

Fall 2019

Distribution and Abundance of *Vibrio Parahaemolyticus* in Surficial Estuarine Sediment From North Inlet, SC, Usa

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Distribution and abundance of *Vibrio parahaemolyticus* in surficial estuarine sediment
from North Inlet, SC, USA

by

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Bachelor of Science
University of South Carolina, 2017

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2019

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Abstract

Vibrio parahaemolyticus is a ubiquitous coastal organism that causes gastroenteritis (vibriosis) after ingestion of mishandled or undercooked seafood. Cases of vibriosis have increased rapidly in the past decade. *V. parahaemolyticus* densities have historically been associated with an increase in sea surface water temperatures, but recent shellfish bed closures in fall and winter months in cooler regions (the Baltic Sea, the Pacific NW, and New England) suggest that there may be additional environmental parameters that affect densities of *V. parahaemolyticus*. The vertical distribution of *V. parahaemolyticus* was assessed in the pristine North Inlet Estuary (SC, USA). The goal of this study was to determine the specific microenvironments that support high densities of *V. parahaemolyticus*. It was determined that surficial sediments act as significant reservoirs for *V. parahaemolyticus*, with densities as high as 10^4 CFU mL⁻¹ in the top three mm of sediment. The highest densities of *V. parahaemolyticus* were found in the fall sampling dates, corresponding to increased shellfish bed closures reported by the Centers for Disease Control and Prevention (CDC). Outbreaks of vibriosis are increasing in size and frequency and this study provides important insight into the preferred environments this organism inhabits and highlights the importance of a broad sampling strategy for assessment of risk.

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Chapter 1:

High abundance of *Vibrio parahaemolyticus* in surficial estuarine sediment

from North Inlet, SC, USA

Introduction

Vibrio parahaemolyticus, the causative agent of vibriosis (seafood associated gastroenteritis), is a ubiquitous coastal organism in diverse marine and estuarine environments. Outbreaks of vibriosis are quite common (FDA, 2005) but cases are often underreported because most cases are not life-threatening and do not require hospitalization. Monitoring of water and oyster samples show that *V. parahaemolyticus* densities in the environment are typically extremely low ($<10^1$ CFU mL⁻¹) (Kaneko and Colwell, 1973); however, a recent study in North Inlet Estuary, SC, USA determined that *V. parahaemolyticus* can become concentrated in oysters. This can produce a “hot” oyster that contains densities of *Vibrio parahaemolyticus* above the FDA safe limit for consumption (DePaola and Kaysner, 2004; Klein and Lovell, 2016). These “hot” oysters occur at low frequencies, suggesting that there are other estuarine environments in which this organism can concentrate and become abundant, consequently infecting the surrounding filter feeding bivalves.

Prediction of a *Vibrio* outbreak relies heavily on the assessment of environmental parameters. Sea surface water temperature (SST) is most commonly used to determine when an outbreak may be possible, with several studies finding a positive correlation between *Vibrio* abundance and SST in the water column (Julie et al., 2010; Johnson et al., 2012; Semenza et al., 2017). However, recent research conducted in the Pacific NW determined no significant correlation between SST and *V. parahaemolyticus* densities in the water column above an oyster bed. This suggests SST is an unreliable indicator of *V. parahaemolyticus* abundance in oysters and that a better risk assessment is enumeration of *V. parahaemolyticus* loads within oyster tissues (Nilsson et al., 2019). Recent

outbreaks at cold water locales Pacific NW, New England, Canada, Baltic Sea) also suggest that this correlation is not as informative as previously thought (Baker-Austin et al., 2013; Taylor et al., 2018). Laboratory measurements examining the relationship between *V. parahaemolyticus* and temperature also suggest a more complex relationship (Burnham et al., 2009; Liu et al., 2016)

