissolved solids (g/L), conductivity (mS/cm), and potential water density (∂t) at each of the three sites.

4.5 Genes Targeted and Generation of Standard Curves

In total, six virulence genes previously reported as Harveyi clade-specific were targeted in this study. Among these genes were the transmembrane transcriptional regulator *toxR*, the quorum sensing master regulator *luxR*, the *vhp* metalloprotease, the *vhh* and vhh_a haemolysins, and the *srp* serine protease. This study also targeted the species-specific $toxR_{vh}$ transcriptional regulator and *hly* haemolysin to evaluate the presence and abundance of *Vibrio harveyi* and *Vibrio campbellii*, respectively. The RNA polymerase A subunit (*rpoA)* housekeeping gene was additionally targeted for further monitoring of these species.

A PCR assay for each target gene was first conducted to generate products that would later be used to make standard curves. Pure cultures of *Vibrio harveyi* (ATCC 14126) and *Vibrio campbellii* (ATCC BAA-1116/BB120) previously reported as carrier of the genes targeted in this study were grown in Marine Broth overnight at 32°C prior to undergoing DNA extraction with the Qiagen DNEasy Microbial Kit. DNA obtained from the extractions were quantified using a NanoDrop ND-1000 Spectrophotometer and subsequently diluted to a concentration of 10 ng/µl. The assay was optimized to include 10 ng of pure *Vibrio harveyi* or *Vibrio campbellii* DNA, 5 µl BioRad PCR Master Mix, 3 µl of nuclease-free molecular grade water, and primers at 250 nM to achieve a total reaction volume of 10 µl. Pure *Escherichia coli* DNA was used as a negative control. The cycling parameters and primers used to amplify *toxR*, *luxR*, *srp*, vhh_a, *vhp*, *vhh*, $toxR_{vh}$, *hly*, and *rpoA* are outlined in Appendix, Table 1 and Table 2.

The resulting PCR products were transferred to sterile 1.5 ml microcentrifuge tubes and labeled. The products were purified using the Qiagen DNEasy PowerClean Clean Up Kit then quantified using a Qubit Spectrophotometer. Concentrations were recorded for each target gene product and the amount of gene copies per tube were calculated using the following formula:

number of copies = (ng of DNA * (6.022×10^{23})) / (size of amplicon in bp * (1.0×10^{9}) * 650). Copy number calculations ranged from 3.90×10^{10} to 1.32×10^{11}, and each DNA sample was serially diluted 10-fold. Detection limits varied by gene and therefore the number of serial dilutions varied for each DNA sample.

The diluted DNA for each gene served as a standard and was ran in triplicates through a dye-based qPCR assay. The assay was optimized to include 1 µl of DNA at the desired dilution, 5 µl BioRad SYBR Green Master Mix, 3 µl of nuclease-free molecular grade water, and 1 µl of forward and reverse primers at 250 nM. The PCR and qPCR assays employed the same primers and cycling conditions. A melt curve step was included at the end of cycling in the RT-qPCR assays. The assays also included a non-template control and serially diluted *E. coli* DNA as a negative control.

The MASTERO BioRad software employs an automated linear regression feature to describe the relationship between the log of the copy number values and the cycle threshold (Ct) values for each assay. The equation provided for each gene's standard curve was further used to calculate the amount of gene copies in environmental samples (see Appendix, Figure 3).

5. RESULTS

5.1 Virulence Gene Detection

Each virulence gene was detected at least once during the course of this study (Table 3), though copy number varied considerably among sampling events and sample type as will be considered later in this chapter. The data presented in this table evidenced that gene profile varied between water and sediments and site-to-site. Their overall incidence frequency was similar for most genes except for *toxR,* which incidence was lower than the other five genes.

Table 3. Virulence gene occurrence and instance of detection at Sites 1-3

Site/Gene	*toxR*	*luxR*	*srp*	*vhh$_a$*	*vhp*	*vhh*
Site 1						
June Sediment	+	+	+	+	+	+
August Sediment	-	+	+	+	+	+
October Sediment	+	-	+	+	+	-
December Sediment	-	+	+	+	+	+
February Sediment	+	+	+	+	+	+
June Water	+	+	+	+	+	+
August Water	-	+	-	+	+	+
October Water	-	+	+	+	+	+
December Water	-	+	-	-	+	-
February Water	-	+	-	-	+	+
Site 2						
June Sediment	+	-	+	+	+	-
August Sediment	+	+	+	+	+	+
October Sediment	+	+	+	+	+	+
December Sediment	-	-	+	+	-	+
February Sediment	-	+	+	+	+	+
June Water	+	+	+	+	+	+
August Water	+	-	+	+	+	+
October Water	-	+	+	+	-	+
December Water	-	+	+	-	-	-
February Water	-	+	-	-	+	-
Site 3						
June Sediment	-	+	+	+	-	+
August Sediment	+	+	+	+	+	+
October Sediment	+	+	+	+	+	+
December Sediment	+	+	+	+	+	+
February Sediment	+	+	+	+	+	+
June Water	+	+	+	+	+	+
August Water	+	+	+	+	+	+
October Water	-	+	+	-	+	+
December Water	-	-	+	-	+	-
February Water	-	-	-	+	+	+
Detection Frequency	15/30	24/30	25/30	24/30	26/30	24/30
Sediment Total	10/15	12/15	15/15	15/15	13/15	13/15
Water Total	5/15	12/15	10/15	9/15	13/15	11/15

5.2 Environmental fate of Virulence Genes in water

All six virulence genes were detected in water samples during June and August with average gene copy numbers of 4.05E+04 and 1.91E+04/mL of water, respectively (Fig. 2). Variations among sites were evaluated with Welch's ANOVA and revealed there was not a significant difference in average gene copies between sites during independent sampling events (data not shown), therefore, gene copies for sites 1-3 were averaged for each month and error bars were excluded. Of the six virulence genes, only two, *luxR* and *vhp*, persisted at all five sampling events with an average copy number of 1.72E+00 and 2.38E+02/mL of water. Three genes, vhh_a, *srp*, and *vhh*, were detected at four sampling events with an average of 2.47E+04, 2.11E+00, and 1.26E+02/mL, respectively. *toxR* was only detected during the June and August sampling events averaging 4.74+05 and 4.52E+03 gene copies/mL, respectively.

