doi: 10.1093/femsec/fix071

Different abundance and correlational patterns exist between total and presumed pathogenic *Vibrio vulnificus* and *V. parahaemolyticus* in shellfish and waters along the North Carolina coast

Tiffany C. Williams[1], Brett A. Froelich[2], Britney Phippen[1], Patricia Fowler[3], Rachel T. Noble[2] and James D. Oliver[1,*]

[1]The University of North Carolina at Charlotte, Department of Biological Sciences, Charlotte, NC 28223, USA, [2]The University of North Carolina at Chapel Hill, Institute of Marine Sciences, Morehead City, NC 28557, USA and [3]The North Carolina Department of Environment and Natural Resources, Division of Marine Fisheries, Morehead City, NC 28223, USA

*Corresponding author: The University of North Carolina at Charlotte, Department of Biological Sciences, 9201 University City Blvd., Charlotte, NC 28223, USA. Tel: +704-687-8516; E-mail: jdoliver@uncc.edu

One sentence summary: We tested environmentally isolated *Vibrio vulnificus* and *V. parahaemolyticus* isolates for the presence of virulence markers and found that pathogenic subpopulations do not necessarily reflect total species abundance and correlation patterns.

Editor: Julie Olson

ABSTRACT

Monitoring of *Vibrio vulnificus* and *V. parahaemolyticus* abundance is pertinent due to the ability of these species to cause disease in humans through aquatic vectors. Previously, we performed a multiyear investigation tracking *Vibrio* spp. levels in five sites along the southeastern North Carolina coast. From February 2013 to October 2015, total *V. vulnificus* and *V. parahaemolyticus* abundance was measured in water, oysters and clams. In the current study, pathogenic subpopulations were identified in these isolates using molecular markers, revealing that 5.3% of *V. vulnificus* isolates possessed the virulence-correlated gene (*vcgC*), and 1.9% of *V. parahaemolyticus* isolates harbored one or both of the virulence-associated hemolysin genes (*tdh* and *trh*). Total *V. parahaemolyticus* abundance was not sufficient to predict the abundance of pathogenic subpopulations. Specifically, pathogenic *V. parahaemolyticus* isolates were more often isolated in cooler waters and were sometimes isolated when no other *V. parahaemolyticus* strains were detectable. *Vibrio vulnificus* clinical (C-) genotypes correlated with total *V. vulnificus*; however, salinity, water depth and total suspended solids influenced C- and E-genotypes differently. Lastly, we documented individual oysters harboring significantly higher *V. vulnificus* levels for which there was no ecological explanation, a phenomenon that deserves closer attention due to the potentially elevated health hazard associated with these 'hot' shellfish.

Keywords: *Vibrio vulnificus*; *Vibrio parahaemolyticus*; North Carolina; oysters; shellfish; virulence markers

Received: 16 February 2017; **Accepted:** 18 May 2017

INTRODUCTION

Vibrios are Gram-negative halophilic bacteria that naturally inhabit estuarine and coastal waters across the globe. Consisting of over 100 species, these heterotrophic bacteria contribute to beneficial ecological processes such as carbon cycling and nutrient recycling (Thompson and Polz 2006; Hunt *et al*. 2008). However, at least a dozen *Vibrio* species have been described in human infections (Daniels and Shafaie 2000), and the annual incidence of *Vibrio* infections in the USA has been increasing (Crim *et al*. 2014). Filter-feeding mollusks concentrate microbes including *Vibrios* in their tissues, some of which become part of the normal microflora in these organisms. Consequently, shellfish such as clams and oysters, which are often consumed raw or undercooked by humans, represent a potential vehicle for foodborne pathogens. According to a recent report by the CDC, an estimated 80 000 people contract foodborne *Vibrio* infections each year in the USA, resulting in over 500 hospitalizations and 100 deaths, the great majority of which are due to *Vibrio vulnificus* and *V. parahaemolyticus* (CDC 2016). Furthermore, these two species are listed by the FDA as 2 (of 12) bacterial pathogens of greatest concern in seafood processing (FDA 2011). In addition to their impact on human health, the presence of these pathogens in shellfish creates a significant problem for the shellfish industry when an outbreak or incidence of infection occurs.