In the water column, *V. parahaemolyticus* is often associated with suspended sediment and other particulate matter (Kaneko and Colwell, 1973; DePaola et al., 1990; Rehnsam-Holm et al., 2010; Johnson et al., 2012; West, 2012). It is well known that *V. parahaemolyticus* is found in sediment at numbers several orders of magnitude higher than in the water column (Kaneko and Colwell, 1977; Shiaris et al., 1987; Hara-Kudo et al., 2003). Densities up to $<10^3$ CFU g⁻¹ have been reported in estuarine sediments along the North Carolina coast (Blackwell and Oliver, 2008). Infaunal burrows also enrich *V. parahaemolyticus* and other *Vibrio* spp. to high abundances in the warmer months, followed by decline during the cooler months, a pattern similar to that in the water column (Gamble and Lovell, 2011). Most studies quantify *V. parahaemolyticus* densities in homogenized bulk sediment collected at locations where human impacts are significant, such as the Chesapeake Bay (Kaneko and Colwell, 1973) and the Pacific NW (Baross and Liston, 1970).

V. parahaemolyticus is commonly associated with marine microalgae (Turner et al., 2009; Seong and Jeong, 2011; Asplund et al., 2011; Turner et al., 2014; Main et al., 2015). Approximately 80% of *V. parahaemolyticus* and other *Vibrio* spp are found attached to plankton or other particulate matter in the water column (Kaneko and Colwell, 1975). In some cases, the attachment to chitin, particularly to copepod

exoskeletons is essential for the annual life cycle of *V. parahaemolyticus* (Kaneko and Colwell, 1975). Research has also been conducted to determine the exact nature of the relationship between *V. parahaemolyticus* and specific algal taxa (Seong and Jeong, 2011; Asplund et al., 2011; Main et al., 2015). Positive correlations have been observed between diatom and *Vibrio* abundance when *V. parahaemolyticus* comprises the majority of the *Vibrio* population (Rehnstam-Holm et al., 2010). Most research conducted to elucidate the nature of these relationships has been conducted in the water column or using fluorescence data collected via satellites while this study utilized extraction methods. The interaction between marine microalgae and *V. parahaemolyticus* in sediment is currently unclear.

To further understand the ecological dynamics of *V. parahaemolyticus*, we examined the niche preferences of this organism in sediment from a relatively pristine salt marsh. A precise sediment coring apparatus was used for precise vertical sampling of the top 1 cm of sediment across distinct microenvironments in the estuary. The densities of *V. parahaemolyticus* were determined for each mm in the top 1 cm of sediment. We observed near infectious dose (10^3 - 10^4 cells) (Martinez-Urtaza et al., 2010) levels of *V. parahaemolyticus* in surficial sediment, particularly in the top 3 mm.

Materials and Methods

Sampling Site

All environmental samples were collected from North Inlet Estuary at the Belle W. Baruch Institute property near Georgetown, SC (33°20'N, 79°12'W). North Inlet Estuary is a pristine estuary that is part of the National Estuarine Research Reserves

System and free of strong anthropogenic influences (Dame et al., 2000; Buzzelli et al., 2004). Salt marshes are important coastal ecosystems that play a significant role in nutrient cycling (Adams, 1963). Semi-diurnal tides facilitate the exchange of nutrients between the water column and salt marsh sediment (Lovell and Davis, 2012).

Vertical Microscale Sampling

A total of 981 sediment samples were collected during biweekly samplings from May-October 2016. Because *V. parahaemolyticus* is a facultative anaerobe, we hypothesized that there would be low densities at depths greater than 1 cm as sediment goes anoxic quickly. Five 1 cm diameter sediment core samples were collected from a low intertidal oyster bed and from a mid-intertidal muddy sand flat for each sampling trip. Samples were immediately transported to Columbia, SC for processing. The top 1 cm of sediment samples were sliced into one mm vertical intervals using a sediment core sectioning apparatus and weighed (Pinckney et al., 1994). Each section was serially diluted in phosphate buffered saline (per L; 23 g NaCl, 4.14 g NaH₂PO₄, pH 7.2, PBS) without enrichment and a 0.1 ml aliquot of each dilution was spread onto a Thiosulfate Citrate Bile salts Sucrose (TCBS) agar plate. The plates were incubated for 48 h at 37°C and green colonies (sucrose non-fermenters, presumptive *V. parahaemolyticus* and/or *Vibrio vulnificus*) were counted. Colony forming units (CFU mL⁻¹) per sediment volume was calculated for each depth interval and mean values were calculated for each sampling date. A non-parametric Spearman's rho test, Mann Whitney U test, and Ryan-Einot-Gabriel-Welsh range test were performed on the resulting data employing SPSS.