Detection rates and average gene copies were highest during the summer sampling events. During October sampling, *toxR* was not detected in water. The genes that were present in October, *vhh*, vhh_a, *srp*, *vhp*, and *luxR*, had a mean copy number of 2.85E+00/mL. December had the lowest detection rate. Only three genes, *vhp*, *srp*, and *luxR*, were detected with an average copy number of 6.12E-01/mL. In February, vhh_a, *vhh*, *vhp*, and *luxR* were detected with an average copy number of 1.78+01/mL, while *toxR* and *srp* were not present.

The water parameters recorded at each sampling event are shown in Table 4. These parameters also spatially and temporally varied, allowing the identification of environmental drivers for the above genes in consequent experiments. Pearson's correlation coefficient was computed and used to analyze the effects of pH, temperature, salinity, turbidity, dissolved oxygen, conductivity, total dissolved solids, and potential water density on gene copy number (Table 5). Overall, all six virulence genes correlated negatively with pH and dissolved oxygen

while correlating positively with temperature. Notably, however, *toxR* and *srp* displayed consistently different correlational trends from those of *luxR*, vhh_a, *vhp*, and *vhh*. Compared to these genes, *toxR* and *srp* had a stronger relationship with pH and potential water density but exhibited weaker correlation with temperature. Additionally, the pair had moderate positive correlation with salinity while the other genes, with the exception of vhh_a, had weak negative correlation. Interestingly, *toxR* and *srp* also had opposite correlational trends with turbidity, conductivity, and total dissolved solids than those of *luxR*, vhh_a, *vhh*, and *vhp*. To visualize the difference in trends between the two groups of genes, the mean copy numbers for each group were averaged by month and plotted against temperature, pH, and salinity (Fig. 3-8).

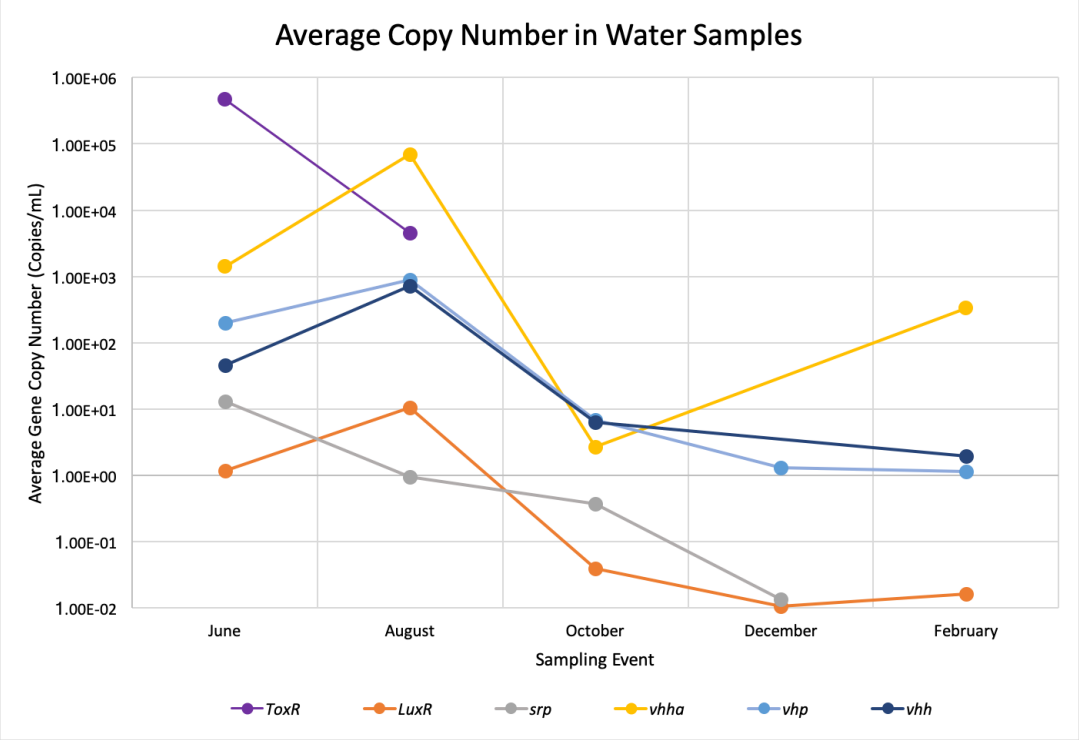

Figure 2. Average gene copies detected in water during all sampling events. There was no statistical difference in gene copies found between sites, therefore the copy numbers for Sites 1-3 were averaged and error bars were excluded.

Table 4. Water parameters recorded at Sites 1-3 during each sampling event

Water Parameters	pH	Temp. (C°)	Salinity (ppt)	Turbidity (NTU)	DO (mg/L)	Conduct. (mS/cm)	TDS (g/L)	Potential Water Density
June								
Site 1	7.10	27.63	18.3	23.6	1.24	31.5	18.1	10.2
Site 2	7.23	26.33	22.4	21.1	1.18	35.7	22.9	11.4
Site 3	7.00	28.05	23.5	22.5	1.26	38.3	23.4	13.6
Average	7.11	27.34	21.40	22.40	1.23	35.17	21.47	11.73
August								
Site 1	7.11	29.42	23.8	15.7	1.50	38.8	24.4	13.8
Site 2	7.10	31.05	24.2	18.4	1.43	39.4	25.1	13.9
Site 3	7.30	30.80	24.9	17.3	0.87	40.0	24.4	14.1
Average	7.17	30.42	24.30	17.13	1.27	39.40	24.63	13.93
October								
Site 1	7.28	20.65	24.3	18.9	2.54	39.5	25.3	15.8
Site 2	7.33	20.95	24.6	20.2	2.30	39.6	25.6	17.3
Site 3	7.37	21.85	25.1	20.3	2.90	39.0	26.1	16.2
Average	7.33	21.15	24.67	19.8	2.58	39.37	25.67	16.43
December								
Site 1	7.43	11.53	23.8	20.4	3.6	43.8	26.7	20.2
Site 2	7.43	11.52	26.6	25.2	3.51	43.5	26.9	20.0
Site 3	7.36	11.32	24.8	21.7	3.22	40.4	24.4	18.1
Average	7.41	11.46	25.07	22.43	3.44	42.57	26.00	19.43
February								
Site 1	7.52	21.29	19.5	17.6	1.5	33.0	20.1	12.8
Site 2	7.37	20.38	21.4	11.2	0.9	35.6	21.7	14.5
Site 3	7.47	19.54	17.0	7.0	1.98	29.6	18.4	11.3
Average	7.45	20.40	19.30	11.93	1.46	32.73	20.07	12.87

Table 5. Heat map depicting the relationship between copy number and water parameter in water samples using Pearson's correlation coefficient.

