LOWER *VIBRIO VULNIFICUS* CONCENTRATIONS OBSERVED IN SUSPENDED, FARMED OYSTERS THAN IN WILD, ON-BOTTOM OYSTERS IN EASTERN NORTH CAROLINA

Rachel Canty

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Approved by:

Brett Froelich

Rachel Noble

Michael Piehler

ABSTRACT

Rachel Canty: Lower *Vibrio vulnificus* concentrations observed in suspended, farmed oysters than in wild, on-bottom oysters in eastern North Carolina
(Under the direction of Brett Froelich and Rachel Noble)

Throughout the United States (US), shellfish aquaculture is growing rapidly in an attempt to meet increasing seafood demands. Shellfish, including oysters, are filter-feeders and can concentrate bacteria from the water column, including two human pathogens, *Vibrio vulnificus* and *Vibrio parahaemolyticus.* Due to roughly 50% of commercial oysters being consumed raw, these bacteria pose a serious risk to consumers and a problem to the growing oyster industry in North Carolina (NC). Oysters can be harvested from the wild or they can be farmed, in which case they are grown using a variety of aquaculture systems. Few studies have been conducted to determine whether oyster aquaculture grow-out methods (e.g. floating bags, floating/submerged cages) influence *Vibrio* concentrations in oyster meats. Of these few studies, none have been conducted in NC. In this study, we compared the total *Vibrio*, total and potentially pathogenic *V. vulnificus*, and total and potentially pathogenic *V. parahaemolyticus* concentrations of wild (on-bottom) and farmed (suspended) oysters harvested during the summer season of 2018. This study found that total *V. vulnificus* and potentially pathogenic *V. vulnificus* concentrations were lower in suspended oysters (unpaired t-test, $p=0.0334$). This was not observed in *V. parahaemolyticus* over the short duration of this study (unpaired t-test, $p=0.2202$). In addition to our intended study results, this study has added to a growing body of literature that shows the proportion of potentially pathogenic *V. parahaemolyticus* found in NC waters and across all types of oysters in NC is very low (less than 1% in this study). Additionally, aside from few discrepancies, culture methods were accurate for quantifying *Vibrio* concentrations, although the range of values observed was high on certain dates and molecular methods were necessary for evaluation of potential pathogenicity. Although this study found some interesting patterns, the results would be best supplemented by another study with a larger geographical range and longer period of study.

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To my mom and dad, who received a phone call every time something went the slightest bit awry, I don’t have words to properly express my thanks. What I can say is: I have never felt closer to you both than through the laughs, tears, and love shared over these past three years. Thank you for your undying support.

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LIST OF ABBREVIATIONS

B	On-bottom oysters
CAV	CHROMAgar *Vibrio*
CDC	Centers for Disease Control and Prevention (US)
CFU	Colony forming unit
CIB	Cedar Island Bay
FDA	Food and Drug Administration (US)
FS	Farmed, suspended
JB	Jarrett Bay
LCD	Local Climate Data
NC	North Carolina
NOAA	National Oceanic and Atmospheric Administration
NR	Newport River
NTC	Non-template control
PCR	Polymerase chain reaction
S	Suspended oysters
TCBS	Thiosulfate-citrate-bile salts-sucrose
TSS	Total suspended solids
US	United States
WB	Wild, on-bottom

INTRODUCTION

Vibrio spp. are Gram-negative, rod-shaped bacteria that are ubiquitous in estuarine and marine environments (Brock 1999, Thompson *et al*. 2004, Oliver 2006) including freshwater habitats, high nutrient coastal regions, oligotrophic open ocean, and even the extreme environments of deep-sea hydrothermal vents (Hasan *et al*. 2015, Pruzzo *et al*. 2005, Yamai *et al*. 1996). The *Vibrio* genus is both diverse and highly adaptable, allowing *Vibrio* spp. to persist in highly variable environments (Thompson *et al*. 2004). Some *Vibrio* spp. have developed evolutionary stable strategies such as biofilm formation and motility (Shinoda *et al*. 1977, Wolfe *et al*. 2004, Yildiz *et al*. 2009). Much of the diversity of the genus is due to the high plasticity of their genomes which confers the ability for lateral gene transfer and the sharing of survival traits across species and strains (Frazer *et al*. 2017, López-Pérez *et al*. 2018, Thompson *et al*. 2004), including some virulence factors (Church *et al*. 2016, Logan *et al*. 2018).

While there are over 100 species of *Vibrio*, only 12 are known to be pathogenic to humans (Martinez-Urtaza *et al*. 2017, Thompson and Polz 2006, Williams *et al*. 2017). Many of the *Vibrio* spp. of interest in estuarine environments are relevant to aquaculture and food safety because they are opportunistic pathogens, and readily attach to particles (Kaneko and Colwell 1975). Shellfish, particularly raw oysters, have posed a significant and increasing public health risk globally for *Vibrio* spp. infections. Because oysters are filter-feeders, they accumulate bacteria and they have been shown to concentrate *Vibrio* spp. concentrations up to 100 times that of the surrounding water column (DePaola *et al*. 2003). This means that pathogenic forms of *Vibrio* spp. can be concentrated in a single oyster to a level that poses a risk to consumers. With the global expansion of shellfish aquaculture and particularly raw product consumption, along with burgeoning coastal tourism and obesity-related diseases, there is a need for understanding the dynamics of *Vibrio* spp. (Andrews 2004, FAO 2004, Rossner *et al*. 2002). The combination of these effects results in a heightened interest in understanding how to raise, harvest, and process oysters for consumption in a way that is most protective of human health.

In the US, an estimated 80,000 people contract *Vibrio* spp. infections, often reported as vibriosis, each year, resulting in over 500 hospitalizations and 100 deaths (CDC 2017). In the US and other developed nations, most *Vibrio* cases (about 54,000 annually in the US) are caused by eating raw or undercooked seafood and oysters infected with pathogenic forms of *V. alginolyticus*, *V. vulnificus*, and *V. parahaemolyticus* (CDC 2017, FDA 2006).

V. parahaemolyticus is the most frequent cause of infections from raw or undercooked seafood, with 60-80% of cases described as gastroenteritis (Levine and Griffin 1993). There are thousands of cases of *V. parahaemolyticus* illnesses each year in the US (Haendiges *et al*. 2015), but the pathogen is also important in other regions of the world. For example, a recent publication reported 10,000 confirmed illnesses and nearly 4,000 hospitalizations due to *V. parahaemolyticus* between 2003 and 2008 in China (Wu *et al*. 2014). Typically following ingestion of undercooked or raw food products, *V. parahaemolyticus* has an incubation period of roughly 15 hours, after which typical symptoms can include diarrhea, abdominal cramping, chills, fever, headache, vomiting, and myalgias (McLaughlin *et al*. 2005). Infections caused by *V. parahaemolyticus* are self-limiting and last about three days in patients with normal immune systems (Velasquez-Roman *et al*. 2013). However, immunocompromised patients can suffer for longer periods of time (Mead *et al*. 1999). Because of the self-limiting nature of the infections, most cases go unreported and the CDC estimates the number of reported cases is only one in twenty (Mead *et al*. 1999). In total, *V. parahaemolyticus* is estimated to cause around 35,000 human illnesses each year in the US (range, 18,000 to 58,000 cases) (Scallen *et al*. 2011), accounting for roughly half of all *Vibrio* spp. infections (Newton *et al*. 2014).

V. parahaemolyticus is also of great concern because of its ability and recent history of pandemic infections. The emergence of the O3:K6 serotype resulted in the first *V. parahaemolyticus* pandemic which rapidly spread throughout Southeast Asia in 1996-97 (Martinez-Urtaza *et al*. 2017, Paranjpye *et al*. 2012). By 1998, the pandemic had reached the US, initially having been associated with oysters harvested from Galveston Bay, TX and affecting 416 people in 12 states. Within the year it had spread to the Pacific Northwest and the Northeast, US (Paranjpye *et al*. 2012). In the last two decades, the O3:K6 strains have spread to almost every other continent (Gonzalez-Escalona *et al*. 2008, Martinez-Urtaza *et al*. 2017, Velazquez-Roman *et al*. 2014). Another pandemic clonal type, ST36, was discovered in 2012

(Gonzalez-Escalona *et al*. 2008, Martinez-Urtaza *et al*. 2017). The spread of *V. parahaemolyticus* is most likely due to natural and anthropogenic climate change and ballast water transport (Martinez-Urtaza *et al*. 2010, Velazquez-Roman *et al*. 2014).

Though *V. vulnificus* infections are much rarer, *V. vulnificus* is the deadliest pathogen in the US when considered on a case-by-case basis, exhibiting a >50% mortality rate (Froelich *et al*. 2012, Mead *et al*. 1999, Oliver 2006). After infection through ingestion, almost always via consumption of raw oysters, *V. vulnificus* is capable of rapidly entering the blood of individuals and disseminating throughout the body resulting in primary septicemia and often death (Linkous *et al*. 1999). *V. vulnificus* is also of concern in recreational waters and fisheries, because it can be contracted through open wounds leading to necrosis and secondary septicemia often resulting in amputation, and/or death. (Linkous *et al*. 1999, Oliver 2005). After flooding events, cases often spike due to contact with contaminated floodwaters. After Hurricane Katrina, there were 22 cases and five confirmed deaths associated with *V. vulnificus* wound infections (Rhoads *et al*. 2006). Although *V. vulnificus* can be contracted through two portals of entry (wound and ingestion), FDA records between 2002 and 2007 report that 92.8% of 180 *V. vulnificus* infections were due to raw oyster consumption (Heng *et al*. 2017). *V. vulnificus* cases are fortunately quite rare, with only around 95 total cases reported, including 85 hospitalizations and 35 deaths per year in the US (CDC, 2013). This is in part due to *V. vulnificus* being a highly opportunistic pathogen; most infections are confined to those with certain preexisting conditions including liver cirrhosis and diabetes (Jones and Oliver 2009).

V. vulnificus, having an optimal temperature range between 16-33°C (Deeb *et al*. 2018), is experiencing an increase in both frequency and range of infection, likely due to rising sea surface temperatures (Baker-Austin *et al*. 2013). In fact, *Vibrio* spp. infections as whole, have been increasing while infections from other foodborne pathogens have generally decreased (Figure 1) (CDC 2009, Henao *et al*. 2015): a report released by the CDC estimated that there was a 43% increase in *Vibrio*-related infections between 2006 to 2012 (CDC 2013). *V. parahaemolyticus* infections associated with oysters have made headlines in recent years for large outbreaks in regions previously unaffected by the pathogen. For example, in 2012, an outbreak of *V. parahaemolyticus* occurred in Massachusetts resulting in hundreds of illnesses. The serovar that caused this outbreak across nine states was typed and was

thought to have come from the Pacific Northwest, and since then the transmission of this serovar has been well-documented (Martinez-Urtaza *et al*. 2013). Another outbreak, in 2004, which occurred on a cruise ship off the coast of Alaska resulted in 62 *V. parahaemolyticus* illnesses (all resulting in gastroenteritis caused by oyster consumption) (McLaughlin *et al*. 2005). Optimal growth temperature for *V. parahaemolyticus* is 37°C and, although it can persist in waters lower than 10°C, infections have not been associated with waters below 15°C (Martinez-Urtaza *et al*. 2010, Raszl *et al*. 2016). The outbreak appears to have been due to abnormally warm waters (>15°C) off the coast of Alaska and became the northern-most recorded *V. parahaemolyticus* outbreak in the region, extending the range of *V. parahaemolyticus* infection by over 1000 km (Martinez-Urtaza *et al*. 2010, McLaughlin *et al*. 2005).