Vibrio vulnificus is responsible for 95% of all seafood-related deaths in the USA earning its name as the most deadly foodborne pathogen in this country (Hlady, Mullen and Hopkin 1993; Oliver 2006a,b; Bross *et al*. 2007). While most *V. vulnificus* cases occur following ingestion of raw or undercooked oysters, clam-associated infections have also been documented (CDC 1999; Slayton *et al*. 2014). Disease onset is often rapid (<24 h post-exposure) and is characterized by symptoms such as abdominal pain, fever, chills, nausea and vomiting, which can progress to severe septic shock, organ failure and the development of necrotizing bullous lesions on the extremities (Jones and Oliver 2009). The mortality rate associated with this pathogen has been reported to be as high as 50%, and survival is largely predicated upon the health of the patient, as immunocompromised individuals have a higher susceptibility for infection (CDC 1996; Oliver 2006a,b; Bross *et al*. 2007; Jones and Oliver 2009). Furthermore, *V. vulnificus* can cause wound infections if allowed to enter exposed cuts or insect bites. These wounds can potentially develop into severe necrotizing fasciitis, which requires extensive tissue debridement and potential amputation at the site of infection. Wound infections can also progress to septicemia and even death (ca. 25% mortality rate) in susceptible individuals (Jones and Oliver 2009).

Given the prevalence of *V. vulnificus* in shellfish and coastal waters, combined with the number of at-risk consumers, it has been predicted that infections should occur more often than is observed. This lower-than-expected incidence of disease is largely due to genetic differences between strains of this species. *Vibrio vulnificus* is grouped into three biotypes with biotype 1 strains being responsible for the majority of human infections. Within biotype 1 strains, genetic polymorphisms in the virulence-correlated gene (*vcg*) serve as a primary feature to distinguish strains of clinical (C-) genotypes from those of environmental (E-) genotypes (Warner and Oliver 1999, 2008b). C-genotypes are highly correlated with disease, with one study revealing that 93% of isolates from clinical cases were of this genotype (Warner and Oliver 1999, 2008a; Rosche, Yano and Oliver 2005); however, genotype is not always a predictor of virulence as E-genotypes are occasionally isolated from clinical cases as well (Thiaville *et al*. 2011; Bier *et al*. 2013).

While it has been consistently demonstrated that salinity and seasonality influence the diversity of *V. vulnificus* in the environment (Kaspar and Tamplin 1993; Motes *et al*. 1998; Arias *et al*. 1999; Randa, Polz and Lim 2004; Thompson and Polz 2006; Johnson *et al*. 2012; Froelich *et al*. 2015, 2016), the proportional abundance of C- and E-genotypes appears to be more complex. Along the Atlantic coastline of the USA, C- and E-genotypes have consistently exhibited an unexplained abundance disproportion within the same samples, with E-genotypes routinely outnumbering C-genotypes by more than 10-fold (Warner and Oliver 2008a; Baker-Austin *et al*. 2009; Bier *et al*. 2013; Froelich and Oliver 2013; Reynaud *et al*. 2014; Froelich *et al*. 2015). E-genotypes attach to chitin and marine aggregates more efficiently relative to C-genotypes, which may partially explain the predominance of E-genotypes within oysters, since incorporation into marine aggregates results in preferential uptake and retention by oysters feeding on these chitin-based conglomerates (Froelich, Ayrapetyan and Oliver 2013; Williams, Ayrapetyan and Oliver 2014, 2015). These studies offer insight into the observed population dynamics of C- and E-genotypes along the US Atlantic coast; however, a few studies have reported an opposite trend, such as Gonzalez *et al*. (2014), who found 68% of *V. vulnificus* isolates collected in mudflats along the Virginia coast to be C-genotype. Additionally, Yokochi *et al*. (2013) found C-genotypes to predominate (>85%) in three different sites along Japan's coastline. These differing studies underscore the need for a better understanding of the biotic and abiotic factors that influence the spatial and temporal distribution of C- and E-genotypes.