Gene/Parameter	pH	Temp. (C)	Salinity (ppt)	Turbid. (NTU)	DO (g/L)	Conduct. mS/cm)	TDS (g/L)	Potent. Water Density
toxR	-0.697	0.403	-0.344	0.465	-0.443	-0.385	-0.44	-0.574
luxR	-0.555	0.692	0.271	-0.156	-0.474	0.185	0.180	-0.240
srp	-0.738	0.450	-0.320	0.467	-0.469	-0.371	-0.42	-0.588
vhh_a	-0.480	0.644	0.294	-0.200	-0.428	0.213	0.214	-0.186
vhp	-0.643	0.747	0.235	-0.101	-0.530	0.142	0.132	-0.310
vhh	-0.515	0.668	0.287	-0.179	-0.449	0.203	0.202	-0.210

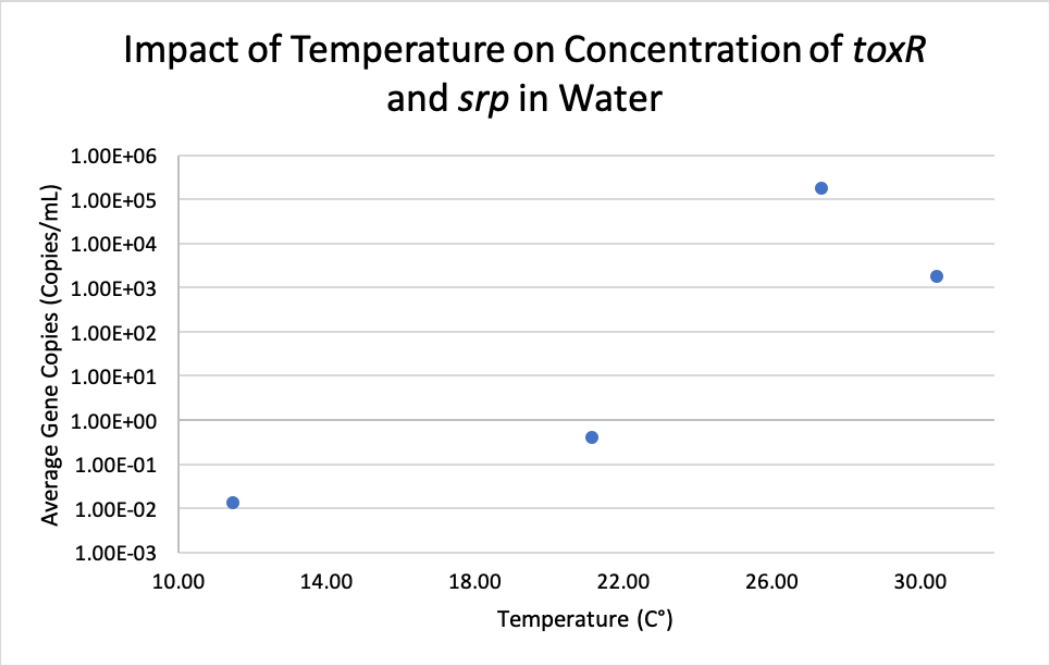

Figure 3. The relationship between temperature and average gene copies in *toxR* and *srp* across sampling events

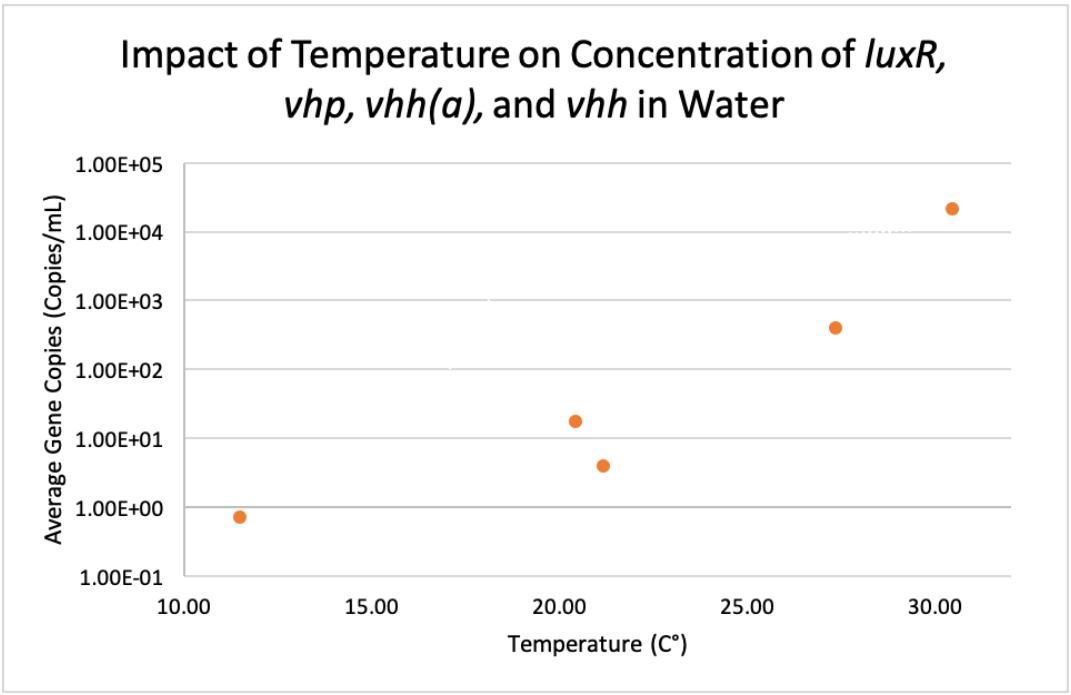

Figure 4. The relationship between temperature and average gene copies in *luxR, vhp, vhh*$_a$ and *vhh* across sampling events