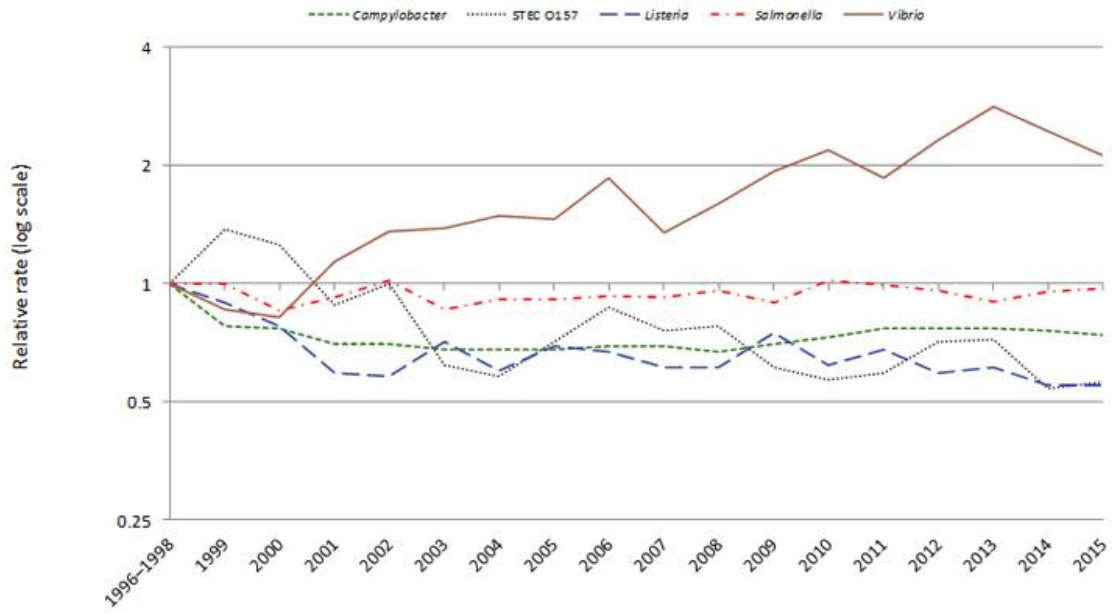


Figure 1. *Vibrio* infections on the rise. Relative rate of culture-confirmed infections with Campylobacter, STEC O157, Listeria, Salmonella, and *Vibrio* compared with 1996-1998 rates, by year, FoodNet 1996-2015 (Henao et al. 2015)

Vibrio concentrations have been increasing locally, as well (Froelich et al. 2013). This increase in environmental *Vibrio* concentrations will only compound with active efforts to increase oyster aquaculture production ten-fold in the next decade in NC (NCSMAC 2018), resulting in increased human risk of seafood-borne illnesses associated with raw oyster consumption. Additionally, because oysters grown in bags and cages by farmers tend to have a deep cup and are considered “cleaner” than on-bottom oysters, farmed oysters are often grown and sold with the intention of being consumed raw (Go Deep

Shellfish Aquaculture). In fact, current risk models indicate that, at all times of the year, 50% of harvested shellfish are consumed raw (NSSP 2017). Prominent NC oyster growers such as Morris Family Farms and Carolina Mariculture Inc. report over 95% raw consumption of their farmed product (Styron and Morris, personal communication). Again, the transition from wild and on-bottom oyster consumption to farmed and suspended oyster consumption will likely lead to increases in raw oyster consumption, which poses an increased risk of bacterial infections.

Despite persistent and increasing issues with bacterial pathogens, oysters have been an important part of NC culture and economy for over 150 years (Grabowski *et al*. 2012). Ecosystem services provided by oysters include carbon sequestration, shore stabilization, augmented fish production, water filtration, diversification of landscape and ecosystem and more (Grabowski and Peterson, 2007). The economic benefit of commercial harvest in NC was estimated at nearly $2 million USD in 2017 (NC Sea Grant 2018). A single *Vibrio* spp*.* infection, however, costs on average $3.6 million USD (Minor *et al*. 2015). Direct exposure to *V. vulnificus, V. parahaemolyticus,* and *V. alginolyticus* costs the US at least $30 million per year, as shown by Ralston *et al*. (2011), with estimates ranging up to ten times that amount given the lack of reporting of the infections. In the same study, *V. vulnificus* was credited with the title of costliest marine-borne pathogen, with annual costs ten times higher than the other pathogens. Additionally, even though *V. vulnificus* only accounted for one third of the *Vibrio* spp*.* infections, it constituted 85% of the *Vibrio* spp*.* direct exposure costs (Ralston *et al*. 2011).

In order to monitor *Vibrio* spp., molecular identification is imperative. *V. vulnificus* is universally identified by the presence of the *vvhA* gene which encodes for an extracellular hemolysin (Hill *et al*. 1991, Panicker and Bej 2005, Wetz 2008). The toxin, although confirmed to be expressed *in vivo*, has recently been shown to be rendered inactive by unbound cholesterol and human serum albumin, lending to the theory that the *vvhA* toxin is not responsible for *V. vulnificus* pathogenicity (Jones and Oliver, 2009). The *vvhA* gene, however, is important for its ubiquity in *V. vulnificus* strains and, therefore, is an effective species identifier (Yamamoto, 1990). Additionally, identification of total *V. parahaemolyticus* abundance is often carried out by isolation of the regulatory gene, *toxR* (Kim *et al*. 1999). The *toxR* gene was first discovered in *V. cholerae* and has been shown to be highly conserved among the *Vibrio* genus (Kim *et al*. 1999).

In addition to identifying *Vibrio* on the species-level, FDA-approved methods have been generated to determine less virulent strains of *V. vulnificus* and *V. parahaemolyticus* from potentially pathogenic (hypervirulent) strains (Kaysner and Depaola 2004). *V. vulnificus* pathogenicity, for example, is identified by the presence of the virulence-correlated gene, *vcgC.* One study found that *vcgC*-positive *V. vulnificus* strains were responsible for more than 93% of *V. vulnificus* infections. In the same study, 87% of environmental isolates contained the *vcgE* polymorphism of the gene (Warner *et al*. 2008). This form of the gene is not totally incapable of infection, as "E-types" are occasionally isolated from clinical cases and are, therefore, capable of causing infection in humans (Oliver 2006). The current accepted determinant of potential pathogenicity in *V. parahaemolyticus* is through molecular identification of genes encoding the thermostable direct hemolysin, *tdh*, and the evolutionarily and functionally similar thermostable-related direct hemolysin, *trh* (Bej *et al*. 1999). Both toxins have hemolytic, cytotoxic, and lethal toxicity in small experimental animals (Nishibuchi *et al*. 1995). These toxins are believed to be involved in human pathogenesis, although the exact mechanism has not been elucidated (Baker-Austin, 2018). While these gene markers are not definitive proof that *Vibrio* strains are pathogenic or not, generally, amplification of the *vcgC* gene in *V. vulnificus* and the *tdh* and/or *trh* gene in *V. parahaemolyticus* are accepted as indicators of potential pathogenicity (Bej *et al*. 1999, Kaysner and Depaola 2004, Warner and Oliver 2008,).

Eliminating risk associated with shellfish consumption is not possible. Mitigation of *Vibrio* infections by the shellfish industry focuses on reducing the concentrations of the bacteria in oysters or preventing preexisting bacterial increases in the product. This has historically been done through post-harvest techniques which include reducing exposure to heat and sunlight, near-immediate refrigeration or placement on ice, secondary filtration, high pressure pasteurization and other approaches (NSSF 2003, Andrews 2004). More recent techniques have included modifications or optimization of oyster farming. One modification is to grow oysters in floating bags suspended off-bottom (termed "suspended" aquaculture). This technique is supported by a 2012 study that found the detection frequency of *V. parahaemolyticus* to be higher in sediments than in the water column (89.7% to 69.5%, respectively) (Johnson *et al*. 2012). With this knowledge, it is inferred that the majority of *Vibrio* spp. are found in the sediment (Johnson *et al*. 2012) and that proximity to sediment may cause increased levels of *Vibrio* spp.

in on-bottom oysters. However, there is a lack of studies showing that on-bottom oysters do, in fact, harbor more *Vibrio* spp. abundances than suspended oysters.

To further understand differences in *Vibrio* spp. concentrations associated with specific oyster farming techniques, much more research is necessary to ensure shellfish safety and reduce the risk associated with this increasingly popular food product. The objective of this study was to quantify the differences in potentially pathogenic *Vibrio* spp. as associated with farmed and wild oysters in a productive shellfishery region of eastern NC. The study was conducted during the warmer summer months in order to better assess the *Vibrio* spp. dynamics associated with farmed and wild oysters. Specifically, *V. vulnificus* and *V. parahaemolyticu*s concentrations, including potentially pathogenic subpopulations identified by the *vcgC* gene in *V. vulnificus* and the *tdh* and/or *trh* genes in *V. parahaemolyticus*, were quantified using a paired study design across shellfish harvested from farmed (suspended) and wild (on-bottom) oysters during summer 2018. The second objective of this study was to assess the relationships between culture-based and molecular confirmation of the two species, *V. vulnificus* and *V. parahaemolyticus* across the study area. This study is the first of its kind to be conducted in NC, and the data generated from this study will assist us in improving our future education and outreach regarding public health risk associated with oysters harvested from the estuarine water of NC.

MATERIALS AND METHODS

Oyster Collection and Processing. Eastern oysters (*Crassostrea virginica*) were collected from three sites in eastern NC: Cedar Island Bay (CIB), Jarrett Bay (JB), and Newport River (NR) (Figure 2). Sampling occurred between late July 2018 and September 2018, which was during the season where wild oyster harvest is closed to the public in NC (April 1st and October 15th each year) (Andrews 2004). Each site contained a wild location and a farm location and they were within no more than 1000m distance and within 3 ppt salinity difference, except during a single extreme rainfall condition. The farm location and its corresponding wild location from each site were sampled on the same day at or within 3 hours of low tide, with oysters harvested typically within an hour of each other. Each site was sampled twice within the two-month period. At each sampling day, 48 farmed oysters and 48 wild oysters were collected (Figure 2). NR was treated as a control site because both the farmed and wild oysters were on-bottom unlike the other two sites that had farmed oysters from floating, on-surface, bags (suspended). The sites will be identified by the location (CIB, JB, or NR), whether it is a farm (F) or wild (W) location, and whether oysters were grown in suspended bags (S) or on-bottom (B). The farm location of Cedar Island Bay, for example, will be termed CIB-FS, while the farm location for Newport River will be NR-FB. Salinity was measured on site with a refractometer. Temperatures, precipitation, and wind speed and direction data were obtained from the National Oceanic and Atmospheric Administration (NOAA) Local Climate Data (LCD). Site latitudes and longitudes were obtained from Google Earth, post sampling.

Shellfish were shucked aseptically, and the hemolymph was drained. Meats of 6 oysters were pooled, weighed, and diluted with sterile phosphate-buffered saline (PBS) at a 1:1 (weight/volume) ratio, resulting in 8 sample bags containing 6 oysters each. Shellfish meats were blended for 10 min in a paddle blender (Fisher Scientific, Waltham, MA) at 280 rpm. Homogenates were diluted 1:10 in PBS, and 100µl aliquots of both diluted and undiluted homogenates were plated on media as described in the next section.

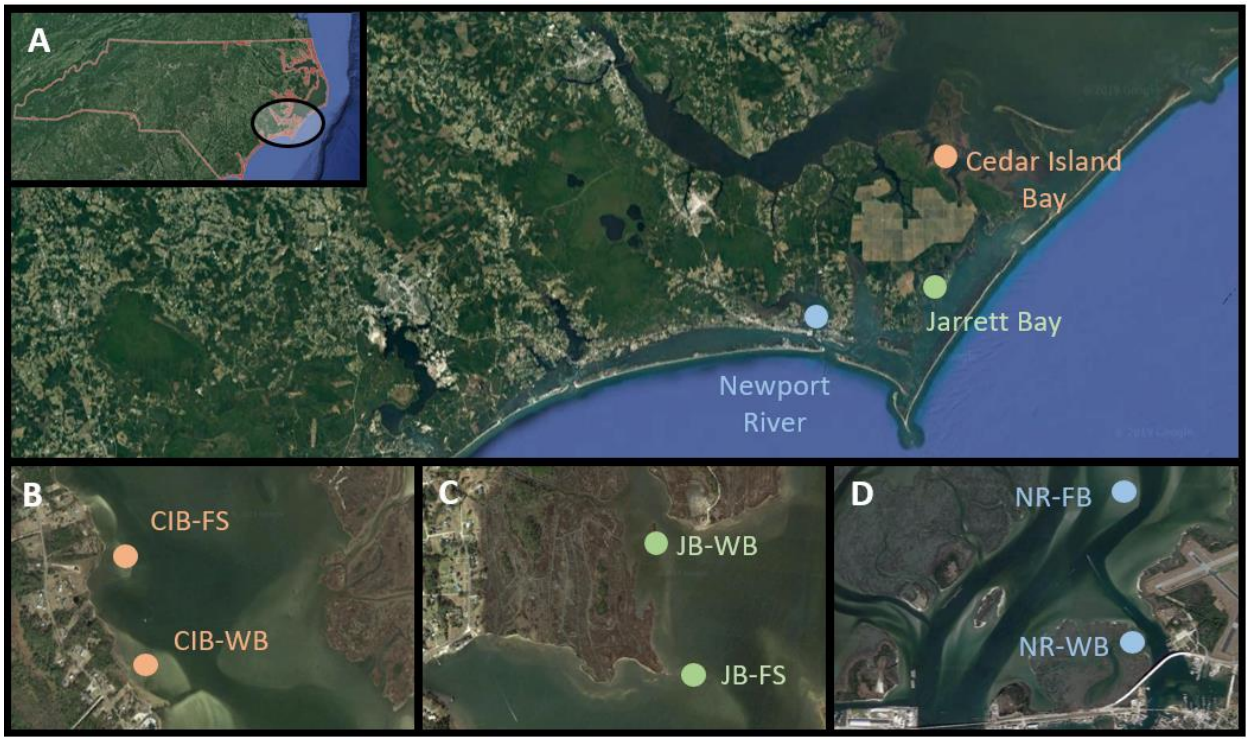


Figure 2. Study area. Study was conducted in eastern NC, with all sampling sites being in Carteret County (A). Close-ups of each site were provided to show proximity of farm and wild sites to one another (B, C, D). Image source: Google Earth.