Vertical Microscale Photopigment Analysis

The vertical distribution of phototrophs in the top 1 cm of sediment in the mid-intertidal muddy sandflat and the low intertidal oyster bed was also examined. Three sediment core samples were collected from each sampling site on each sampling date for photopigment analysis. Photopigment analysis was used to detect photopigments produced by diatoms (fucoxanthin), cyanobacteria (zeaxanthin), and total phototroph biomass (chlorophyll *a*) because each of these organisms are often associated with *V. parahaemolyticus*. Sediment core samples were sectioned vertically, as described above (Vertical Microscale Sampling), weighed, and frozen at -70° C pending processing. Sediment extraction protocols and HPLC Photopigment Analysis procedures from Dr. James Pinckney's lab website (<https://phytoninja.com/lab-protocols/>) were employed.

Results

Vertical Microscale Sampling

Presumptive *V. parahaemolyticus* densities up to 10⁴ CFU mL⁻¹ were found in the upper 3 mm of sediment at both the low intertidal oyster bed and the mid-intertidal muddy sandflat (Figures 1.1, 1.2, 1.3). At depths of 9-10 mm, presumptive *V. parahaemolyticus* densities were the lowest, at 10¹-10² CFUs mL⁻¹. *V. parahaemolyticus* abundance significantly decreased with increasing depth in the low intertidal oyster bed (Spearman's rho=-0.501, p<0.01, n=445) and the mid-intertidal muddy sandflat (Spearman's rho=-0.316, p<0.01, n=440). The 27 September sampling date contained the highest presumptive *V. parahaemolyticus* densities in the low intertidal oyster bed at

3.60×10^4 CFU mL⁻¹ followed closely by the 16 September sampling date in the same location (3.00×10^4 CFU mL⁻¹) (Figures 1.2 and 1.3).

A Non-parametric Mann Whitney U test was employed to determine if location was a significant driver of *V. parahaemolyticus* abundance. *V. parahaemolyticus* densities in the low intertidal oyster bed were significantly different than densities in the mid-intertidal muddy sandflat (U=98913, p<0.01, n=985). In addition, the low intertidal oyster bed contained higher mean densities ranks than the mid-intertidal muddy sandflat.

Lastly, we determined that sampling date was a significant driver of *V. parahaemolyticus* abundance. The highest densities were found in the fall (9/27/16 and 10/25/16) sampling dates in both the low intertidal oyster bed (p=0.02) (Table 1.1) and the mid-intertidal muddy sandflat (p=0.54) (Table 1.2) and were significantly different from the summer months. Water temperature and salinity are environmental parameters commonly used to predict when shellfish harvesting is high risk. Meteorological data from the National Estuarine Research Reserve (NERRs) website were accessed to determine if there were any significant environmental factors (water temperature and salinity) that correlated with elevated density dates (Table 1.3). Sampling dates 9/27 and 10/25 had lower water temperature and salinity values in comparison to other sampling dates that yielded lower *V. parahaemolyticus* abundance.

Vertical Microscale Photopigment Analysis

Each photopigment (total chl *a*, fucoxanthin, and zeaxanthin) significantly decreased with increasing depth of sediment in the low intertidal oyster bed (rho=-0.354, -0.355, -0.226, p<0.01, n=247). Total chl *a* biomass and fucoxanthin followed the same trend in the mid intertidal sandflat (rho=-0.593, -0.390, p<0.01, n=247); however,

zeaxanthin concentration increased with increasing depth ($\rho=0.213$, $p<0.01$, $n=247$). A multiple regression analysis determined there were significant positive correlations between each photopigment concentration and *V. parahaemolyticus* densities in the low intertidal oyster bed ($n=247$, $\text{adj } r^2=0.08$, $p<0.01$) and the mid-intertidal muddy sandflat ($n=247$, $\text{adj } r^2=0.09$, $p<0.01$).