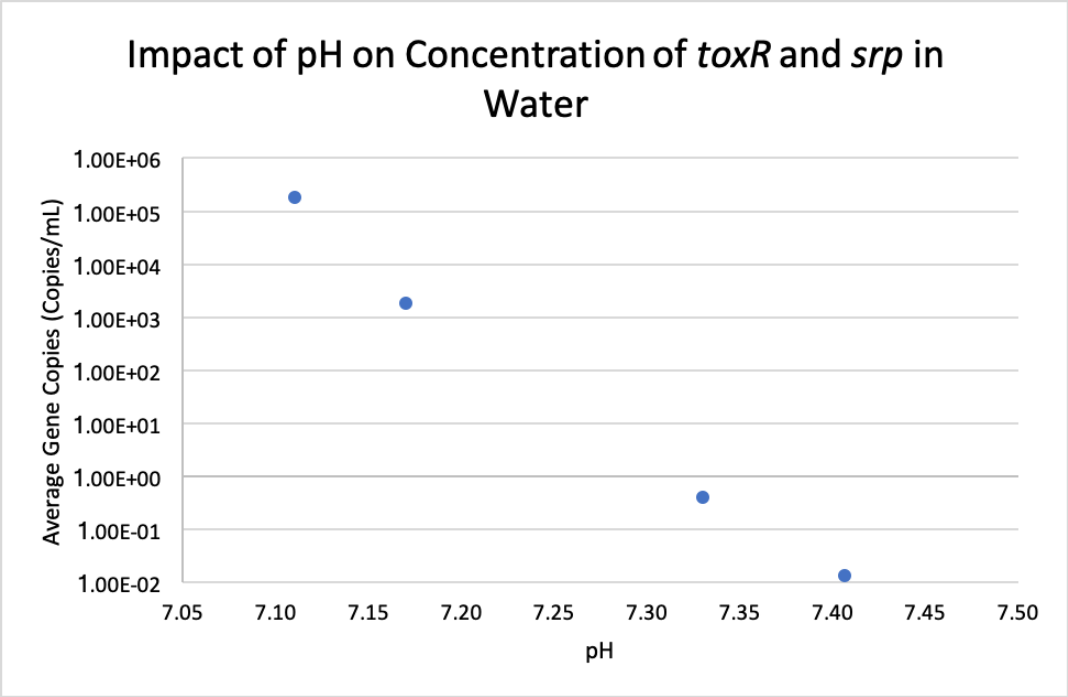

Figure 5. The relationship between pH and average gene copies in *toxR* and *srp* across sampling events

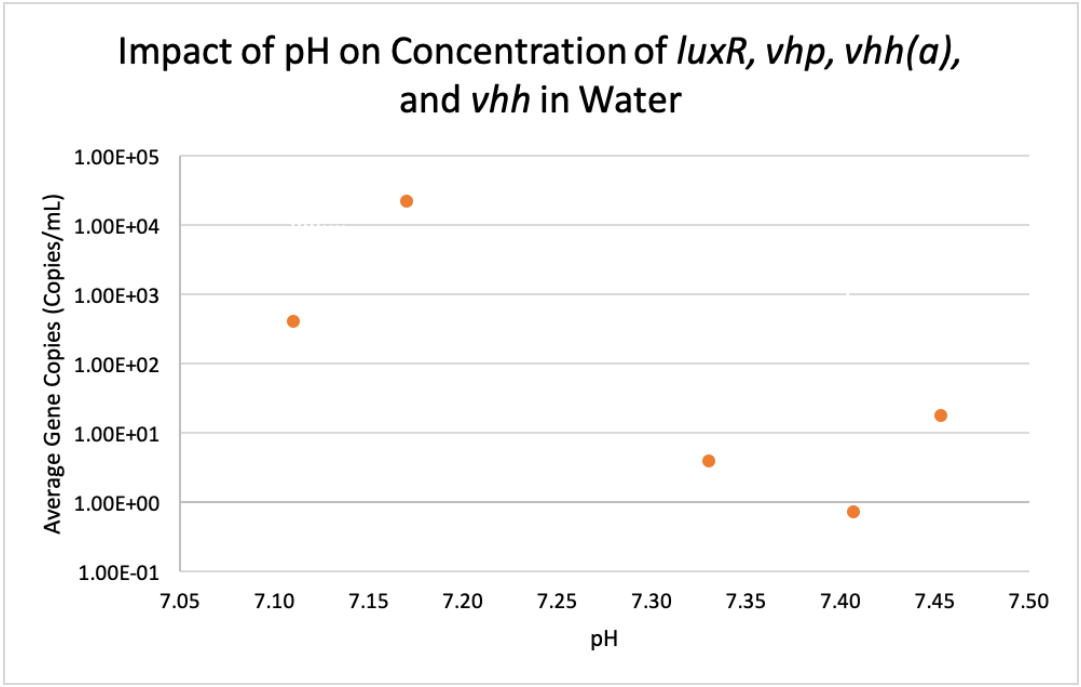

Figure 6. The relationship between pH and average gene copies in *luxR, vhp, vhh*$_a$ and *vhh* across sampling events