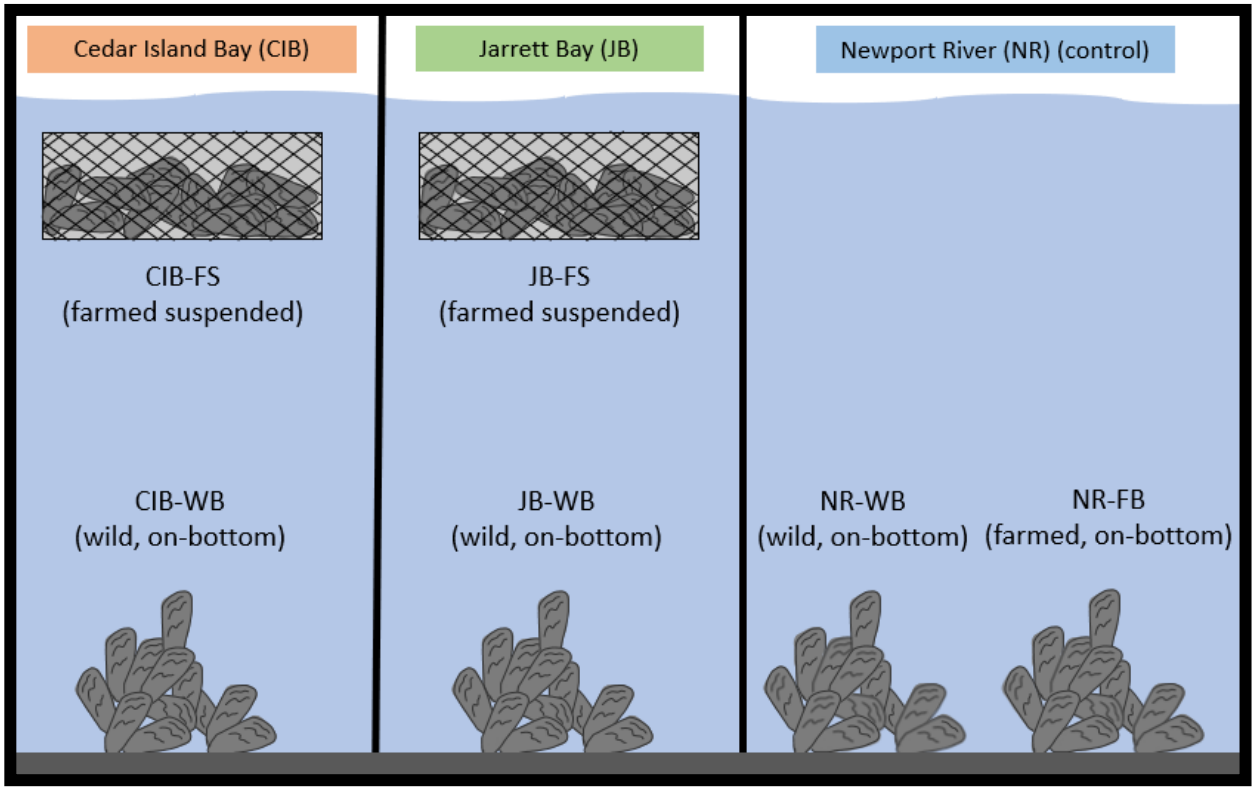


Figure 3. Sample design. This study had three sites, two of which were treated as experimental sites (Cedar Island Bay and Jarrett Bay) and one which was treated as a control site (Newport River).

Colony growth and isolation. For all samples, 100µL of both undiluted and diluted homogenate were spread plated on thiosulfate-citrate-bile salts-sucrose (TCBS) and CHROMagar *Vibrio* (CAV) (CHROMagar, Paris, France). For total *Vibrio* spp. quantification, TCBS plates were prepared as by manufacturer's instructions (Himedia, Mumbai, India), were used to enumerate total *Vibrio*. Green and yellow colonies were counted, as described by Froelich *et al*. (2016), and values were summed to determine total *Vibrio* colony forming unit (CFU) abundance per unit shellfish tissue mass. CAV plates, prepared as instructed, were used to isolate presumptive colonies of *V. parahaemolyticus* (purple) and *V. vulnificus* (blue). All plates were incubated at 37°C for 24 hours. After incubation, colonies on plates were counted and the data were converted to CFU per gram of oyster. From each plate up to five colonies each of presumed *V. vulnificus* and *V. parahaemolyticus* were isolated using sterile approaches and placed into 100µL of heart-infusion (HI) broth and incubated at room temperature overnight. Following this, cells were lysed to release DNA by incubation at 100°C for 10 minutes. Centrifugation at 10,000 x *g* for 10 min separated the aqueous DNA from cellular material. Supernatants, to be used as polymerase chain reaction (PCR) templates, were diluted in nuclease-free water and the undiluted, 10^{-1}, and 10^{-2} isolate subaliquots were stored at -80°C until they were prepared for PCR amplification as described below**.**

Molecular confirmation of isolates and determination of potential pathogenicity. Molecular species identification of both *V. vulnificus* (*vvhA*) and *V. parahaemolyticus* (*toxR*) was performed by conventional PCR on the C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, US) using GoTaq® Green Master Mix (Promega, Madison, WI, US) with subsequent gel (1.5% agarose) electrophoresis in tris-acetate (TAE) buffer at 140 mAmps for 25 minutes. The resultant bands were visualized via ethidium bromide staining and subsequent exposure to UV light.

All five assays (*vvhA*, *toxR*, *vcgC*, *trh*, and *tdh*) were analyzed via PCR amplification on the BioRad CFX96 ™ Real-Time System (Bio-Rad) using the PowerUp™ SYBR® Green Master Mix (ThermoFisher Scientific, Pittsburgh, PA, US). Following SYBR® Green qPCR (quantitative PCR), a melt curve was generated (Table 2) in order to confirm amplification of only the target amplicon and only those peaks that matched the positive control (see below) were considered positive for the corresponding gene.

Gene target name	Direction	Sequence (5'-3')	Amplicon Size (bp)	Source
vvhA[1]	F	TTCCAACTTCAAACCGAACTATGAC	205	Panicker and Bej 2005
	R	ATTCCAGTCGATGCGAATACGTTG		
vvhA[2]	F	CCGGCGGTACAGGTTGGCGC	519	Hill *et al*. 1991
	R	CGCCACCCACTTTCGGGCC		
vcgC[1]	F	AAAACTCATTGARCAGTAACGAAA	146	Warner and Oliver, 2008
	R	AGCTGGATCTAAKCCCAATGC		
toxR[3]	F	GTCTTCTGACGCAATCGTTG	368	Kim *et al*. 1999
	R	ATACGAGTGGTTGCTGTCATG		
tdh[1]	F	GTAAAGGTCTCTGACTTTTGGAC	269	Bej *et al*. 1999
	R	TGGAATAGAACCTTCATCTTCACC		
trh[1]	F	TTGGCTTCGATATTTTCAGTATCT	500	Bej *et al*. 1999
	R	CATAACAAACATATGCCCATTTCCG		

Table 1. Primer sequences. All isolates were typed for *toxR* and *vvhA* via both conventional PCR and SYBR® Green qPCR. While *toxR* used the same primer set for both methods, *vvhA* analysis used two primer sets, due to difficulties in transitioning the conventional *vvhA* primer set to SYBR® Green qPCR. Isolates were also typed for three other genes (*vcgC*, *tdh*, *trh*) but were only done so via SYBR® Green qPCR

[1] Primers were used for SYBR® Green qPCR only
[2] Primers were used for conventional PCR only
[3] Primers were used for both PCR methods

	SYBR® Green qPCR	Conventional PCR	
Assay	*vvhA*[1], *toxR*[3], *vcgC*[1], *trh*[1], *tdh*[1]	*vvhA*[2]	*toxR*[3]
Primer Concentrations	500 nM	400nM	400nM
Initialization	50°C, 2:00		
1) Initial Denaturation	95° C, 2:00	95° C, 2:00	95° C, 2:00
2) Denaturation	95° C, 0:15	95° C, 1:00	95° C, 0:30
3) Annealing	60°C, 0:45	53°C, 1:00	50°C, 0:30
4) Extension	72°C, 0:45	72°C, 0:30	72°C, 0:30
Cycles (Steps 2-4)	40	30	40
5) Final Extension		72°C, 5:00	72°C, 5:00
Melt Curve	60°C-90°C, increments of 5°C for 0:05		

Table 2. Conventional PCR and SYBR® Green qPCR parameters. All five SYBR® Green qPCR assays had the same cycling parameters. The melt curve was created by climbing from 60°C to 95°C increasing by 5°C for 5 seconds at each interval.
Conventional PCR cycling parameters differed between *vvhA* and *toxR* assays, with different annealing temperatures (53°C and 50°C, respectively). Although conventional methods do not require a melt curve, it was followed by gel electrophoresis and bands were visualized with ethidium bromide staining and UV exposure.

[1] Primers were used for SYBR® Green qPCR only
[2] Primers were used for conventional PCR only
[3] Primers were used for both PCR methods

Two positive controls were used for this study. For *V. parahaemolyticus* assays, *toxR*, *tdh*, and *trh*, the positive control was the *tdh*-positive/*trh*-positive environmental isolate, F11-3A, which is an ST36 pandemic strain (Nordstrom *et al*. 2007, Gonzalez-Escalona *et al*. 2008). The positive control for *vvhA* and *vcgC* assays was the septicemia isolated, *vcgC*-positive strain, MO6 (Wright *et al*. 1990). Both positive controls were inoculated from freezer stocks in heart infusion broth at 37°C overnight, then boiled-lysed for 10 minutes. Extracted controls were stored at -20°C. A non-template control (NTC) was used for all analyses. All negative controls were negative for data generated for this study.

Quantification of *V. vulnificus* and *V. parahaemolyticus* was performed as described by Williams *et al*. (2017) where concentrations in CFU/gram obtained from culture data were multiplied by the percentage of *vvhA*-positive and *toxR*-positive isolates, respectively. The same process was used in quantifying abundance and percent potentially pathogenic *V. vulnifics* (*vcgC*-positive) and *V. parahaemolyticus* (*tdh*/*trh*-positive).

Statistical analysis. All *Vibrio* concentrations (CFU/gram) were combined in JMP® Pro 14 and normality was tested using both the non-transformed and log-transformed data. Skewness and kurtosis were both reduced when data was transformed into logarithmic form. Therefore, non-parametric statistical analyses were utilized. In addition, a quartile range outlier test on the log data removed the single outlier from the non-log transformed data. Non-detects from diluted homogenate were given a value of 1. Statistical comparisons between *Vibrio* populations of suspended and on-bottom oyster values were conducted using Graphpad Prism software. Comparisons were determined significant with 95% confidence ($p=<0.05$).

Correlations between *Vibrio* populations and environmental parameters were determined by calculating Spearman's rank correlation coefficients (R Studio). Total *Vibrio* concentration values were tested against daily temperature, 7-day, 3-day, and 24-hour rainfall, 4-day and 24-hour wind speed, salinity. Correlation plots were also created in R Studio (not shown). Further analyses correlating *Vibrio* populations against each other were conducted in Excel Professional Plus 2019 (Figure 5).

RESULTS

Total *Vibrio* concentrations. Using culture-based methods, the average concentration of total *Vibrio* throughout the entire study was $1.2x10^4$ CFU/gram, with an observed range of $3.8x10^2$-9.5810^4 CFU/gram. Total *Vibrio* concentrations did not vary statistically from site to site (Table 3A) nor from sampling date to sampling date (Table 3B). Lastly, there was no difference in concentration of total *Vibrio* between suspended and on-bottom oysters (unpaired t-test, p=0.5426) (figure not shown).

A.

Site	W/F	S/B	Average total *Vibrio* log (CFU/gram)
CIB-FS	F	S	4.3 ± 1.1
CIB-WB	W	B	4.2 ± 0.9
JB-FS	F	S	4.1 ± 0.9
JB-WB	W	B	4.2 ± 1.1
NR-FB	F	B	3.5 ± 1.3
NR-WB	W	B	3.1 ± 1.1

B.

Date of Harvest	Site	W/F	S/B	Average total *Vibrio* log (CFU/gram)
7/22/2018	CIB-FS	F	S	3.9 ± 1.2
	CIB-WB	W	B	4.2 ± 1.3
8/3/2018	JB-FS	F	S	4.1 ± 1.3
	JB-WB	W	B	4.3 ± 1.6
8/7/2018	NR-FB	F	B	3.5 ± 1.3
	NR-WB	W	B	3.1 ± 1.1
8/13/2018	CIB-FS	F	S	4.6 ± 1.6
	CIB-WB	W	B	4.2 ± 1.3
8/24/2018	JB-FS	F	S	4.2 ± 1.4
	JB-WB	W	B	4.0 ± 1.3
9/4/2018	NR-FB	F	B	3.7 ± 1.3
	NR-WB	W	B	3.7 ± 1.3

Table 3. Log total *Vibrio* concentrations in farmed and wild oysters. Total *Vibrio* concentrations were separated by site (A) and by date of harvest (B). Total *Vibrio* concentrations were obtained from culture-based analyses data. “F” indicates farmed oysters and “W” indicates wild oysters.

Evaluation of culture methods at the species-level. Out of a total number of 394 isolates selected as presumed *V. parahaemolyticus*, the SYBR® Green qPCR method confirmed 351 as *toxR*-positive indicating that 89.1% of the colonies appearing on the plates were accurately characterized. When separated by sampling date, eight of the ten sampling events had >92% agreement, with the two other sampling events having 55.9% and 72.5% agreement (Table 4). As for *V. vulnificus*, 266 of the 378 gathered isolates were confirmed *vvhA*-positive via SYBR® Green qPCR, indicating that 70.4% of the

isolates selected were accurately characterized. This range widened significantly when separated by sampling date, with the lowest value being 35.0% and the highest being 100% (Table 4).