Vibrio parahaemolyticus is the leading cause of bacterial seafood poisoning in the USA and produces a variety of syndromes, of which gastroenteritis represents ca. 60%–80% of infections (Levine and Griffin 1993). Infection can result from the ingestion of raw or undercooked oysters, clams and mussels. Initial symptoms are similar to those of *V. vulnificus* and include diarrhea with abdominal cramps, nausea, vomiting, headache, chills and low grade fever (Yeung and Boor 2004). Infections are typically self-limiting; however in rare cases, fatal septicemia may occur in immunocompromised patients. Whereas *V. vulnificus* cases appear to be sporadic, *V. parahaemolyticus* has caused several major outbreaks of disease and has even become pandemic (Ceccarelli *et al*. 2013; Velazquez-Roman *et al*. 2013; Pazhani *et al*. 2014). Of special concern is the increasing number of *V. parahaemolyticus* outbreaks occurring in regions of the world with typically cooler water temperatures, which has been strongly linked to the trending increases in surface seawater temperatures as a result of climate change (Baker-Austin *et al*. 2010, 2013, 2017; Martinez-Urtaza *et al*. 2010; Vezzulli *et al*. 2016).

Virulence markers associated with disease-causing strains of *V. parahaemolyticus* include two hemolysin genes: thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) (FAO/WHO 2011). Pathogenic strains harbor one or both of these genes; however, it is important to note that other virulence factors such as the type III secretion system are possibly involved in the pathogenic mechanism of this bacterium, since roughly 10% of infections in the USA have been caused by strains that lack the virulence-associated hemolysin genes (Park *et al*. 2004; Jones *et al*. 2012; Ceccarelli *et al*. 2013).

Vibrio parahaemolyticus densities in shellfish and waters are strongly influenced by water temperature (DePaola *et al*. 2003; Zimmerman *et al*. 2007; Martinez-Urtaza *et al*. 2008; Parveen

Table 1. Summary data for *V. vulnificus* abundance by site.

	Date range	Average salinity (ppt)	Min/max salinity (ppt)	Total presumptive *V. vulnificus*	Total confirmed *V. vulnificus*	No. of V.v. E-types confirmed			No. of V.v. C-types confirmed		
						Oyster	Water	Clams	Oyster	Water	Clams
Harlowe Creek	2/4/13 – 10/16/15	24.8 ± 7.9	8/36	695	235 (33.8%)	106	48	53	11	8	1
South River	3/11/13– 9/11/15	16.8 ± 3.9	10/22	662	475 (71.8%)	369	84		6	7	
North River	2/28/13– 12/18/13	30.3 ± 4.8	21/37	124	24 (19.4%)	10	13	0	0	1	0
Hoop Pole Creek	2/14/13– 3/27/15	35.5 ± 2.8	28/40	190	14 (7.4%)	7	3	0	1	3	0
Jumping Run Creek	3/20/14– 11/10/14	34.0 ± 4.1	12/27	61	5 (8.2%)		2	1		0	0
Total	2/4/13– 10/16/15			1900	770 (40.5%)	499	156	54	19	21	1

et al. 2008; Johnson *et al.* 2010, 2012; Julie *et al.* 2010; Cruz, Hedderley and Fletcher 2015); however, there have been conflicting reports regarding the relationship between *V. parahaemolyticus* abundance and salinity. Some studies have identified a relationship between *V. parahaemolyticus* and salinity (Zimmerman *et al.* 2007; Martinez-Urtaza *et al.* 2008; Yu *et al.* 2013; Tey *et al.* 2015), whereas other have not (Deepanjali *et al.* 2005; Parveen *et al.* 2008; Kirs *et al.* 2011; Johnson *et al.* 2012; Cruz, Hedderley and Fletcher 2015; Froelich *et al.* 2015); thus, the role salinity plays in *V. parahaemolyticus* abundance is difficult to generalize. Furthermore, a number of studies on the abundance of total and potentially pathogenic *V. parahaemolyticus* abundance have found that pathogenic subpopulations are not proportional to the total *V. parahaemolyticus* population (Zimmerman *et al.* 2007; Julie *et al.* 2010; Johnson *et al.* 2012); thus, high total counts do not necessarily indicate that pathogenic isolates will more likely be present.