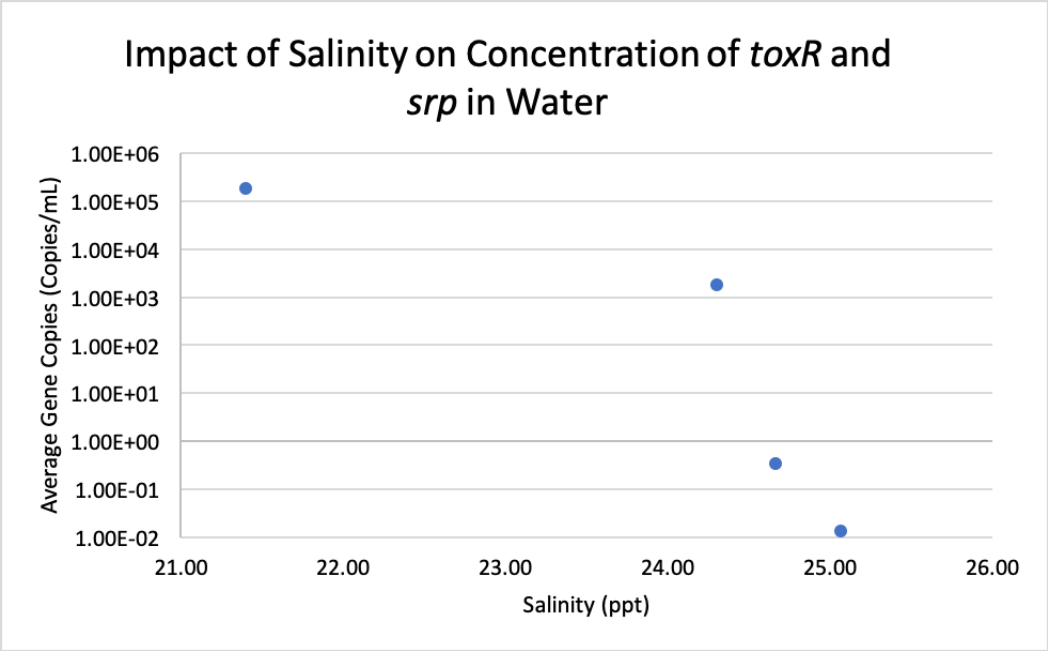

Figure 7. The relationship between salinity and average gene copies in *toxR* and *srp* across sampling events

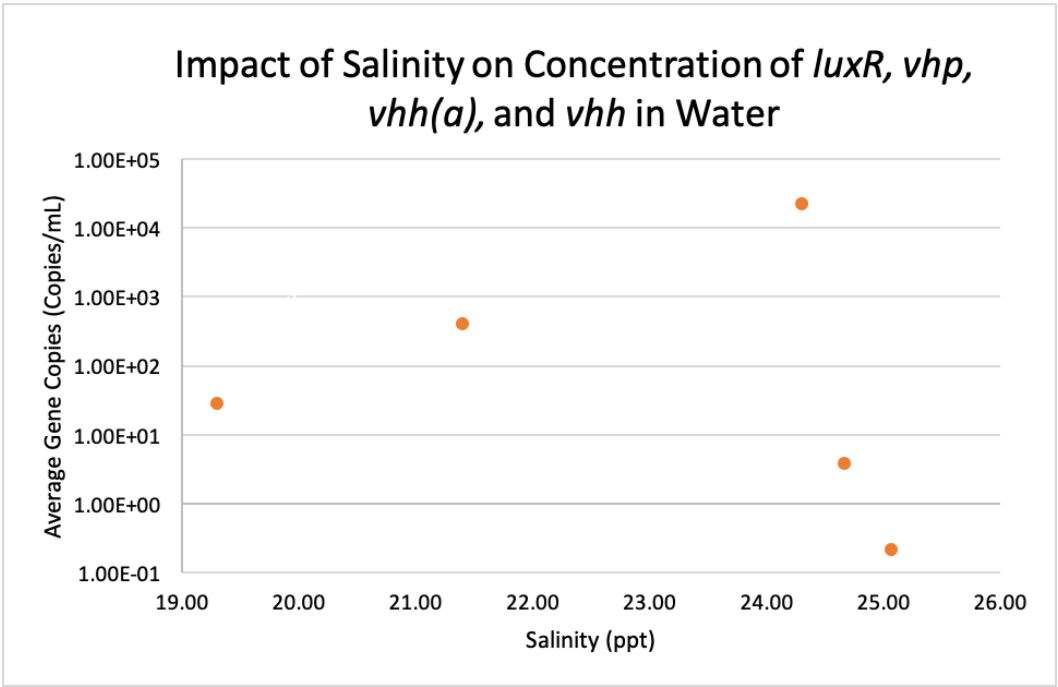

Figure 8. The relationship between salinity and average gene copies in *luxR, vhp, vhh*$_a$ and *vhh* across sampling events.

5.3 Environmental fate of Virulence Genes in sediment

Each of the six virulence genes were detected in sediments during the June, August, October, December, and February sampling events with an average copy number of 4.37E+07, 4.39E+07, 8.63E+05, 4.66E+05, and 1.11E+05 per gram of sediment, respectively (Figure 9). Likewise to the water samples, the highest copy numbers were seen in June and August. December also had the lowest detection rate of all sampling events. In contrast, however, the average copy number for all genes detected in sediments during February was lower than those in December.

Figure 9 shows the average copy number for each gene during independent sampling events. The copy numbers for each gene detected at sites 1-3 were evaluated with Welch's ANOVA, and the variation in copy number was not significant. Furthermore, copy numbers for sites 1-3 were averaged and error bars were excluded in Figure 9. The highest gene concentration detected in this study was found in vhh_a, overall averaging 1.13E+08 copies per gram of sediment. The second highest copies were detected in *toxR* with an average of 4.11E+06/g, followed by *srp* with 2.04E+05, *vhh* with 1.65E+05, *vhp* with 1.35E+05, and *luxR* with 1.41E+04/g. On average, sediment samples had gene concentrations 3-6 orders of magnitude greater than those in water.

Correlation between copy number and water parameters were analyzed with Pearson's coefficient and shown in Table 6. When analyzed individually, the correlation to both pH and temperature was overall stronger in the genes detected in sediment samples than in water samples. All six genes had a strong to very strong negative correlation with pH and a moderate to very strong positive correlation with temperature. Compared to *toxR*, *srp*, vhh_a, and *vhh*, a slightly weaker negative correlation to pH was seen with *luxR* and *vhp*. Among the outlier genes

observed in water samples, stronger negative correlation to pH corresponded to stronger positive correlation with temperature. This pattern was not observed with *luxR* and *vhp* in sediments. Interestingly, the correlational trends among *toxR*, *luxR*, and *vhp* with other parameters were similar to those of the outlier genes in water. These genes had weak positive correlation with salinity, conductivity, and total dissolved solids while *srp*, *vhh*, and vhh_a had weak negative correlation. Additionally, *toxR*, *luxR*, and *vhp* had weaker negative correlation with potential water density than the other genes. In contrast to the distinct groups observed in water samples, there was no obvious separation in the two virulence gene groups in their relationships to water parameters in sediments.