Date of Harvest	Site	W/F	S/B	Percent *V. parahaemolyticus* isolates confirmed	Percent *V. vulnificus* isolates confirmed
7/22/2018	CIB-FS	F	S	55.9%	41.9%
	CIB-WB	W	B	92.5%	67.5%
8/3/2018	JB-FS	F	S	95.0%	100.0%
	JB-WB	W	B	72.5%	97.5%
8/7/2018	NR-FB	F	B	94.9%	60.5%
	NR-WB	W	B	95.1%	68.6%
8/13/2018	CIB-FS	F	S	92.5%	88.2%
	CIB-WB	W	B	97.5%	70.0%
8/24/2018	JB-FS	F	S	92.5%	35.0%
	JB-WB	W	B	97.5%	70.0%
9/4/2018	NR-FB	F	B	NA	NA
	NR-WB	W	B	NA	NA

Table 4. Percent confirmed *V. parahaemolyticus* and *V. vulnificus* concentrations. Percentages were not applicable (NA) for sampling date 9/4/2018. This was due to difficulties in recovering DNA from isolates generated on this date.

Comparison of molecular methods. When both PCR methods (SYBR® Green qPCR and conventional PCR), were conducted using the same *toxR* primers for *V. parahaemolyticus* species analysis, the results demonstrated 96.19% agreement. For *V. vulnificus*, the two methods were in 98.15% agreement with each other even though two different *vvhA* primer sets were used (Hill *et al*. 1991, Panicker and Bej 2005). For the remainder of the reporting in this document, we have used the data from the SYBR® Green qPCR method for *toxR* and *vvhA* because conventional PCR was not performed across all three of the pathogenicity assays (*vcgC*, *tdh*, and *trh*).

Total *V. parahaemolyticus* concentrations. Average concentrations of confirmed *V. parahaemolyticus* were 9.7×10^2 CFU/gram which is only 0.2 log less from the presumptive value (not shown), indicating close agreement in approaches. The maximum concentration was almost an order or magnitude greater than the average at 9.5×10^3 CFU/gram. When separated by the type of grow-out scenario, i.e. farmed suspended versus wild on-bottom oysters, there was agreement between suspended and on-bottom average and maximum values.

A.

Site	W/F	S/B	Average concentration *V. parahaemolyticus* log (CFU/gram)	Average concentration *V. vulnificus* log (CFU/gram)
CIB-FS	F	S	2.5 ± 0.6	2.6 ± 0.7
CIB-WB	W	B	3.2 ± 0.8	3.0 ± 0.8
JB-FS	F	S	3.2 ± 0.8	3.3 ± 0.8
JB-WB	W	B	2.8 ± 0.7	3.4 ± 0.9
NR-FB	F	B	3.1 ± 1.2	2.3 ± 0.8
NR-WB	W	B	2.6 ± 0.9	2.9 ± 1.1

B.

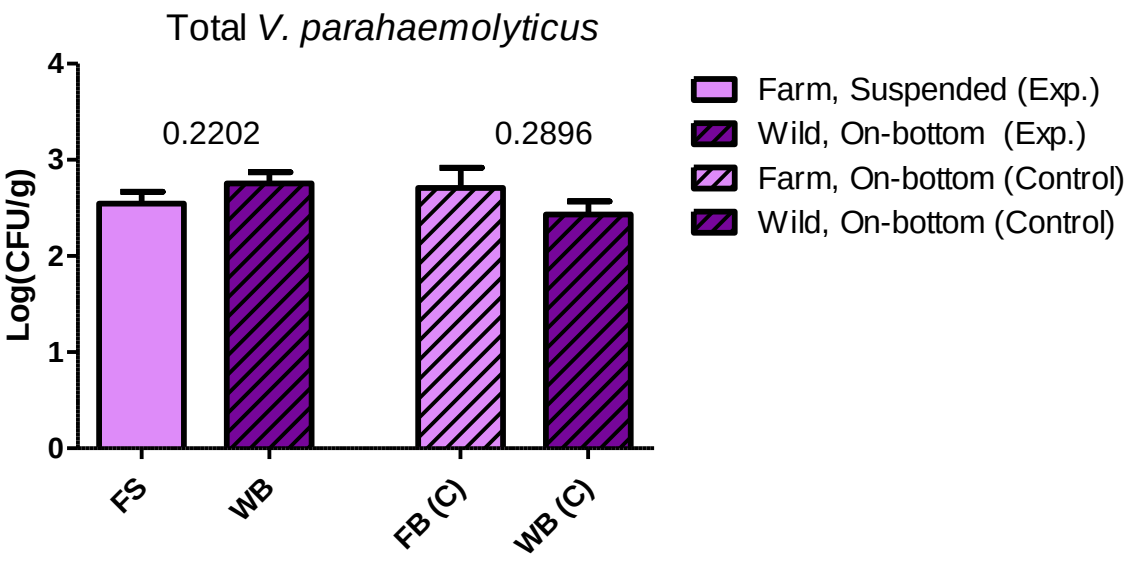
Date of Harvest	Site	W/F	S/B	Average concentration *V. parahaemolyticus* log (CFU/gram)	Average concentration *V. vulnificus* log (CFU/gram)
7/22/2018	CIB-FS	F	S	1.9 ± 0.7	1.8 ± 0.6
	CIB-WB	W	B	2.7 ± 0.9	2.5 ± 0.8
8/3/2018	JB-FS	F	S	3.4 ± 1.2	3.5 ± 1.2
	JB-WB	W	B	2.7 ± 0.9	3.7 ± 1.4
8/7/2018	NR-FB	F	B	3.1 ± 1.2	2.3 ± 0.8
	NR-WB	W	B	2.6 ± 0.9	2.9 ± 1.1
8/13/2018	CIB-FS	F	S	2.8 ± 0.9	2.9 ± 1.0
	CIB-WB	W	B	3.4 ± 1.1	3.2 ± 1.2
8/24/2018	JB-FS	F	S	2.8 ± 1.0	2.4 ± 0.8
	JB-WB	W	B	2.8 ± 0.9	2.6 ± 0.8

Table 5. Log *V. parahaemolyticus* and *V. vulnificus* concentrations in farmed and wild oysters. Concentrations were separated by site (A) and by date of harvest (B). "W" = wild, "F" = farmed.

V. parahaemolyticus was found in 98.8% of the oyster samples in this study: 97.9% in on-bottom and 100% in suspended oysters. Total *V. parahaemolyticus* concentrations did not show any statistical difference between suspended and on-bottom oysters at experimental sites (unpaired t-test, p=0.2202), nor at the control site (unpaired t-test, p=0.2896) (Figure 4A). Lastly, total *V. parahaemolyticus* did not correlate with total *Vibrio* in neither suspended (R^2=0.0999, n=32) nor on-bottom oysters (R^2=0.1482, n=48) (Figure 5A).

Total *V. vulnificus* concentrations. Average confirmed *V. vulnificus* concentrations were $1.3x10^3$ CFU/gram, which is roughly the same as the culture-based concentrations (not shown), again, indicating close agreements in approaches. The maximum concentration was over an order of magnitude greater than the average at $2.4x10^4$ CFU/gram. *V. vulnificus* was confirmed in 87.5% of the oyster

A.



B.

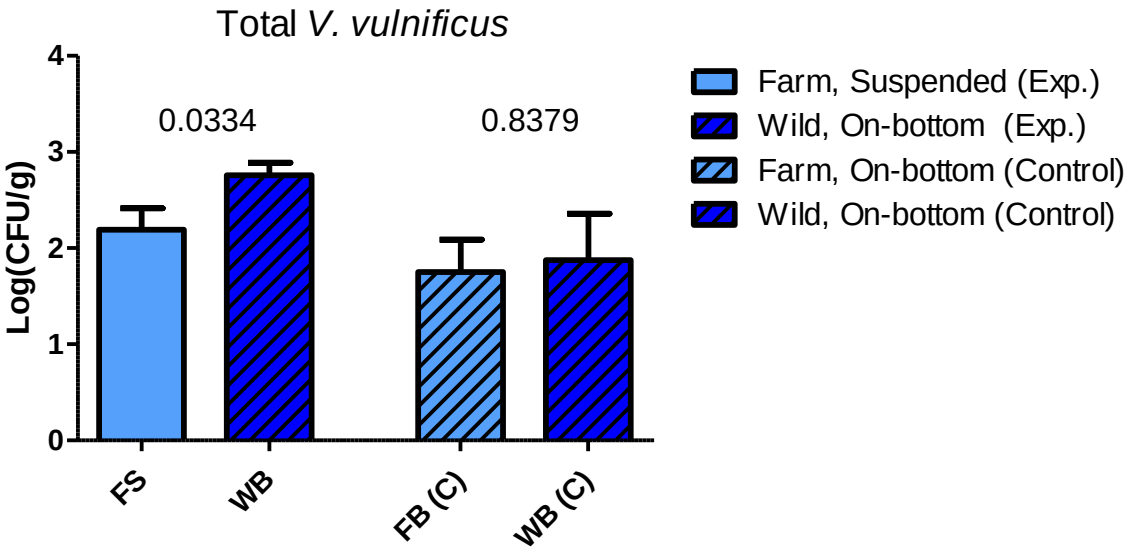


Figure 4. Comparison of *V. parahaemolyticus* and *V. vulnificus* concentrations in farmed, suspended and wild, on-bottom oysters. As the legend to the right of the figures show, lavender indicates farmed oysters and dark purple indicates wild oysters for total *V. parahaemolyticus* (A). Light blue indicates farmed oysters and dark blue indicates wild oysters for *V. vulnificus* (B). Additionally, hatched bars indicate on-bottom oyster grow-out methods while no markings in bars indicate suspension oyster grow-out approaches. The values are unpaired t-test p values using a 95% confidence interval.

samples in this study: 91.7% of on-bottom oysters had *V. vulnificus* and 81.3% of suspended oysters had *V. vulnificus*. Ten samples were devoid of confirmed *V. vulnificus*, four from on-bottom oysters and six

from suspended oysters. Half of the suspended oyster samples that were devoid of *V. vulnificus* came from sampling at JB-FS on 8/24/2018, meaning that three of the eight suspended oyster samples from that date did not have any confirmed *V. vulnificus*. The corresponding on-bottom site (JB-WB) had confirmed *V. vulnificus* in ten out of ten oyster samples for that date. Oyster samples taken from waters with salinities lower than 20 ppt all had confirmed *V. vulnificus*.

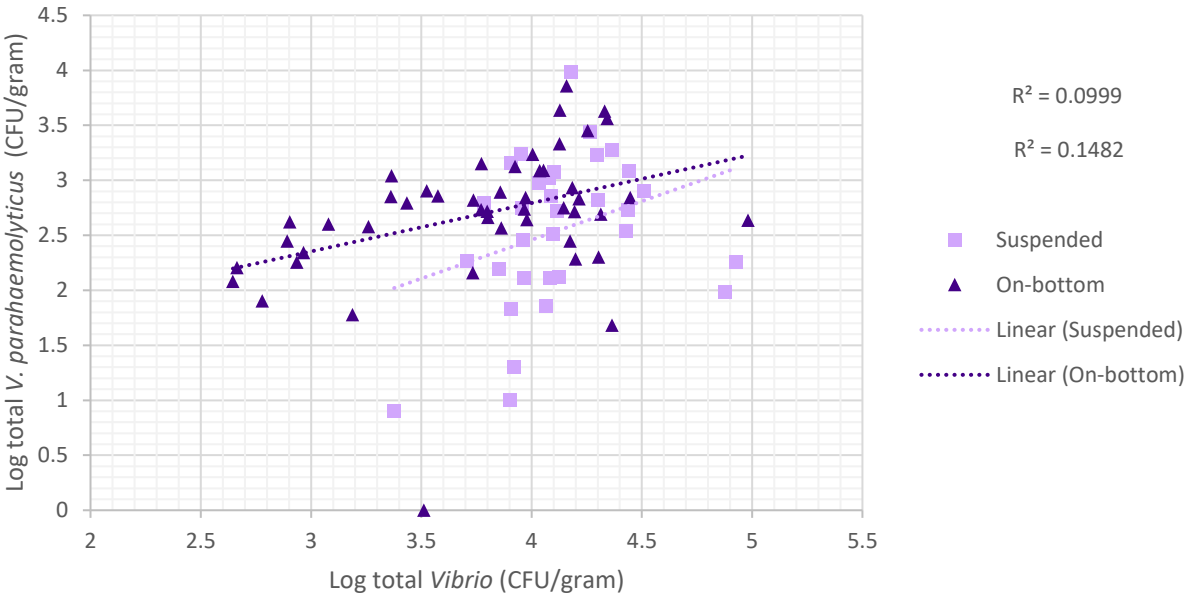
When analyzing CIB and FB (experimental sites), there were significantly lower concentrations of confirmed *V. vulnificus* in the farmed, suspended oyster samples than in their wild, on-bottom counterparts (unpaired t-test, p=0.0334). This difference was not observed at the control site (unpaired t-test, p=0.9379), where both farmed and wild oysters were grown and harvested on-bottom (Figure 4B). Lastly, total *V. vulnificus* did not correlate with total *Vibrio* in neither suspended (R^2=0.0048, n=32) nor on-bottom oysters (R^2=0.1964, n=48) (Figure 5B).