Despite the worldwide ubiquity of these two pathogens, abundance in the environment and shellfish varies across geographical locations and seasons (Motes *et al.* 1998; Zimmerman *et al.* 2007; Parveen *et al.* 2008; Warner and Oliver 2008a; Baker-Austin *et al.* 2010; Froelich *et al.* 2015, 2016). Furthermore, we recently reported that clam and oysters collected from the same site harbored substantially different levels of *V. vulnificus* and *V. parahaemolyticus* (Froelich *et al.* 2016), indicating that shellfish biology can influence resident *Vibrio* populations. Lastly, differences in pathogenic potential within strains of these two species raises the question as to whether simply assessing abundance of total populations is sufficient for risk assessment. The current study elaborates on a multiyear dataset in which we have continually documented the abundance and distribution of *V. vulnificus* and *V. parahaemolyticus* in five environmentally distinct sites along the N.C. coastline (Froelich *et al.* 2015, 2016). The goal of the current study was to assess *V. vulnificus* and *V. parahaemolyticus* isolates for virulence markers to determine if total *V. vulnificus* and *V. parahaemolyticus* reflect the same abundance and correlational patterns as their pathogenic subpopulations. The findings reported herein add to the growing assemblage of studies striving to tease apart the environmental factors that influence pathogenic *Vibrio* abundance. Collectively, these studies contribute relevant information to regulatory agencies, the shellfish industry and public health officials in order to identify high-risk environmental conditions.

METHODS

Sampling locations and environmental parameters

Oysters (*Crassostrea virginica*), clams (*Mercenaria mercenaria*) and water samples were collected from five sites along the eastern North Carolina coast (see Froelich *et al.* 2016 for map). These harvest sites were chosen to represent the range of high and low salinity environments, some of which experience large variations in salinity fluctuations, while others have a very small salinity fluxes (Tables 1 and 2). Environmental parameters were collected as previously described (Froelich *et al.* 2016). Briefly, at each collection event, water temperature was measured and salinity was measured with an HI 96 822 digital refractometer (Hanna Instruments, Carrollton, TX, USA). Depth of shellfish was measured by hand at the time of each collection. Dissolved oxygen (DO) was measured using an Orion 5 Star handheld probe (Thermo Scientific, Waltham, MA, USA), and pH measurements were taken with a Denver Instrument (Bohemia, NY, USA) UB-5 pH meter. To determine total suspended solids (TSS), water samples were vacuum filtered through a pre-dried, pre-weighed 25 mm glass microfiber filter (GE Life Sciences, Pittsburgh, PA, USA), with a minimum of 100 ml of water. Filters were oven-dried and then reweighed.

Sample collection and processing

Clams and oysters were collected routinely from February 2013 to October 2015. Detailed methods for sample collection and processing can be found in the study by Froelich *et al.* (2016). Briefly, at each sampling event, five oysters and/or five clams were collected, and were transported on ice and processed within 5 h of collection. Shellfish were aseptically shucked and the meats of all five samples were combined, weighed and diluted with sterile phosphate-buffered saline (PBS) at a 1:1 w:v ratio and blended in a paddle blender (Fisher Scientific, Waltham) for 10 min at 280 rpm. Diluted (1:10 in PBS) and undiluted homogenate were spread plated onto *Vibrio* selective media as described below.

Each time shellfish were collected, 1 l water samples were also collected and transported on ice along with shellfish. Water samples of 1 to 10 ml were vacuum-filtered through a 47-mm diameter, 0.45-μm pore-size mixed cellulose ester filter (Pall, Port Washington, NY) and filters were placed on selective media as described below.

Table 2. Summary data for *V. parahaemolyticus* abundance by site.

	Date range	Average salinity (ppt)	Min/max salinity (ppt)	Total presumptive *V.p.*	Total confirmed *V.p.*	*V.p.* confirmed		
						Oyster	Water	Clams
Harlowe Creek	2/4/13–10/16/15	24.8 ± 7.9	8/36	426	293 (68.8%)	129	109	55
South River	3/11/13–9/11/15	16.8 ± 3.9	10/22	221	100 (45.2%)	46	54	
North River	2/28/13–12/18/13	30.3 ± 4.8	21/37	112	57 (50.9%)	27	30	0
Hoop Pole Creek	2/14/13–3/27/15	35.5 ± 2.8	28/40	211	128 (60.7%)	60	67	1
Jumping Run Creek	3/20/14–11/10/14	34.0 ± 4.1	12/27	71	28 (39.4%)		14	14
Total	2/4/13–10/16/15			1093	640 (58.0%)	272	289	79