Discussion

V. parahaemolyticus abundance was correlated with depth and the highest densities were found in the upper 3 mm of sediment at both the mid-intertidal muddy sandflat and the low intertidal oyster bed sampling sites. While *V. parahaemolyticus* densities were occasionally relatively high (10^3 CFU mL⁻¹) deeper in the top 1 cm of sediment, it is likely attributed to bioturbation (Pinckney et al., 1994). Our results are consistent with observations of elevated abundances of *Vibrio* spp and other potentially pathogenic organisms, such as *Escherichia coli* and *Salmonella* spp, in estuarine surficial sediment (Berthe et al., 2008). Increased *V. parahaemolyticus* abundance in the upper 3 mm could result in resuspension during tidal changes and other times of strong water flow, which would result in uptake by filter-feeding oysters.

Previous research has demonstrated a direct correlation between *V. parahaemolyticus* abundance and turbidity (Blackwell and Oliver, 2008; Julie et al., 2010; Davis et al., 2017). Increased water column turbidity results from resuspension of surficial sediment, where we determined *V. parahaemolyticus* is abundant. This sediment resuspension would facilitate transport of the organism and enhance its potential to colonize filter feeding bivalves. *Vibrio* densities in water and sediment samples from the Neuse River estuary after Hurricane Ophelia and Tropical Storm Ernesto were orders of

magnitude higher than those predicted by models (Wetz et al., 2008). *Vibrio* densities in the water column are typically low suggesting that significant resuspension from major storms can result in an influx of vibrios. Climate change has contributed to increases in tropical storm and hurricane numbers and intensities (Michener et al., 1997; Brooks et al., 2017; Jesser and Noble, 2018) and it is likely that this will have a significant impact on the transportation of vibrios, both within and among coastal systems. The length of time required for the vibrios to settle back into a more typical distribution is currently unknown. Sediment resuspension will likely have a significant impact on outbreaks of vibriosis as the east coast of the US endures increasingly more active hurricane seasons.

Coastal economies rely heavily on the commercial shellfish industry and are significantly impacted by vibrios. Oysters can become enriched with *V. parahaemolyticus* indicating that an oyster bed provides nutrients and opportunities for surface attachment (Klein and Lovell, 2016). The low intertidal oyster bed sampling site in our study contained significantly greater *V. parahaemolyticus* densities in comparison with the mid-intertidal muddy sandflat. This is likely due to settling of particulate sediment and greater organic matter content in the oyster bed sediment. Sediment grain size is positively correlated with organic matter content and has a significant effect on pathogen indicator abundance such as *E. coli*, *Salmonella* spp, and *Vibrio* spp (Watkins and Cabelli, 1985). Fine sand grains have significantly lower organic matter content when compared to silt and clay rich sediments. (Perkins et al., 2014).

Elevated *V. parahaemolyticus* abundance in a relatively pristine oyster bed presents a significant concern for commercial shellfish beds. States bordering the highly polluted northern Gulf of Mexico produce the greatest numbers of vibriosis cases, in the

US, on average per year (Levine et al., 1993). In 2005, Louisiana was impacted by Hurricanes Katrina and Rita between late August and late September. While vibriosis outbreaks in the area are commonly associated with *V. parahaemolyticus* and *V. vulnificus*, that year there were two reports of toxigenic *V. cholerae* O1 cases after a couple consumed contaminated seafood (CDC, 2006). *V. cholerae* associated illness is rare in the US and infections from consumption of seafood after hurricanes will be a pressing issue in coastal regions as climate change produces more frequent and severe storms.