Potentially pathogenic *V. parahaemolyticus*. Only two of the 351 isolates (0.57%) possessed one or both hemolytic virulence genes (two isolates were *tdh*-positive, none were *trh*-positive). Both *tdh*-positive isolates came from farmed sites, one from suspension farming at JB-FS and the other from the on-bottom control, NR-FB. However, given the low number of potentially pathogenic isolates detected in the current study, correlation analyses could not be performed.

Potentially pathogenic *V. vulnificus*. Of the 266 confirmed *V. vulnificus* (*vvhA*-positive) isolates throughout the entire study, 44 contained the virulence correlated gene, *vcgC*, constituting 16.5% of the sample population. When analyzed according to growing approach, i.e. by suspended and on-bottom oysters, however, 20.1% of on-bottom oysters were *vcgC*-positive and only 10.3% of suspended oysters were potentially pathogenic.

Similar to confirmed *V. vulnificus,* potentially pathogenic *V. vulnificus* and *percent* potentially pathogenic *V. vulnificus* were also lower in suspended farmed oysters than on-bottom wild oysters at the experimental sites (unpaired t-test, p=0.0366 (Figure 6) and 0.0342 (not shown), respectively). Again, this was not demonstrated at the control site (unpaired t-test, p=0.7832 and 0.8924 (not shown), respectively).

A.



B.

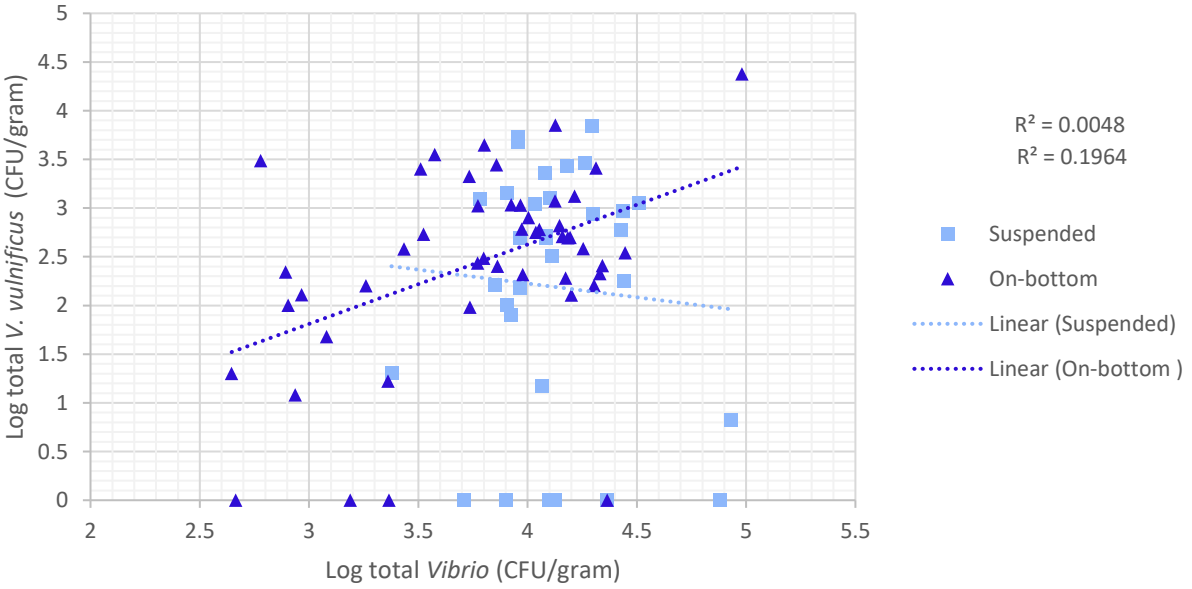


Figure 5. *Vibrio* species (*V. parahaemolyticus* and *V. vulnificus*) versus total *Vibrio* concentrations suspended and on-bottom oysters. Total *Vibrio* neither correlates with *V. parahaemolyticus* in floating, suspended oysters (lavender, R^2=0.0999, n=32) nor on-bottom oysters (dark purple, R^2=0.1482, n=48) (A). Total *Vibrio* also neither correlates with *V. vulnificus* in floating, suspended oysters (light blue, R^2=0.0048, n=32) nor on-bottom oysters (dark blue, R^2=0.1964, n=48).

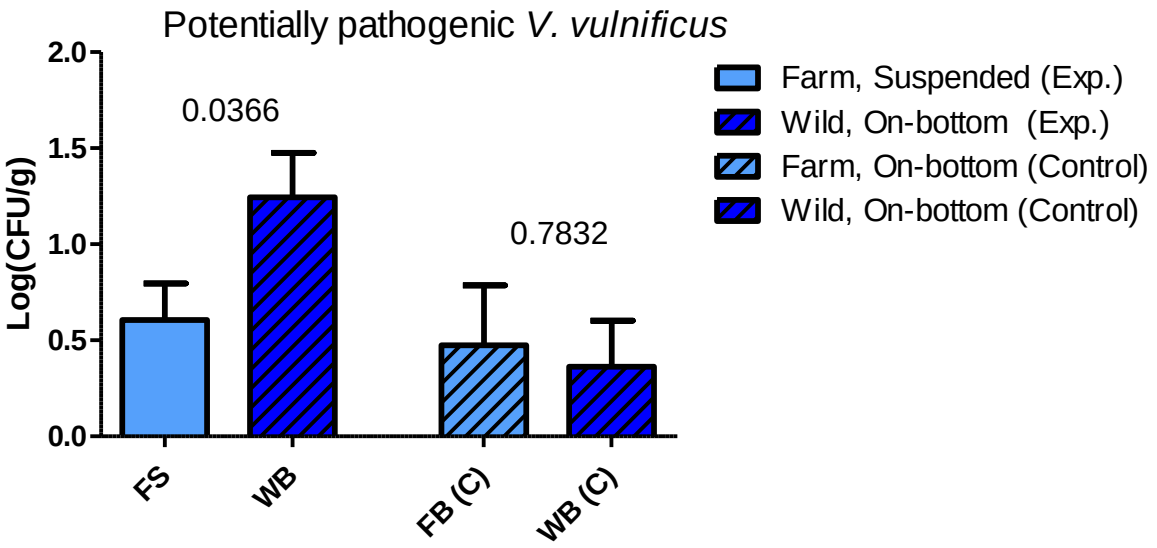


Figure 6. Comparison of potentially pathogenic *V. vulnificus* concentrations in farmed, suspended and wild, on-bottom oysters. As the legend to the right of the figures show, light blue indicates farmed oysters and dark blue indicates wild oysters for potentially pathogenic *V. vulnificus*. Additionally, hatched bars indicate on-bottom grow-out method while no markings in bars indicate suspension grow-out. The values are unpaired t-test p values using a 95% confidence interval.

Potentially pathogenic *V. vulnificus* was found in 35.0% of the oyster samples in this study: 41.7% of on-bottom oysters contained *vcgC*-positive *V. vulnificus* and 25% of suspended oysters contained *vcgC*-positive *V. vulnificus*. Two samples contained 100% *vcgC*-positive *V. vulnificus*, both from the same sample site and day (NR-FB and NR-WB on 8/7/2018). Salinity was 23 ppt and daily air temperature was 28°C.

Environmental parameters and correlations with total *Vibrio*. Daily air temperatures during this time period averaged at 27°C, with a range of 24°C-29°C (Table 8). Throughout this study period, temperature and salinity exhibited very weak correlations with total *Vibrio* concentrations. Low salinities observed early in the study period (August 3, 2018 (Table 6)) were due to heavy rainfall in July. In Carteret County, NC, rainfall total for the month of July was between 11.47 inches and 12.95 inches making it the wettest July on record (NOAA, NC Coastal Fed. 2018). July 24, 2018, alone, had 3.51 inches of rain (Table 8). Heavy rainfall frequently results in shellfish harvest closures due to high concentrations of fecal indicator bacteria (FIB) in harvest waters as a result of stormwater runoff (Converse et al. 2010). Shellfish harvesting closures were implemented sporadically between July 8-August 20 in and near the sampling area of this project due to rainfall (North Carolina Division of Marine

Fisheries 2018). Although salinity did not correlate with total *Vibrio*, weak correlations were observed between total *Vibrio* and rainfall. Specifically, although total *Vibrio* in wild, on-bottom oysters correlated weakly with 24-hour rainfall (R^2=0.329, n=64, (figure not shown)), total *Vibrio* in suspended oysters had weak, negative correlations with three-day and seven-day rainfall (R^2=-0.618, R^2=-0.439, respectively, n=32).

Date of Harvest	Site	W/F	S/B	Lat.	Long.	Salinity (ppt)	Description
7/22/2018	1	F	S	34°59'59" N	76°18'32" W	24	Floating bags
		W	B	34°59'58" N	76°18'36" W	23	Clusters, intertidal
		W	B	34°59'46" N	76°18'30" W	23	Clusters, intertidal
8/3/2018	2	F	S	34°47'10"N	76°29'48"W	12	Floating bags
		W	B	34°47'27"N	76°29'51"W	3	Oyster bed, intertidal
8/7/2018	3	F	B	34°44'32"N	76°40'18"W	20	Sandbar, intertidal
		W	B	34°43'41"N	76°40'16"W	23	Oyster bed, intertidal
8/13/2018	1	F	S	34°59'59" N	76°18'32" W	26	Floating bags
		W	B	34°59'58" N	76°18'36" W	28	Clusters, intertidal
8/24/2018	2	F	S	34°47'10"N	76°29'48"W	34	Floating bags
		W	B	34°47'27"N	76°29'51"W	34	Oyster bed, intertidal
9/4/2018	3	F	B	34°44'32"N	76°40'18"W	34	Sandbar, intertidal
		W	B	34°43'41"N	76°40'16"W	34	Oyster bed, intertidal

Table 6. Sampling and environmental parameters. Wild and farmed oysters from the same site were collected on the same day within 2 hours of each other and were within 1000 meters of each other. CIB has two wild locations because oyster clusters were scarce in the original wild area we chose to sample. Salinities were within 3 ppt of each other except during a single extreme rainfall condition (JB, August 3, 2018). “W” = wild, “F” = farmed, “S” = suspended, “B” = on-bottom.

Prevailing wind direction across coastal NC is along the SW-NE trajectory. Additionally, the shallow estuaries in North-Eastern NC are largely freshwater and wind dominated (tidal influence is dampened by presence of barrier islands) (Reynolds-Fleming and Luettich, 2004). During the study period, winds came predominantly from SW/SSW (Table 8). Average wind speed was 9.7 mph. On each day that the wind direction was not SW/SSW, the wind speed was below average, except for a WSW wind on August 9 that was just above the average (10.0 mph). The maximum wind speed was 18.2 mph coming from the SW. Daily wind speeds negatively correlated with total *Vibrio* concentrations in surface oysters (R^2=-0.617, n=32 (figure not shown)). There was no correlation between wind and total *Vibrio* in on-bottom oysters.

DISCUSSION

Total *Vibrio* dynamics and concentrations of total *V. parahaemolyticus* and total *V. vulnificus*. Total *Vibrio*, total *V. parahaemolyticus,* and total *V. vulnificus* average and maximum concentrations from across the entire study were similar to those previously reported in eastern NC. The concentrations of *Vibrio* spp. observed for this study were within an order of magnitude of values gathered from a similar study in NC conducted on wild (on-bottom) oysters just three years prior (Froelich *et al*. 2015). Even considering suspended and on bottom separately, average and maximum concentrations for total *Vibrio*, *V. parahaemolyticus*, and *V. vulnificus* were still within one order of magnitude agreement with Froelich *et al*. (2015). Variances between suspended and on-bottom oyster concentrations were not observed for total *Vibrio*. This indicates that the complexity and opportunistic nature of specific species within the genus allows the overall concentrations to remain stable even while species dynamics are shifting (e.g. Jesser and Noble 2018).

***V. vulnificus* and *V. parahaemolyticus* dynamics**. *Vibrio parahaemolyticus* was confirmed in almost all oysters samples (in 97.9% of on-bottom and 100% of suspended oysters) and variances between suspended and on-bottom oyster concentrations were not observed for total *V. parahaemolyticus*. This is in contrast to a study conducted in 2012 (Cole *et al.* 2015) that, like our study, focused on the effects of suspension farming on *Vibrio* populations in oysters in a shallow, estuarine location. Unlike our study, the Cole *et al*. (2015) study was conducted in the Gulf Coast, over a longer time-scale (one year), and deployed their own oysters for both suspended oysters (in floating bags) and on-bottom oysters (in on-bottom cages) indicating they did not use wild oysters for their on-bottom oysters. The study found higher total *V. parahaemolyticus* concentrations in suspended oysters than on-bottom oysters (48% higher) in suspended oysters (not statistically significant). This result was not corroborated by our study.