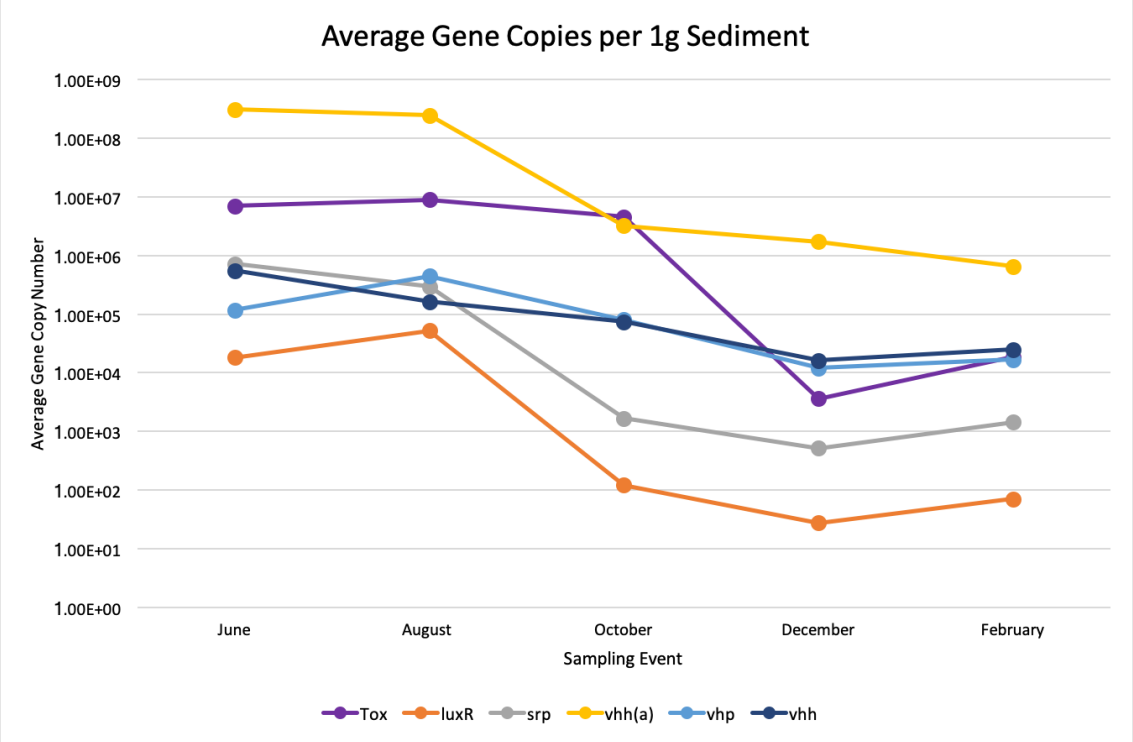

Figure 9. Average gene copies detected in sediments during all sampling events. There was no statistical difference in gene copies found between sites, therefore the copy numbers for Sites 1-3 were averaged and error bars were excluded.

Table 6. Heat map depicting the relationship between copy number and water parameter in sediment samples using Pearson's correlation coefficient

Gene/Parameter	pH	Temp. (C°)	Salinity (ppt)	Turbid. (NTU)	DO (g/L)	Conduct. mS/cm)	TDS (g/L)	Potent. Water Density
toxR	-0.927	0.893	0.221	0.227	-0.584	0.029	0.098	-0.472
luxR	-0.724	0.791	0.188	-0.045	-0.583	0.092	0.074	-0.380
srp	-0.897	0.666	-0.228	0.390	-0.619	-0.302	-0.359	-0.654
vhh$_a$	-0.957	0.809	-0.092	0.278	-0.689	-0.185	-0.235	-0.635
vhp	-0.653	0.772	0.292	-0.087	-0.510	0.169	0.193	-0.291
vhh	-0.852	0.600	-0.251	0.440	-0.559	-0.335	-0.370	-0.636

5.4 Detection of Vibrio harveyi and Vibrio campbellii in Water and Sediments

A qPCR assay with primers for t*oxR*$_{vh}$ and *hly* was used to identify *Vibrio harveyi* and *Vibrio campbellii* in environmental samples, respectively. Neither t*oxR*$_{vh}$ nor *hly* were detected in water or sediment throughout the duration of this study (results not shown). Species-specific primers for *rpoA* were additionally utilized for detection of these species. In water samples, *rpoA* was present in low concentrations during June and August (9.88E+00 and 1.04E+00 copies/mL, respectively). October, December, and February sampling failed to elude *rpoA* in water. In sediment samples, *rpoA* was detected in June, August, and October averaging 5.57E+03, 1.69E+02, and 5.09E-02 copies/g, though it was not present in December or February.

6. DISCUSSION

The six virulence genes targeted in this study (*toxR*, *luxR*, *srp*, vhh_a, *vhp*, and *vhh*) have been well-documented in pathogenic species belonging to the Vibrio Harveyi clade. Though several pathogenic Vibrio have been isolated from the southeastern United States, there are no previous studies documenting the presence and abundance of these virulence genes. While their presence does not necessarily indicate a high acute virulence of their carriers, it does indicate the presence of *Vibrio* Harveyi clade pathogens; and their concentrations reflect the abundance of those pathogens Furthermore, this research sought to assess the temporal and spatial distribution of these virulence genes- and possibly their carriers- in Coastal Georgia.

All six genes were consistently detected in water and sediments. Gene concentration was significantly higher in sediments than water, indicating greater persistence of Harveyi clade *Vibrios* in sediment. Sediments act as a natural filter for water and provide protection against environmental stressors, so these results were unsurprising. These findings are consistent with recent reports of virulence gene and tetracycline resistance gene abundance in coastal Georgia tidal creeks, where gene concentrations in sediment were up to three orders of magnitude higher than those of water (Jones and Barkovskii, 2017).

The generally observed positive relationship between temperature, presence, and concentrations of these genes in both water and sediment was not surprising. Since *Vibrio* are preferential to warmer waters and exhibit optimal growth at 20-35°C, it was expected that gene copy numbers would be greater during summer months than during winter (Sheikh et al., 2022). Previous studies in the region documenting virulence gene abundance in *Vibrio*

parahaemolyticus have shown similar seasonality trends as outlined in this research (Prescott and Barkovskii, 2021).