Outbreaks of vibriosis have sharply increased in the past decade (Newton et al., 2012), likely as a result of increasing sea surface water temperatures (SST) across the globe (Baker-Austin et al., 2013; Moore et al., 2017). Increased SST at higher latitudes has expanded the geographic range of environments that *V. parahaemolyticus* can occupy. As a result, there have been increased shellfish bed closures at high latitudes outside the normal *V. parahaemolyticus* high risk season (Kaneko and Colwell, 1973). In our study, we found the highest *V. parahaemolyticus* densities in the fall (9/27/16 and 10/25/16) sampling dates and these did not coincide with a higher SST or salinity, environmental parameters commonly used to predict risk of an outbreak. Even laboratory measurements have demonstrated growth of this organism at temperatures that were previously considered too low to support growth (Burnham et al., 2009; Liu et al., 2016). Vezzulli et al. (2016) showed that climate change is having a significant impact on SST around the globe with coastal areas seeing an average increase in SST by $\sim 1.5^{\circ}$ C, coinciding with lengthier warm periods globally. The European Centre for Disease Control and Prevention (ECDC) predicts that the areas suitable for *Vibrio* growth will

expand even further in future decades (Semenza et al., 2017). Clearly SST will no longer be a reliable indicator for an outbreak of this organism and other environmental parameters must be examined.

V. parahaemolyticus is also commonly associated with benthic microalgae in the environment. Correlations between specific algal taxa and *V. parahaemolyticus* have been examined in order to determine if benthic microalgae abundance might be used to predict when a *V. parahaemolyticus* bloom is possible. We hypothesized that the bacteria utilize the excreta from exopolymer sloughing and/or microalgal destruction as a carbon and energy source in the salt marsh environment where labile carbon and energy sources are often not readily available (Moran and Hodson, 1990; Amon and Benner, 1996; Kaiser and Benner, 2009). While we found a slight positive correlation between total phototroph biomass (total chl *a*), diatoms (fucoxanthin), and cyanobacteria (zeaxanthin), the nature of the relationship between these organisms and *V. parahaemolyticus* is unclear. Previous laboratory experiments revealed *V. parahaemolyticus* can stimulate some phototrophs *in vitro* and prey on others (Klein et al., 2019). Based on our data, presence of phototrophs in estuarine sediment is indicative that *V. parahaemolyticus* is present but does not provide a reliable measure of its abundance. Further research examining the relationship between *V. parahaemolyticus* and each phototroph in estuarine sediment should be conducted.

While previous research has determined a correlation between sediment resuspension and *V. parahaemolyticus* abundance, the exact nature of that relationship has not been determined. How sediment resuspension affects the transport of this organism is also currently unknown. This study identified a significant *V.*

parahaemolyticus niche, surficial estuarine sediment. With *V. parahaemolyticus* reaching near infectious dose levels in the top 3 mm of sediment, we hypothesize there will be an influx of *V. parahaemolyticus* cells into the water column. This would facilitate the transport of *V. parahaemolyticus* throughout the estuary and into surrounding filter feeding invertebrates. Therefore, it is imperative that we examine, the effect that frequent resuspension due to tropical storms and hurricanes will have on transport and subsequent redistribution of this pathogen throughout coastal ecosystems. Determining the minimum water velocity required for saltation of sediment particles and quantifying *V. parahaemolyticus* abundance in the water column during this process would provide insight into *V. parahaemolyticus* transport. Further research examining this relationship between sediment resuspension and *V. parahaemolyticus* abundance and transport should be done to determine the impacts that increasingly turbulent weather will have on the commercial shellfish industry in coastal regions.