Our study was, however, in agreement with Cole *et al*. (2015) *V. vulnificus* dynamics. Cole *et al*. found higher total *V. vulnificus* in on-bottom oysters than suspended oysters (3% lower in suspended

oysters (no statistically significant)). The data collected in our study found a statistically significant decrease in total *V. vulnificus* concentrations in suspended oysters, giving this trend more scientific credibility. Additionally, *V. vulnificus* was confirmed in more on-bottom oysters than suspended oysters (91.7% and 81.3%, respectively). Six suspended oyster samples were devoid of confirmed *V. vulnificus*, half of which came from a single sampling location on a single date (JB-FS on 8/24/2018). The corresponding on-bottom site (JB-WB) for that date had confirmed *V. vulnificus* in ten out of ten oyster samples, showing contrasting on-bottom and surface dynamics on the same date. In the weeks before this sampling date, there was heavy rainfall which brought expectedly low surface salinities and, likely, an influx of suspended solids, both of which have been shown to have dramatic effects on *V. vulnificus* populations in the water column (CDC 2005, Eiler *et a*l. 2007, Jesser and Noble 2018, Shaw *et al.* 2014, Wetz *et al.* 2008). However, *Vibrio* concentrations by day or even by week in water samples have been shown to be poor indicators of *Vibrio* concentrations in oysters on the same time-scale (Cole *et al.* 2015). The environment inside the oysters is likely not as affected by these changes as in the water column and, when it is, it is likely on longer timescales than days (Audemard et al. 2011, Froelich et al. 2012 (b), Froelich and Noble 2014, Motes et al. 1996). So, although salinity on this sampling date (8/24/2018) shifted to very high salinity (34ppt - the upper limit of *V. vulnificus* survival in salinity (Kaspar and Tamplin 1993, Tan *et al*. 2010)), it is likely that the population dynamics we observed on this date and throughout much of this study period were lingering effects of this rainfall event. Because of this, predicting *Vibrio* populations in oysters based off of water measurements and water column ecology is something of which to be wary. Environmental trends over week- and month-long time-scales in the study area are likely more accurate predicters. Given the patterns observed at this particular location, it may be useful to conduct further studies here in order to understand the likelihood that environmental trends, such as large rainfall events, are playing a role in *V. vulnificus* populations.

Diving further into how rainfall may be affecting *V. vulnificus* concentrations in on-bottom and suspended oysters, large influxes of less-dense, freshwater into more-dense estuarine and marine water cause stratification events which are common in NC estuaries (Paerl et al. 1998). It is possible that surface salinities were too low for *V. vulnificus* survival during this persistent plume of rainfall. However, it is likely not as simple as one factor explaining what we have observed in this study and parameters such

as TSS, chlorophyll-A, etc., that were brought into the estuary by rainfall, may be at play in this *V. vulnificus* dynamic in on-bottom and suspended oysters. For example, in a study conducted on *Vibrio* concentrations in suspended and on-bottom oysters in the Chesapeake Bay (another shallow, often stratified estuary) after Hurricane Irene, although there were no differences in *V. vulnificus* nor *V. parahaemolyticus* concentrations in suspended versus on-bottom oysters, *V. vulnificus* in all oyster tissue correlated with TSS (r=0.41, P=0.04). Perhaps our study was just beginning to observe the effects of the large rainfall event on *Vibrio* populations in oysters as the suspended solids from the watershed were flushing out of the estuary. Whatever the direct mechanism, this may be a trend applicable to many other highly-stratified estuaries with large watersheds and frequent flushing events as the effects appear to last weeks after the initial rainfall event.

An alternative, but not contrasting, theory as to the *V. vulnificus* population dynamics observed in this study is due to proximity to sediments. Cole *et al*. (2015) suggested that the discrepancies they observed in *V. vulnificus* in suspended and on-bottom oysters was due to higher *Vibrio* spp. concentrations in sediments (Johnson *et al.* 2012) and, therefore, also in on-bottom oysters due to proximity. This theory is further supported by research previously conducted by Fries *et al.* (2008) in the nearby Neuse River Estuary. This study demonstrated that *Vibrio* spp. attached to sediment were a prominent proportion of the total *Vibrio* spp. in their samples and demonstrated that during resuspension events *Vibrio* spp. concentrations were higher in the water column. In our study, we found that total *Vibrio* negatively correlated with daily wind speed, an indicator of potential sediment resuspension events. However, the factors explaining these patterns may be more complex than just daily wind speed (i.e. sustained wind speed, gust speed, sustained wind direction, water column depth, etc.) and the timing of the data pairing may be inappropriate. However, to date, no lag analysis has been conducted of any sort on *Vibrio* spp. concentrations in eastern NC systems.

Again, this study was conducted over both short temporal and spatial scales making assessments of relationships between environmental conditions and other *Vibrio* populations besides total *Vibrio* (i.e. total *V. vulnificus*, total *V. parahaemolyticus*, and potentially pathogenic version of these species) difficult to analyze. As we demonstrated above, neither *V. vulnificus* nor *V. parahaemolyticus* correlated with total *Vibrio*, so even though total *Vibrio* correlated negatively with daily wind speed and

rainfall, these correlations may not also be observed in *V. vulnificus* and/or *V. parahaemolyticus*. This is important because, previous to the most recent decade, many researchers were content to simply model *V. vulnificus* and *V. parahaemolyticus* off of total *Vibrio* spp. using temperature and salinity. We can observe from this study and others that modeling the entire genus does not permit accurate prediction of the behavior and ecology of the specific species of concern (Jesser and Noble 2018, Shaw *et al*. 2014). Because of this, we have not used total *Vibrio* correlations to explain what we have observed here for *V. vulnificus* and *V. parahaemolyticus*.

Potentially pathogenic *V. vulnificus* and *V. parahaemolyticus* dynamics. Interestingly, 16.5% of the confirmed *V. vulnificus* isolates contained the virulence correlated gene, *vcgC*. This proportion is on the high end of a range from previous studies conducted in NC that found proportions of *vcgC*-positive isolates to be 5-15% of the population of *V. vulnificus* in oyster tissue (Warner *et al*. 2007, Williams *et a*l. 2017). This is surprising but given that this study was conducted only during the warm summer season, there are seasonal characteristics that could have attributed to these differences.

Similar to the total *V. vulnificus* findings, potentially pathogenic *V. vulnificus* was found to be lower in suspended oysters than on-bottom oysters. Considering the salient findings generated in this study regarding *V. vulnificus* concentrations, it would, at first, appear that the discrepancies in potentially pathogenic *V. vulnificus* between suspended and on-bottom grown oysters are due to the fluctuations observed across the entire *V. vulnificus* population (i.e. as total *V. vulnificus* concentrations decrease, so do the concentrations of *vcgC*-positive *V. vulnificus*). However, we have also demonstrated that the *proportion* of potentially pathogenic *V. vulnificus* (percent *vcgC*-positive) also declined in oysters suspended in the water column. This information indicates that there are other factors, besides total *V. vulnificus* concentrations, that play a role in *vcgC*-positive *V. vulnificus* dynamics in oysters. This is also the first study of its kind conducted on *potentially pathogenic V. vulnificus* in NC and possibly in the US.

Additionally, more on-bottom oysters contained potentially pathogenic *V. vulnificus* than suspended oysters (in 20.1% of on-bottom and 10.3% of suspended oysters). The discrepancy in these two values may, however, be due to CIB, in which 40.0% of the on-bottom isolates (CIB-WB) were *vcgC*-positive on both sampling days. The farmed, suspended oysters at CIB-FS also had no *vcgC*-positive *V. vulnificus* isolates on the first day of sampling. Discrepancies like these were not observed at the other

experimental site, nor the control site, indicating that there may be specific characteristics of the location or on-bottom bed-type causing this increase in potentially pathogenic *V. vulnificus*.

The lack of potentially pathogenic *V. parahaemolyticus* observed over the course of this study is a major finding for NC shellfish aquaculture. Only two of the 351 confirmed *V. parahaemolyticus* isolates were confirmed *tdh*-positive (0.57%) and none were identified as *trh*-positive. This finding is interesting, firstly, in that we only found *tdh*-positive strains. This eliminates the possibility that these strains are the pandemic ST36 strain (*tdh*-negative, *trh*-positive) that has wreaked havoc on the oyster industry on the east coast of the US since its arrival in 2012 (Gonzalez-Escalona *et al.* 2008, Martinez-Urtaza *et al*. 2017). Secondly, this finding is interesting because this proportion of potentially pathogenic *V. parahaemolyticus* is in agreement with previous studies conducted in NC waters and oysters that have found potentially pathogenic *V. parahaemolyticus* to be only a fraction of a percent of the total *V. parahaemolyticus* population (Oliver *et al*. 2007, Williams *et al.* 2017). Studies conducted in other locations, such as the Gulf Coast, have found much higher levels of *tdh*/*trh*-positive *V. parahaemolyticus* (DePaola *et al*. 2003, Johnson *et al.* 2012, Velazquez-Roman *et al*. 2012, Zimmerman *et al*. 2007). With the high concentrations of total *V. parahaemolyticus* and incredibly low numbers of potentially pathogenic *V. parahaemolyticus* in this study and in other studies in the region, we have shown that NC shellfish aquaculture environments are preferentially selecting for non-pathogenic forms of the species. Given that the management criteria for *Vibrio* spp. have largely been developed in the Gulf and South Atlantic states, and simply applied en masse to other regions, there may be a further interest in identifying some of the region-specific characteristics about NC strains of *V. parahaemolyticus* that are different from the Gulf Coast.

Because potentially pathogenic strains of both *Vibrio* species are such a small portion of the total population in this study and in previous studies performing statistical analyses has proven difficult (Chatry *et al*. 1983, Froelich *et al*. 2013, NCEZID (CDC) 2013, Tarr *et al.* 2007). Because of this, correlations between environmental parameters could not be conducted for neither potentially pathogenic *V. vulnificus* nor potentially pathogenic *V. parahaemolyticus* due to small sample size. This is unfortunate because in large-scale studies and meta-analyses, potentially pathogenic *V. vulnificus* and *V. parahaemolyticus* have, interestingly, been found to not adhere to the same drivers as at the species level (salinity and

temperature, primarily). Some of these drivers include TSS, dissolved oxygen, and pH (Froelich and Noble 2016). Again, the large rain events during this study period could prove to have had a large influence on *Vibrio* spp. dynamics and measurements of the aforementioned drivers could help elucidate that influence. Even so, the potentially pathogenic populations of both species are consistent with previous studies conducted in NC.

Again, a study design for larger-scale sampling, especially across a wider range of temperature and salinity conditions, would benefit the comparison of interactions between *Vibrio* spp. and suspended and on-bottom oysters. Furthermore, a study that identified the range of *Vibrio* strains and concentrations of *Vibrios* in the oyster tissue without the use of the intermediary enrichment step (culturing isolates on selective media plates) would be preferred (qPCR and/or digital droplet PCR (ddPCR), for example, do not use require an enrichment step). This type of study would allow researchers to further understand the dynamics of the *Vibrio* spp. in the complex estuarine environment, in the absence of the confounding issues associated with enrichment.

Evaluation of culture methods via molecular identification. In addition to analyzing suspended and on-bottom *Vibrio* concentrations across oysters grown in different grow-out scenarios, this study sought to compare traditional, culture-based techniques with molecular techniques. In total, 660 isolates were gathered for molecular evaluation, 394 of which were presumed to be *V. parahaemolyticus* and 266 were presumed to be *V. vulnificus*. The SYBR® Green qPCR method that was utilized here identified that 89.1% of the presumed *V. parahaemolyticus* were accurately characterized. This value came with a significant range when separated by day: between 55.9%-97.5% accuracy. The range and frequency of false-positives increased for *V. vulnificus* with a range of 35.0%-100% (Table 4) when separate by day and an overall accuracy of 70.4%. However, these percentages of confirmed *V. parahaemolyticus* were higher than published previously, while the confirmation rate across *V. vulnificus* was similar to that published previously. Previous work has shown that quantification of *Vibrio* spp. via culture methods has between 20-40% false positives (Nigro and Steward 2015, Pfeffer and Oliver 2003). The number of false positives in this study was well within this value (10.9% in *V. parahaemolyticus* and 29.6% in *V. vulnificus*). While culture-based quanitification using CAV media is cost-efficient and relatively accurate as shown in this study, it comes with a potentially large range of error. Additionally, molecular

methods are necessary for determining potential pathogenicity. Overall, the data generated via culture-based concentrations have yielded a similar and accurate picture of the dynamics across *Vibrio* spp. concentrations in this study.