Of particular interest, however, is the evidenced heterogeneity with respect to the temperature among these virulence genes that likely reflects heterogeneity among their carriers. This heterogeneity is clearly evidenced in water. Whereas *luxR*, *vhp, vhh*$_a$, and *vhh* consistently positively responded to the increase of temperature up to 30°C, the *toxR* and *srp* genes exhibited limited adaptability at temperatures greater than 30°C. Previous studies assessing virulence of *Vibrios* have shown that elevated temperature correlates with increased virulence gene expression and pathogenicity (Hernandez-Cabanyero et al., 2020; Gu et al., 2016; Li et al., 2022). The contradictory trend exhibited by *toxR* and *srp* in water in this study suggests that the primary carriers of these genes are less adapted to high temperatures than other Harveyi clade *Vibrios.* There are no prior reports assessing the temperature-dependent abundance or expression of these specific virulence genes, therefore further research regarding this relationship is needed.

Many members of the *Vibrio* genus are preferential to alkaline environments (pH ≥ 7.4), therefore it was expected that gene concentrations (and therefore, carriers) would increase with rising pH. Instead, there was no statistically relevant correlation between pH and concentrations of *luxR*, *vhp, vhh*$_a$, and *vhh.* Surprisingly, there was a strong negative correlation between pH and *toxR* and *srp* gene copies. This again suggested heterogeneity in their bacterial carriers, and, likely in Harveyi clade in general that has not been reported before.

There was no such heterogeneity among virulence genes in sediments with respect to the temperature and pH. In fact, these results contradict previous studies assessing the abundance and distribution of *V. parahaemolyticus* and *V. vulnificus* virulence and species-specific genes in

coastal Georgia (Prescott and Barkovskii, 2021). In that study, gene copies in sediments and water exhibited no significant difference in correlation with pH. Based on the above, we can hypothesize that Harveyi clade bacteria in Georgia waters are split into two groups, one of which represented by *luxR, vhp, vhh$_a$,* and *vhh g*enes that is well-adapted to increasing temperature, and the other represented by *toxR* and *srp* that is not. The first group is not directly driven by pH in contrast to the second that is neutrophilic at best and is inhibited by alkali pH. This heterogeneity was not obvious in sediments, likely due to different interactions of these groups in these microenvironments.

This heterogeneity between gene groups in water was also obvious in relation to salinity. Harveyi clade *Vibrios* are halophilic and require high salt concentrations in their environments (0.5 and 2.5% salinity), therefore it was expected that salinity would play a factor in gene detection rates (Baker-Austin et al., 2018). The lack of detection of *toxR* and *srp* in February when salinity was lowest indicated that these carriers are less adapted to low salinity than those of *luxR, vhp, vhh$_a$,* and *vhh.* Surprising, however, was the inconsistent correlation to salinity observed among the virulence genes detected in sediments. Similar to *toxR* and *srp* in water, the *srp, vhh$_a$,* and *vhh* genes in sediment exhibited an overall negative correlation with salinity. Though this relationship was not observed in *toxR, luxR,* and *vhp,* the magnitude at which the correlation varied among these virulence genes in sediment was not as significant as that in water Furthermore, it is unlikely that two or more distinct groups of bacteria are responsible for this variation observed in sediment.

Surprisingly, salinity had one of the weakest and most variable correlations of all parameters in both sediment and water copy numbers. This overall implies that Harveyi clade *Vibrios* are, in general, not as greatly affected by seasonal changes in salinity compared to those

effects of temperature and pH. Increased virulence gene expression in *V. harveyi, V. parahaemolyticus,* and *V. vulnificus* has previously been linked with high salinity, though this relationship with virulence gene abundance and presence has yet to be explored.

The correlation with other water parameters considered in this study were more variable than those with pH, temperature, and salinity. While still valuable, the relationships with turbidity, dissolved oxygen, conductivity, total dissolved solids, and potential water density were not explored in depth. Climate change is altering the pH, temperature, and salinity of the world's oceans at a global scale and will directly impact virulence gene abundance and distribution in the future (Cavicchioli et al., 2019). Furthermore, the relationships between gene copies and temperature, pH, and salinity were more significant for the scope of this study.

Moreover, the second aim of this study was to determine the presence of *Vibrio harveyi* and *Vibrio campbellii* in Coastal Georgia. *V. harveyi* and *V. campbellii* are notorious aquaculture pathogens in Southeast Asia, Southern Europe, and South America, however, they have never been detected in the United States (Montánchez and Kaberdin, 2020). The southeastern United States has seen an increase in climate and SST's making it a potentially accommodating environment for the emergence of new bacteria like *V. harveyi* and *V. campbellii* (EPA, 2017). Previous studies have indicated the presence of *V. parahaemolyticus* and *V. alginolyticus* in the Southeastern US, and given the ubiquitous nature of the genus, it was hypothesized that other members of the Harveyi clade would be present.

The species-specific genes used to monitor for *V. harveyi* and *V. campbellii* in environmental water and sediment samples were $toxR_{vh}$ and *hly*, respectively. The *toxR* gene in *V. harveyi* and *hly* gene in *V. campbellii* have high sequence similarity with other vibrios such as

V. parahaemolyticus at ~84% and *V. cholera* at ~85%, respectively, however, the primers used in this study have reliably shown to detect *V. harveyi* and *V. campbellii* with high specificity (Pang et al., 2006). The qPCR assay for $toxR_{vh}$ was optimized to detect $\geq 10^1$ gene copies, therefore it is unlikely that *V. harveyi* was present below the detectable limit in our samples. The assay for *hly*, however, had a high detection limit ($\geq 10^4$ copies) and therefore served as a poor identification tool for *V. campbellii*. In the meantime, both *harveyi* and c*ampbellii*-specific primer for the *rpoA* gene detected the above gene in two (water) and three (sediment) sampling events at low concentrations. Additional studies with lower detection limits for these two species are needed to conclusively determine the presence of *V. campbellii* and *V. harveyi* in the region. While the methods used in this study did not elude the presence of *V. harveyi* or *V. campbellii*, it is important to monitor for these pathogens in the future. Early detection will be essential for mitigating the inevitable effects on the aquaculture industry in the SE United States.