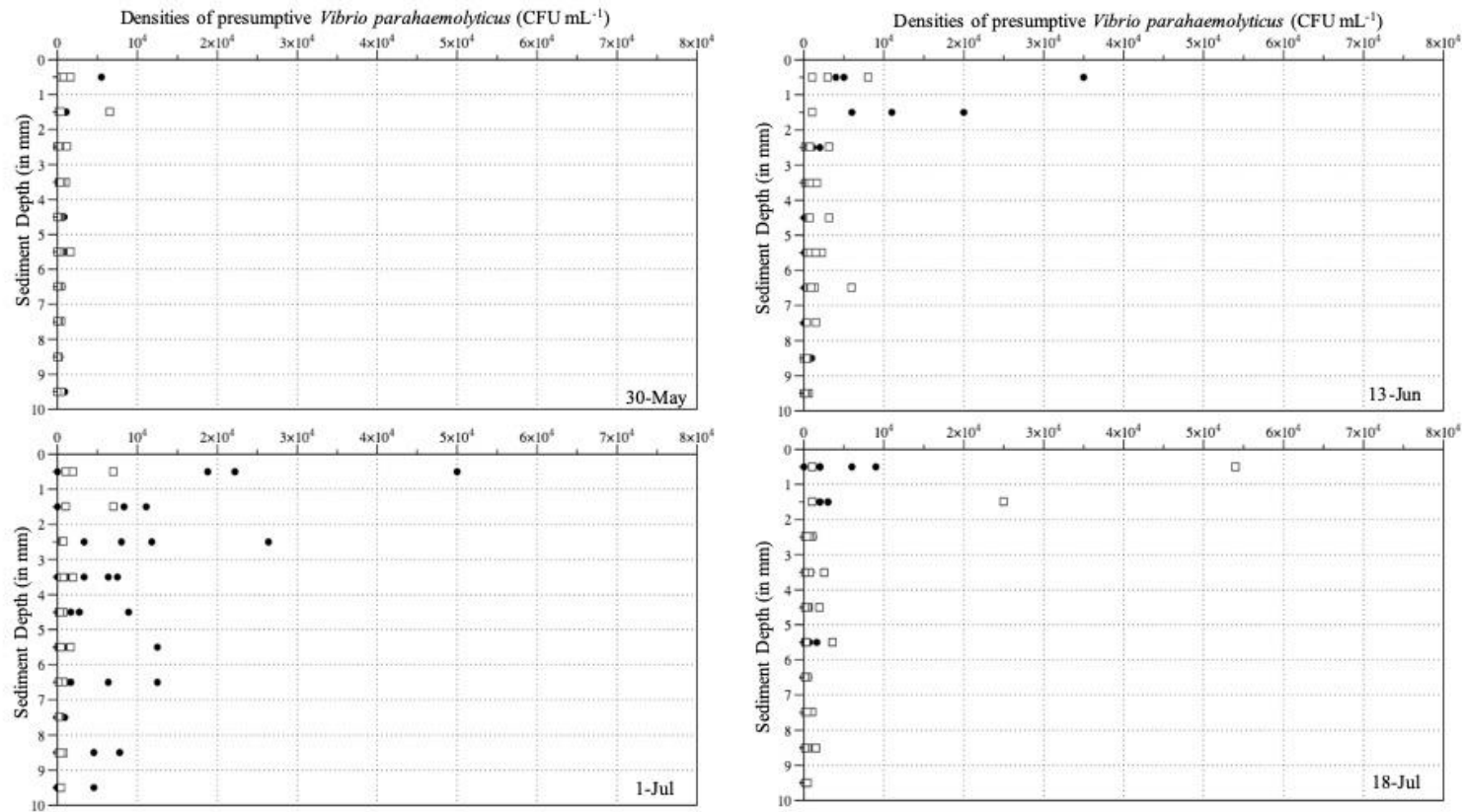


Figure 1.1. Vertical distribution of presumptive *Vibrio parahaemolyticus* in the microscale 2016 sampling dates 30-May, 13-Jun, 1-Jul, and 18-Jul. Black circles are samples collected from the low intertidal oyster bed. Gray squares are samples collected from the mid intertidal muddy sandflat. Each data point is indicative of a single sample. Five samples were collected at each depth interval for each sampling locale and date