Comparison of two molecular identification methods. The SYBR® Green qPCR approach used for this study were unconventional. SYBR® Green qPCR dye is typically used in a semi-quantitative way, as part of qPCR, where the amount of fluorescence in a sample compared to a standard curve is indicative of a relative amount of corresponding DNA template. During this study, we attempted to conduct qPCR and digital droplet PCR on whole oyster tissue homogenates. However, due to the low recovery of DNA during the sample processing and extraction procedure, we were not able to generate data using this approach. Instead, we adapted a method, using SYBR® Green qPCR dye to determine presence/absence of the five genes in isolates. Through the use of the SYBR® Green qPCR approach we were able to compare this method to conventional PCR paired with gel electrophoresis and UV exposure. Comparing the conventional PCR methods to SYBR® Green qPCR, there was 96.19% agreement in *V. parahaemolyticus* isolate confirmation and 98.15% agreement in *V. vulnificus* isolate confirmation. Our data indicates very close agreement with accepted conventional PCR methods, but we believe the SYBR® Green qPCR method is a time- and cost-effective method for determining presence/absence in isolates over conventional PCR. This is because it allows for a higher throughout of samples (96 samples can be analyzed at a single time) and removal of the subsequent gel electrophoresis step that is necessary in conventional PCR and which is time-consuming, tedious, and prone to user-error. Additionally, the melt curve analysis allows users to observe and analyze artifacts such as primer dimers, non-specific amplification, and bimodal peaks, all valuable information for understanding the quality of DNA generated for analysis.

One limitation of this study is a lack of environmental measurements including in field measurements of water temperature (air temperatures were gathered post-harvest from NOAA stations), dissolved oxygen, total suspended solids and turbidity, and chlorophyll-A and phytoplankton species analysis. In addition to the lack of in-field measurements, this study was conducted over a short period of time, during one season, and did not allow for assessment of a wide array of conditions and widely ranging environmental parameters such as salinity and air temperature. Lastly, the study design was

created for a small geographic location and would have benefitted from the inclusion of more study sites over a larger geographic range.

This study confirms previous findings that have demonstrated differences in *Vibrio* spp. concentrations as associated with suspended versus on-bottom grow out conditions. We demonstrated a difference in *V. vulnificus* according to grow out conditions, with higher rates of potentially pathogenic *V. vulnificus* observed in on-bottom grown oysters. However, overall the incidence of pathogenic forms of *Vibrio* spp. were very, very low compared to those reported in other regions of the United States during summer months. Molecular confirmation of colonies of *V. parahaemolyticus* and *V. vulnificus* were reliably generated, and a high-throughput approach was used to type the potentially pathogenic subpopulation of each species. Even though interesting findings were generated, a further controlled sets of field experiments to address *Vibrio* spp. dynamics in the estuarine waters of NC will continue to reduce the risks associated with oyster consumption.

CONCLUSION

- Culture based methods for both *V. parahaemolyticus* and *V. vulnificus* showed strong agreement with molecular confirmation methods.
- The incidence of potentially pathogenic *V. parahaemolyticus* in all samples, regardless of growth and harvest at wild or farmed sites, was extremely low.
- *V. vulnificus* concentrations appeared to be higher in wild, on-bottom oysters, but the low concentrations of potentially pathogenic isolates made statistical analysis difficult.
- SYBR® Green qPCR based molecular confirmation is a high throughput accurate and cost-effective approach for confirming *Vibrio* spp.

REFERENCES

Anonymous, 2005. Quantitative risk assessment on the public health impact of pathogenic *Vibrio parahaemolyticus* in raw oysters. Washington, DC: US Food and Drug Administration

Andrews LS, 2004. Strategies to control *Vibrios* in molluscan shellfish. Food Prot Trends 24, pp. 70-76.

Audemard C, Kator HI, Rhodes MW, Gallivan T, Erskine AJ, Leggett AT, Reece KS, 2011 High salinity relay as a postharvest processing strategy to reduce *Vibrio vulnificus* levels in Chesapeake Bay oysters (*Crassostrea virginica*). J Food Prot 74, 1902 – 1907. DOI: 10.4315/0362-028X.JFP-11-152

Baker-Austin C, Trinanes JA, Taylor NGH, Hartnell R, Siitonen A, Martinez-Urtaza J, 2013. Emerging *Vibrio* risk at high latitudes in response to ocean warming. Nat. Clim. Change 3, 73–77 DOI:10.1038/nclimate1628doi:10.1038/nclimate1628

Baker-Austin C, Gore A, Oliver JD, Rangdale R, McArthur JV, Lees DN, 2010. Rapid in situ detection of virulent *Vibrio vulnificus* strains in raw oyster matrices using real-time PCR. Environmental Microbiology Report. 2(1), 76-80. DOI: 10.1111/j.1758-2229.2009.00092

Baker-Austin C, Oliver JD, Alam M, Ali A, 2018. *Vibrio* species infections. Primer. DOI: 10.1038/s41572-018-0005-8

Baker-Austin C, Oliver JD, 2018. *Vibrio vulnificus*: new insights into a deadly opportunistic pathogen. Environ. Microbiol., 20, pp. 423-430. DOI: 10.1111/1462-2920.13955

Bej AK, Patterson DP, Brasher CW, Vickery MC, Jones DD, Kaysner CA, 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh*, *tdh*, and *trh*. J Microbiol Methods 36, 215-225.

Brock TD, 1999. Robert Koch. A life in medicine and bacteriology. ASM Press, Washington, D.C.

Centers for Disease Control and Prevention (CDC), 2005. *Vibrio* illnesses after Hurricane Katrina – multiple states, August-September 2005. Morb Mortal Wkly Rep, 54:928-931.

Center for Disease Control and Prevention (CDC), 2009. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food – 10 states, 2008. Morb Mortal Wkly Rep, 58(22), 333– 337.

Centers for Disease Control and Prevention (CDC), 2013. *Vibrio vulnificus*. Atlanta, GA, USA. Available online at: http://www.cdc.gov.libproxy.lib.unc.edu/vibrio/vibriov.html

Centers for Disease Control and Prevention (CDC), 2017. *Vibrio* species causing vibriosis. Atlanta, GA, USA. Available online at: https://www.cdc.gov/vibrio/index.html

Chatry M, Dugas R, Easley KA, 1983. Optimum salinity regime for oyster production on Louisiana's state seed grounds. Contrib Mar Sci 26:81–94.

CHROMagar. The Chromogenic Media Pioneer. Paris, France: Food & Water Q.C., 2009.

Church SR, Lux T, Baker-Austin C, Buddington SP, Michell SL, 2016. *Vibrio vulnificus* type 6 secretion system 1 contains anti-bacterial properties. PLoS ONE 11:e0165500. DOI:10.1371/journal.pone.0165500

Cole KM, Supan J, Ramirez A, Johnson CN, 2015. Suspension of oysters reduces the populations of *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Lett Appl Microbiol 61:209- 13. DOI: 10.1111/lam.12449.

Converse RR, Piehler MF, Noble RT, 2011. Contrasts in concentrations and loads of conventional and alternative indicators of fecal contamination in coastal stormwater. Water Research 45: 5229–5240. DOI: 10.1016/j.watres.2011.07.029

Deeb R, Tufford D, Scott GI, 2018. Impact of climate change on *Vibrio vulnificus* abundance and exposure risk. Estuaries and Coasts 41: 2289. DOI: 10.1007/s12237-018-0424-5

DePaola A, Nordstrom JL, Bowers JC, Wells JG, Cook DW, 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl. Environ. Microbiol. 69, 1521–1526. DOI :10.1128/AEM.69.3.1521-1526.2003

Eiler A, Gonzalez-Rey C, Allen S, Bertilsson S, 2007. Growth response of Vibrio cholera and other Vibrio spp. to cyanobacterial dissolved organic matter and temperature in brackish water. FEMS Micobiol Ecol 60:411-418. DOI: 10.1111/j.1574-6941.2007.00303.x.

Food and Agriculture (FAO) Fisheries Department, 2004. The state of world fisheries and aquaculture (SOFIA). Rome Italy: FAO.

Food and Drug Administration (FDA), 2006. Fresh and frozen seafood: selecting and serving it safely. Rockville, MD, USA. Available online at: http://www.fda.gov/Food/ResourcesForYou/Consumers/ucm077331.htm.

Fraser C, Hanage WP, Spratt BG, 2007. Recombination and the nature of bacterial speciation. Science 315, 476–480. DOI: 10.1126/science.1127573

Fries JS, Characklis GW, Noble RT, 2008. Sediment-water exchange of *Vibrio* spp. and fecal indicator bacteria: implications for persistence and transport in the Neuse River Estuary, North Carolina, U.S.A. Water Res 42:941–950. DOI: 10.1016/j.watres.2007.09.006

Froelich BA, Ayrapetyan M, Oliver JD, 2012. Integration of *Vibrio vulnificus* into marine aggregates and its subsequent uptake by *Crassostrea virginica* oysters. Appl Environ Microbiol 79, 1454–1458. DOI: 10.1128/AEM.03095-12

Froelich BA, Williams TC, Noble RT, Oliver JD, 2012. Apparent loss of *Vibrio vulnificus* from North Carolina oysters coincides with a drought induced increase in salinity. Appl Environ Microbiol 78:3885–3889. DOI: 10.1128/AEM.07855-11.

Froelich B, Bowen J, Gonzalez R, Snedeker A, Noble R, 2013. Mechanistic and statistical models of total *Vibrio* abundance in the Neuse River Estuary. Water Res 47:5783–5793. DOI: 10.1016/j.watres .2013.06.050.

Froelich BA, Noble RT, 2014 Factors affecting the uptake and retention of *Vibrio vulnificus* in oysters. Appl. Environ. Microbiol. 80, 7454– 7459. DOI: 10. 1128/AEM.02042-14

Froelich BA, Ayrapetyan M, Fowler P, Oliver JD, Noble RT, 2015. Development of a matrix tool for the prediction of *Vibrio* species in oysters harvested from NC. Appl Environ Microbiol 81, 1111-1119. DOI: 10.1128/AEM.03206-14

Froelich BA, Noble RT, 2016. *Vibrio* bacteria in raw oysters: managing risks to human health. Philos Trans R Soc Long Biol Sci, 371,1698. DOI: 10.1098/rstb.2015.0209

Froelich BA, Phippen B, Fowler P, Noble RT, Oliver JD, 2016. Differences in total *Vibrio* spp., *V. vulnificus*, and *V. parahaemolyticus* abundance between clams and oysters in NC. Appl Environ Microb. DOI: https://doi.org/10.1128/aem.02265-16

Go Deep Shellfish Aquaculture. Floating Bag Oyster Culture. http://godeepaquaculture.com/oysters/suspension-culture-in-floating-systems/

González-Escalona N, Martinez-Urtaza J, Romero J, Espejo RT, Jaykus LA, Depaola A, 2008. Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. J. Bacteriol. 190, 2831–2840. DOI: 10.1128/JB.01808-07

Gonzalez RA, Conn KE, Crosswell JR, Noble RT, 2012. Application of empirical predictive modeling using conventional and alternative fecal indicator bacteria in eastern NC waters. Water Res 46:5871– 5882. DOI: 10.1016/j.watres.2012.07.050

Grabowski JH, Peterson CH, 2007. Restoring oyster reefs to recover ecosystem services. Pages 281–298 In Cuddington K Byers J Wilson W Hastings A, eds. *Ecosystem Engineers: Plants to Protists*. Academic Press.

Grabowski JH, Brumbaugh RD, Conrad RF, Keeler AG, Opaluch JJ, Peterson CH, Piehler MF, Powers SP, Smyth AR, 2012. Economic valuation of ecosystem services provided by oyster reefs. BioScience 62 (10), 900−909. DOI: 10.1525/bio.2012.62.10.10

Haendiges J, Timme R, Allard MW, Myers RA, Brown EW, Gonzalez-Escalona N, 2015. Characterization of *Vibrio parahaemolyticus* clinical strains from Maryland (2012-2013) and comparisons to locally and globally diverse *V. parahaemolyticus* strains by whole genome sequences analysis. Front Microbiol 6 1-11. DOI: 10.3389/fmicb.2015.00125

Hasan NA, Grim CJ, Lipp EK, Rivera IN, Chun J, Haley BJ, *et al.,* 2015. Deep-sea hydrothermal vent bacteria related to human pathogenic *Vibrio* species. Proc Natl Acad SciUSA 112:E2813–E2819. DOI: 10.1073 /pnas.1503928112.

Henao OL, Jones TF, Vugia DJ, Griffin PM, Network FDAS, 2015. Foodborne diseases active surveillance network - 2 decades of achievements, 1996-2015. Emerging Infect Dis 21, 1529–1536. DOI: 10.3201/eid2109.150581

Heng SP, Letchumanan V, Deng CY, Ab Mutalib NS, Khan TM, Chuah LH, *et al*., 2017. *Vibrio vulnificus*: an environmental and clinical burden. Front Microbiol 8:997, DOI:10.3389/fmicb.2017.00997.

Hill WE, Keasler SP, Trucksess MW, Feng P, Kaysner CA, Lampel KA, 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Appl Environ Microbiol 57:707–711

Jesser KJ, Noble RT, 2018. *Vibrio* Ecology in the Neuse River Estuary, North Carolina, Characterized by Next-Generation Amplicon Sequencing of the Gene Encoding Heat Shock Protein 60 (hsp60). Appl Environ Microbiol 84(13). DOI: 10.1128/AEM.00333-18

Johnson CN, Flowers AR, Noriea NF, Zimmerman AM, Bowers JC, DePaola A, Grimes DJ, 2010. Relationships between environmental factors and pathogenic *vibrios* in the Northern Gulf of Mexico. Appl Environ Microbiol 76:7076 –7084. DOI: 10.1128/AEM.00697-10.