As previously stated, this is the first study to examine *Vibrio harveyi*, *Vibrio campbellii*, and the temporal dynamics of *toxR*, *luxR*, *srp*, vhh_a, *vhp*, and *vhh* in the Southeastern United States. Despite the absence of *V. harveyi* and *V. campbellii,* it is evident that other members of the Harveyi clade are present in the environment. Our findings evidence that these clade-members, particularly those in water, exhibit different adaptations to temperature, pH, and salinity. Further analysis of these bacteria should be conducted as they are likely agents of disease in both aquaculture products and humans, and there are no previous reports of heterogeneity among Harveyi clade *Vibrios* in respect to their virulence genes and environmental adaptations. The overarching goal of this research is to help assess the effects of climate change on pathogenic *Vibrio*. Furthermore, the results provided here will serve as a basis for future studies related to Harveyi clade bacteria in the region.

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8. APPENDIX

Table 1. Target gene primer sequence and amplicon size

Gene	Primer Sequence	Amplicon Size (bp)	Reference
luxR	FW: TCAATTGCAAAGAGACCTCG RV: AGCAAACACTTCAAGAGCGA	84	Ruwandeepika et al. (2011)
toxR	FW: CGACAACCCAAAATACGGAA RV: AGAGCAATTTGCTGAAGCTA	131	Ruwandeepika et al. (2011)
srp	FW: TGCACGACCAGTTGCTTTAG RV: AAGTGGTCGTCAGCAAATCC	232	Ruwandeepika et al. (2011)
vhh	FW: TTCACGCTTGATGGCTACTG RV: GTCACCCAATGCTACGACCT	234	Ruwandeepika et al. (2011)
vhh_a	FW: GCGCTTGGTATCTTCTCTGA RV: CAGACAGCTCATCACGCATT	226	Ruwandeepika et al. (2011)
vhp	FW: CTGAACGACGCCCATTATTT RV: CGCTGACACATCAAGGCTAA	201	Ruwandeepika et al. (2011)
rpoA	FW: CGTAGCTGAAGGCAAAGATGA RV: AAGCTGGAACATAACCACGA	197	Ruwandeepika et al. (2011)
$toxR_{vh}$	FW: GAAGCAGCACTCACCGAT RV: GAAGCAGCACTCACCGAT	382	Pang et al. (2006)
hly	FW: CTATTGGTGGAACGCAC RV: GTATTCTGTCCATACAAAC	328	Halder et al. (2010)

Table 2. PCR and qPCR cycling parameters for target genes

Gene	Initial Activation	Denaturation	Annealing	Elongation	Cycles	Reference
luxR	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
toxR	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
srp	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
vhh	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
vhh_a	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
vhp	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
rpoA	2 min at 50°C	10 min at 95°C 15 sec at 95°C	20 sec at 55°C	30 sec at 72°C	45	Ruwandeepika et al. (2011)
$toxR_{vh}$	-	10 min at 95°C 1 min at 94°C	1 min at 55°C	1 min at 72°C	30	Pang et al. (2006)
hly	-	10 min at 95°C 1 min at 94°C	30 sec at 54°C	30 sec at 72°C	30	Halder et al. (2010)

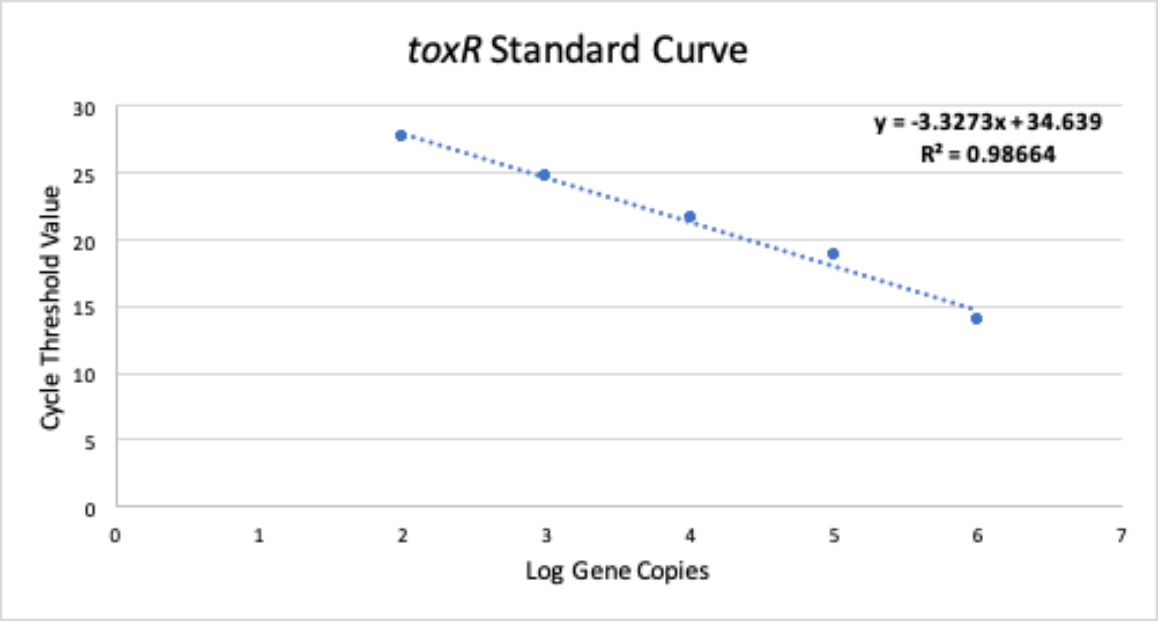

Figure 1. Example standard curve (*toxR)*. The slope and y-intercept provided from each standard curve was first used to calculate gene copies in 1 µl of sample DNA (the amount used in each qPCR reaction). This was done using the following equation:

Equation 1: Gene Copies per 1 µl of sample DNA = $10^{((\text{Ct value} - \text{y-intercept}) / (\text{slope}))}$

To translate the amount of gene copies in 1 µl of sample DNA to 1 gram of sediment, a series of conversions were performed. The extraction kit for sediment samples eluded 100 µl of DNA in elution buffer, therefore, the value computed in Equation 1 was first multiplied by 100 to find the total number of gene copies in 100 µl of environmental sediment DNA. Since the kit required 0.25 g of sediment for extraction, this value was further multiplied by 4 to represent the total number of copies in 1 gram of sediment. The same method was used to calculate gene copies in water, however, the DNA extraction kit used for these samples eluded 50 µl of DNA from 1 liter of environmental water samples. Furthermore, to find the number of copies in 50 µl of DNA, the

value computed in Equation 1 was first multiplied by 50. The resulting value was then divided by 1000 to convert from copies/liter to copies/mL.