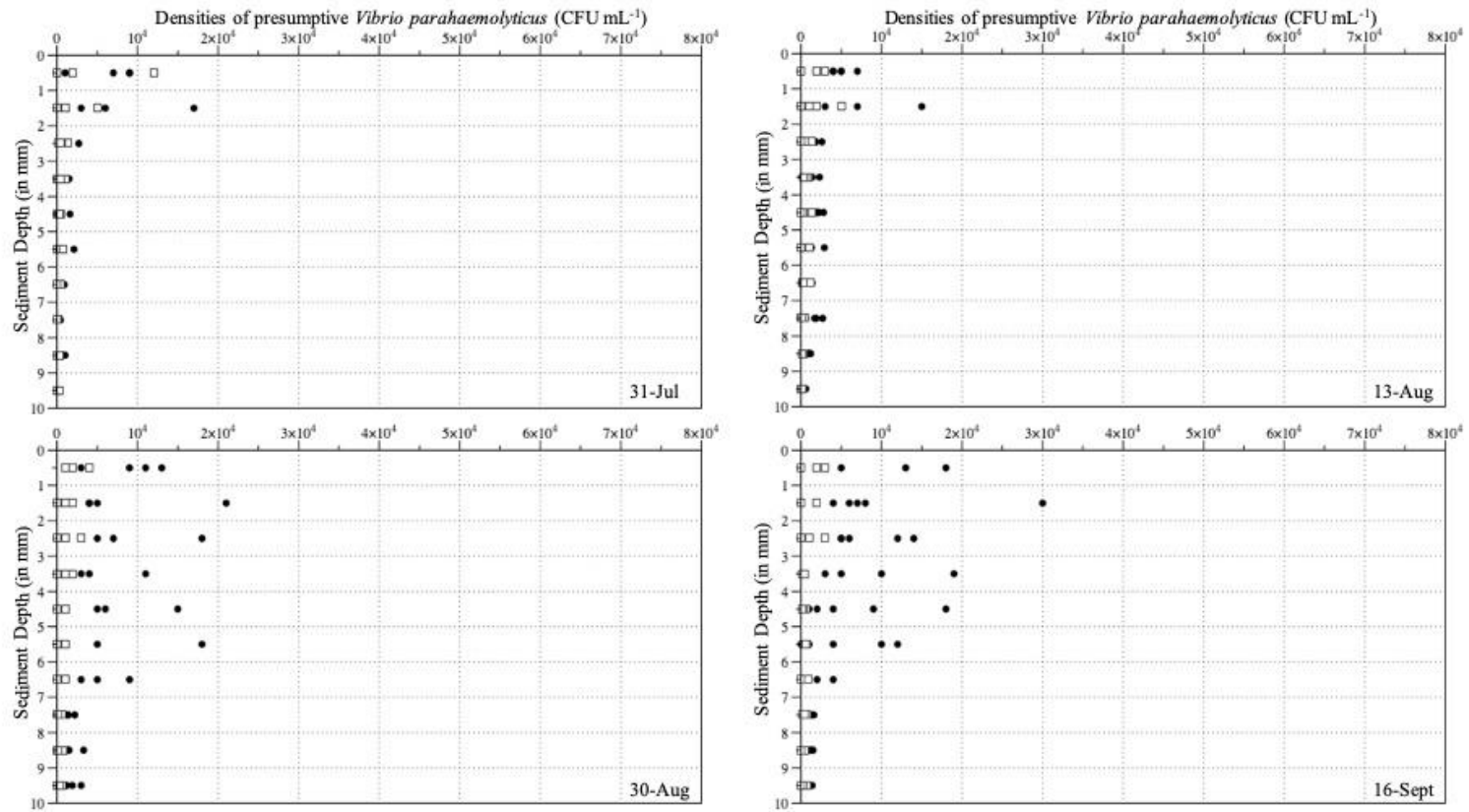


Figure 1.2. Vertical distribution of presumptive *Vibrio parahaemolyticus* in the microscale 2016 sampling dates 31-Jul, 13-Aug 30-Aug, and 16-Sept. Black circles are samples collected from the low intertidal oyster bed. Gray squares are samples collected from the mid intertidal muddy sandflat. Each data point is indicative of a single sample. Five samples were collected at each depth interval for each sampling locale and date.