Johnson CN, Bowers JC, Griffitt KJ, Molina V, Clostio RW, Pei S, Laws E, Paranjpye RN, 2012. Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). Appl Environ Microbiol 78, 7249–7257. DOI: 10.1128/AEM.01296-12

Jones MK, Oliver JD, 2009. *Vibrio vulnificus*: disease and pathogenesis. Infect Immun 77:1723–33. DOI: 10.1128/IAI.01046-08

Kaneko T, Colwell RR, 1975. Adsorption of *Vibrio parahaemolyticus* onto chitin and copepods. Appl Microbiol 29(2):269–274

Kaspar CW, Tamplin ML, 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl Environ Microbiol 59**:**2425-2429. PMID: 8368832

Kaysner CA, DePaola AJ, 2004. BAM: *Vibrio*. Available online at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M, 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J Clin Microbiol 37: 1173–1177. PMID: 10074546

Levine WC, Griffin PM, 1993. *Vibrio* infections on the Gulf Coast: results of first year of regional surveillance. Gulf Coast *Vibrio* Working Group. J Infect Dis. 167:479–83. PMID: 8421186

Lin M, Payne DA, Schwarz JR, 2003 Intraspecific diversity of *Vibrio vulnificus* in Galveston Bay water and oysters as determined by randomly amplified polymorphic DNA PCR. Appl Environ Microbiol 69, 3170– 3175. DOI: 10.1128/AEM.69.6. 3170-3175.2003

Linkous DA, Oliver, JD, 1999. Pathogenesis of *Vibrio vulnificus*. FEMS Microbiol Lett 174: 207-214. DOI: 10.1111/j.1574-6968.1999.tb13570.x

Logan SL, Thomas J, Yan J, Baker RP, Shields DS, Xavier JB, Hammer BK, Parthasarathy R, 2018. The *Vibrio cholerae* type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. Proc Natl Acad. Sci.USA. DIO: 10.1073/pnas.1720133115

López-Pérez M, Jayakumar JM, Haro-Moreno JM, Zaragoza-Solas A, Reddi G, Rodriguez-Valera F, Shapiro OH, Alam M, Almagro-Moreno S, 2018. Evolutionary Model of Cluster Divergence of the Emergent Marine Pathogen *Vibrio vulnificus*: From Genotype to Ecotype. mBio, 10 (1) e02852-18; DOI: 10.1128/mBio.02852-18

Martinez-Urtaza J, Bowers JC, Trinanes J, DePaola A, 2010. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses. Food Res Int 43: 1780–1790. DOI: 10.1016/j.foodres.2010.04.001

Martinez-Urtaza J, Baker-Austin C, Jones JL, Newton AE, Gonzalez-Aviles GD, *et al*., 2013. Spread of Pacific Northwest *Vibrio parahaemolyticus* strain. The New England journal of medicine 369: 1573–1574.DOI: 10.1056/NEJMc1305535

Martinez-Urtaza JM, van Aerle R, Abanto M, Haendiges J, Myers RA, Trinanes J, Baker-Austin C, Gonzalez-Escalona N, 2017. Genomic variation and evolution of *Vibrio parahaemolyticus* ST36 over the course of a transcontinental epidemic expansion. mBio 8, e01425–17.DOI: 10.1128/mBio.01425-17

McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napolilli NP, Allison C, *et al*., 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. New England Journal of Medicine, 353, 1463–1470. DOI: 10.1056/NEJMoa051594

Mead PS, Slutsker L, Dietz V, McGaig LF, Bresee JS, Shapiro C, *et al*., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases, 5, 607–625.

Minor T, Lasher A, Klontz K, Brown B, Nardinelli C, Zorn D. The per case and total annual costs of foodborne illness in the United States. Risk Anal. 2015;35(6):1125–39. DOI: 10.1111/risa.12316

Motes ML, DePaola A, 1996 Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). App Environ Microbiol 62, 3875 – 3877.

Motes ML, DePaola A, Cook DW, Veazey JE, Hunsucker JC, Garthright WE, Blodgett RJ, Chirtel SJ, 1998 Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. 64, 1459– 1465

National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 2013. *Vibrio parahaemolyticus*. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc .gov/vibrio/vibriop.html.

National Oceanic and Atmospheric Administration (NOAA), National Centers for Environmental Information. Local Climatological Data (LCD), Data Tool. Accessed and ordered data sheet at: https://www.ncdc.noaa.gov/cdo-web/datatools/lcd

National Shellfish Sanitation Program (NSSP), 2017 Revision. Guide for the Control of Molluscan Shellfish. Available at: http://www.fda.gov/Food/GuidanceRegulation/FederalStateFoodPrograms/ucm2006754.htm

Newton AE, Garrett N, Stroika SG, Halpin JL, Turnsek M, Mody RK, 2014. Notes from the field: increase in *Vibrio parahaemolyticus* infections associated with consumption of Atlantic coast shellfish - 2013. MMWR Morb Mortal Wkly Rep 63, 335–336. PMID: 24739344

Nigro OD, Steward GF, 2015. Differential specificity of selective culture media for enumeration of pathogenic *vibrios*: advantages and limitations of multi-plating methods. J Microbiol Methods 111:24 – 30. DOI: 10.1016/j.mimet.2015.01.014

Nordstrom JL, Vickery MC, Blackstone GM, Murray SL, DePaola A, 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl Environ Microbiol 73: 5840–5847. DOI: 10.1128/AEM.00460-07

North Carolina Coast Federation (NC Coastal Fed.), August 22, 2018. Closures result of record rainfall in July. Coastal Review Online. Accessed at: https://www.coastalreview.org/2018/08/closures-result-of-record-rainfall-in-july/

North Carolina Division of Marine Fisheries (NCDMF), 2018. Polluted area proclamation archives. Accessed at: http://portal.ncdenr.org/web/mf/2018-polluted-area-proclamations

North Carolina Sea Grant (NC Sea Grant), 2018. NC Cultured Shellfish. Available online at: https://ncseagrant.ncsu.edu/aquaculture/north-carolina-cultured-shellfish/

North Carolina Shellfish Mariculture Advisory Committee (NCSMAC), 2018. North Carolina strategic plan for shellfish mariculture: a vision to 2030. Available online at: https://www.nccoast.org/wp-content/uploads/2019/01/NC-Strategic-Plan-for-Shellfish-Mariculture-Final-20181230.pdf

Oliver JD, 2005. Wound infections caused by *Vibrio vulnificus* and other marine bacteria. Epidemiol Infect 133:383–391. PMID: 15962544

Oliver JD, 2006. *Vibrio vulnificus*, p 349 –366. In Thompson FL, Austin B, Swings J (ed), The biology of *Vibrios*. ASM Press, Washington, DC.

Oliver JD, Warner RA, Cleland DR, 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting *vibrios* in the marine environment. Appl Environ Microb 45:985–98.

Paerl HW, Pinckney JL, Fear JM, Peierls BL, 1998.Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA. Mar Ecol Prog Ser 166:17–25

Panicker G, Bej AK, 2005. Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vvhA*. Appl Environ Microbiol 71:5702–5709. DOI: 10.1128 /AEM.71.10.5702-5709.2005

Paranjpye R, Hamel OS, Stojanovski A, Liermann M, 2012. Genetic diversity of clinical and environmental *Vibrio parahaemolyticus* strains from the Pacific Northwest. Appl Environ Microbiol 78:8631– 8638. DOI: 10.1128/AEM.01531-12

Pfeffer C, Oliver JD, 2003. A comparison of thiosulphate-citrate-bile salts-sucrose (TCBS) agar and thiosulphate-chloride-iodide (TCI) agar for the isolation of *Vibrio* species from estuarine environments. Lett Appl Microbiol 36: 150–151.

Pruzzo C, Huq A, Colwell RR, Donelli G, 2005. Pathogenic *Vibrio* species in the marine and estuarine environment. In: Belkin S, Colwell RR (eds). Ocean and Health Pathogens in the Marine Environment. Springer-Verlag: New York, pp 217–252.

Ralston EP, Powell HK, Beet A, 2011. An estimate of the cost of acute health effects from food- and water-borne marine pathogens and toxins in the USA. J. Water Health 680–694. DOI: 10.2166/wh.2011.157

Raszl SM, Froelich BA, Vieira CRW, Blackwood AD, Noble RT, 2016. *Vibrio parahaemolyticus* and *Vibrio vulnificus* in South America: water, seafood and human infections. J Appl Microbiol 121:1201-1222. DOI 10.1111/jam.13246

Reynolds-Fleming JV, Luettich RA, 2004. Wind-driven lateral variability in a partially mixed estuary. Estuar Coast Shelf Sci 60:395–407. DOI 10.1016/j.ecss.2004.02.003

Rhoads J, 2006. Post-Hurricane Katrina challenge: *Vibrio vulnificus*. J Am Acad Nurse Pract. 18:318-324. DOI: 10.1111/j.1745-7599.2006.00139.x

Rosche TM, Yano Y, Oliver JD, 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical and environmental isolation. Microbiol Immunol 49:381–389. DOI: 10.1111/j.1348-0421.2005.tb03731.x.

Rossner S, 2002. Obesity: the disease of the twenty-first century. Int J Obes Relat Metab Disord 26:Suppl 4:S2-S4. DOI: 10.1038/sj.ijo.0802209

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM, 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17:7–15. DOI: 10.3201/eid1701.P11101

Shaw KS, Jacobs JM, Crump, BC, 2014. Impact of Hurricane Irene on *Vibrio vulnificus* and *Vibrio parahaemolyticus* concentrations in surface water, sediment and cultured oysters in the Chesapeake Bay, Maryland, USA. Front Microbiol 5:204. DOI: 10.3389/fmicb.2014.00204

Shinoda S, Okamoto K, 1977. Formation and function of *Vibrio parahaemolyticus* lateral flagella. J Bacteriol 129:1266–1271.

Tan HJ, Liu SH, Oliver JD *et al*, 2010. Role of *RpoS* in the susceptibility of low salinity-adapted *Vibrio vulnificus* to environmental stresses. Int J Food Microbiol 137:137–42.

Tarr CL, Patel JS, Puhr ND, Sowers EG, Bopp CA, Strockbine NA, 2007. Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. J Clin Microbiol 45:134 –140. DOI: 10 .1128/JCM.01544-06.

Thompson JR, Polz MF, 2006. Dynamics of *Vibrio* populations and their role in environmental nutrient cycling. In: Thompson FL (ed.) The Biology of *Vibrios*. Washington, DC: American Society for Microbiology Press, 190–203

Thompson FL, Iida T, Swings J, 2004. Biodiversity of *Vibrios*. Microbiol Mol Biol Rev 68:403–431. DOI: 10.1128/MMBR.68.3.403-431.2004

Velazquez-Roman J, Leon-Sicairos N, Flores-Villasenor H et, 2012. Association of pandemic *Vibrio parahaemolyticus* O3:K6 present in the coastal environment of Northwest Mexico with cases of recurrent diarrhea between 2004 and 2010. Appl Environ Microb 78:1794–803.

Velazquez-Roman J, León-Sicairos N, de Jesus Hernández-Díaz L, Canizalez-Roman A, 2014. Pandemic *Vibrio parahaemolyticus* O3:K6 on the American continent. Front Cell Infect Microbiol 3:110. DOI: 10.3389/fcimb.2013.00110

Warner E, Oliver JD, 2007. Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. Appl Environ Microb 74:80–5. DOI: 10.1128/AEM.01434-07

Wetz J, Blackwood AD, Fries JS, Williams ZF, Noble RT, 2008. Trends in total *Vibrio* spp. and *Vibrio vulnificus* concentrations in the eutrophic Neuse River Estuary, NC, during storm events. Aquat Microb Ecol 53, 141–149. DOI: 10.3354/ame01223

Wolfe AJ, Millikan DS, Campbell JM, Visick KL, 2004. *Vibrio fischeri* sigma(54) controls motility, biofilm formation, luminescence, and colonization. Appl Environ Microbiol 70:2520–2524 DOI: 10.1128/AEM.70.4.2520-2524.2004

Wright AC, Simpson LM, Oliver JC, Morris JG, 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infect Immun 58:1769–1773. PMID: 2160432

Wu Y, Wen J, Ma Y, Ma XC, Chen Y, 2014. Epidemiology of foodborne disease outbreaks caused by *Vibrio parahaemolyticus*, China, 2003–2008. Food Control 46 197–202. DOI: 10.1016/j.foodcont.2014.05.023

Yamai, S, Okitsu, T, Katsube, Y, 1996. Isolation and incidence of *Vibrio cholerae* from river water. Kansenshogaku Zasshi 70, 1234–1241. DOI: 10.11150/kansenshogakuzasshi1970.70.1234

Yamamoto K, Wright AC, Kaper JB, Morris JG, 1990. The cytolysin gene of *Vibrio vulnificus*: sequence and relationship to the *Vibrio cholerae* E1 Tor hemolysin gene. Infect Immun 58(8):2706-9.

Yildiz FH, Visick KL, 2009. *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17:109 – 118. DOI: 10.1016/j.tim.2008.12.004

Zimmerman AM, Depaola A, Bowers JC, Krantz JA, Nordstrom JL, Johnson CN, Grimes DJ, 2007. Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Mexico water and oysters. Appl Environ Microbiol 73, 7589–7596. DOI: 10.1128/AEM.01700-07