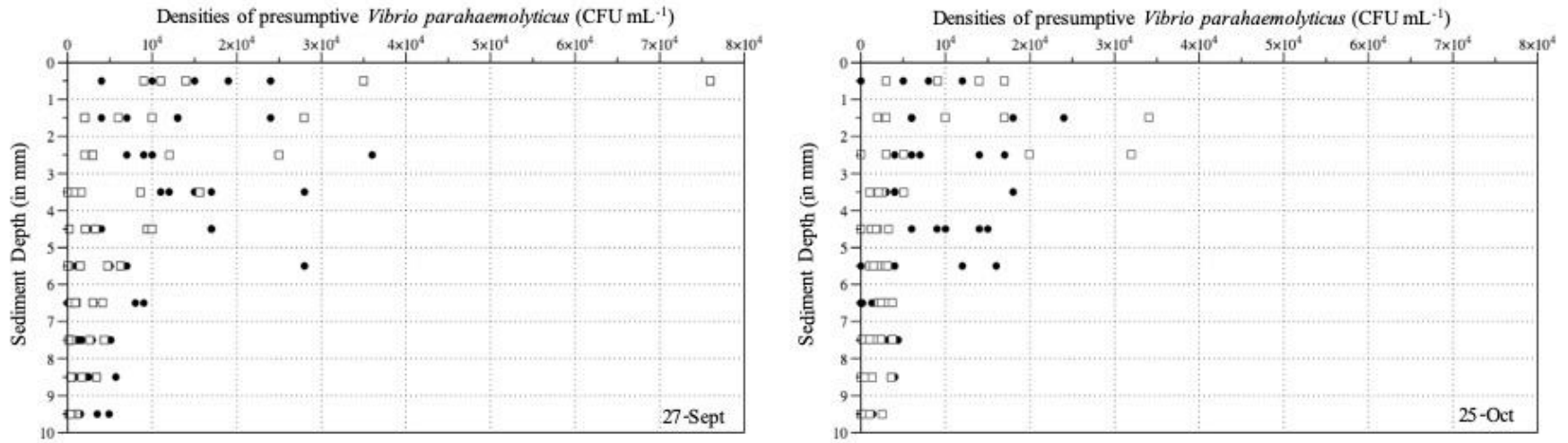


Figure 1.3. Vertical distribution of presumptive *Vibrio parahaemolyticus* in the microscale 2016 sampling dates 27-Sept and 25-Oct. Black circles are samples collected from the low intertidal oyster bed. Gray squares are samples collected from the mid intertidal muddy sandflat. Each data point is indicative of a single sample. Five samples were collected at each depth interval for each sampling locale and date.

Table 1.1. Results of REGW range test for total presumptive *Vibrio parahaemolyticus* densities in the low intertidal oyster bed. Homogenous and statistically significant subsets are displayed. Values presented are based on observed means. Alpha = 0.01.

Ryan-Einot-Gabriel-Welsh range score for isolates in subset:						
Date	No. of samples	1	2	3	4	5
30-May	50	494.4				
18-Jul	47	925.8	925			
1-Jul	50	1130	1130			
31-Jul	48	1510	1510			
13-Jun	50	1922	1922			
13-Aug	50	2088	2088	2088		
30-Aug	50		4294	4294	4294	
16-Sep	50			5088	5088	
25-Oct	50				5590	5590
27-Sep	50					8404
Sig.		0.76	0.02	0.02	0.83	0.02

Table 1.2. Results of REGW range test for total presumptive *Vibrio parahaemolyticus* densities in the mid-intertidal muddy sandflat. Homogenous and statistically significant subsets are displayed. Values presented are based on observed means. Alpha = 0.01.

Ryan-Einot-Gabriel-Welsh range score for isolates in subset:				
Date	No. of samples	1	2	3
30-May	50	472.2		
16-Sep	40	625.2		
30-Aug	50	638.4		
31-Jul	50	660.4		
13-Aug	50	664.2		
1-Jul	50	764.3		
13-Jun	50	979.1		
18-Jul	50	2038	2038	
25-Oct	50		4970	4970
27-Sep	50			6562
Sig.		0.94	0.04	0.54

Table 1.3. Recorded meteorological data from the National Estuarine Research Reserve System (NERRs) website for each microscale sampling date during the sampling time in 2016.

Date	Water Temp (°C)	Air Temp (°C)	Salinity (ppt)
30-May	25	24	32
13-Jun	29	27	34
1-Jul	28	27	33
18-Jul	27	30	33
31-Jul	30	30	36
13-Aug	31	32	34
30-Aug	28	29	36
16-Sep	27	28	32
27-Sep	27	27	27
25-Oct	20	19	22

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