Isolation media and molecular confirmation of isolates

Shellfish homogenates and filtered water samples were plated onto CHROMagar *Vibrio* medium (CHROMagar, Paris, France) and incubated at 37°C for 24 h. This medium allows for the selective and differential growth of *Vibrio vulnificus* (dark blue colonies) and *V. parahaemolyticus* (dark purple colonies) along with other *Vibrio* species (CHROMagar 2009; Williams, Froelich and Oliver 2013) and is used regularly for the primary isolation of these two enteric pathogens (Froelich *et al.* 2012, 2015; Gonzalez *et al.* 2014; Shaw *et al.* 2014). After incubation, the number of presumptive *V. vulnificus* and *V. parahaemolyticus* colonies was documented, and 10–25 colonies of each presumptive species were further cultivated in heart infusion (HI) broth (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) for molecular confirmation. Pure cultures were boiled for 10 min followed by centrifugation at 10 000 × g for 10 min to separate aqueous DNA from cellular material. Supernatants, to be used for PCR templates, were stored at –20°C until examined. Using primers described by Tarr *et al.* (2007), presumptive *V. parahaemolyticus* isolates were confirmed using the pollar flagellin E (*flaE*) gene. Isolates that positively amplified the *flaE* gene were further typed for the virulence markers TDH and TRH using primers previously designed (Nishibuchi and Kaper 1985; Honda *et al.* 1991; Honda and Iida 1993). *Vibrio parahaemolyticus* strains SAK11 (*tdh+/trh–*), AQ4037 (*tdh–/trh+*) and F11–3A (*trh+/tdh+*) were used as positive controls and nuclease-free water was used as a negative control. Presumptive *V. vulnificus* isolates were confirmed based on the presence of the hemolysin/cytolysin gene (*vvhA*) using primers detailed by Warner and Oliver (2008b). Isolates that positively amplified *vvhA* were further genotyped using a multiplex PCR based on the identification of one of two alleles of the virulence-correlated gene (*vcg*), using primers and protocols previously published by Warner and Oliver (2008b), with slight modifications as previously described by Froelich *et al.* (2015). *Vibrio vulnificus* strains CMCP6 (*vcgC+*) and JY1305 (*vcgE+*) were used as positive controls.

To quantify *V. vulnificus* and *V. parahaemolyticus* abundance, two steps were taken. First, the number of presumptive colonies was adjusted to presumptive CFU per gram of shellfish tissue or CFU per milliliter of water (to account for dilutions and differences in oyster weight or volume of seawater filtered). Second, to account for the presence of false-positive isolates growing on CHROMagar *Vibrio*, CFU/g or CFU/ml values were multiplied by the percentage of isolates confirmed to be *V. vulnificus* or *V. parahaemolyticus*.

Correlations between *Vibrio* populations and environmental parameters were determined by calculating Spearman's rank correlation coefficient (Graphpad Prism software). Total *V. vulnificus*, *V. vulnificus* C-genotype and E-genotype subpopulations and total *V. parahaemolyticus* abundance values were tested against temperature, salinity, TSS and water depth. Resulting r-correlation coefficients and associated *P*-values are shown in Table 4. Note, correlational analyses were not performed on pathogenic *V. parahaemolyticus* subpopulations due to the low number of positive samples.

RESULTS AND DISCUSSION

Total and pathogenic *Vibrio parahaemolyticus* abundance and distribution

Of 1011 *V. parahaemolyticus* presumptive isolates, 57.6% were molecularly confirmed to be this species. A total of 11 isolates (1.9%) possessed one or both of the hemolytic virulence genes (*trh* and *tdh*) indicating the presence of potentially pathogenic strains (Fig. 1 and Table 2). This percentage is in agreement with a number of studies on pathogenic *V. parahaemolyticus* levels along the Atlantic coastline (Baker-Austin *et al.* 2008; Parveen *et al.* 2008) and across the globe (Robert-Pillot *et al.* 2004; Martinez-Urtaza *et al.* 2008; Kirs *et al.* 2011; Yu *et al.* 2013; Cruz, Hedderley and Fletcher 2015; Tey *et al.* 2015; Caburlotto *et al.* 2016), which have shown that pathogenic levels represent only a small fraction of the total *V. parahaemolyticus* in shellfish at the time of harvest. However, other studies have reported relatively high percentages of strains harboring virulence-associated *tdh/trh* genes (DePaola *et al.* 2003; Deepanjali *et al.* 2005; Johnson *et al.* 2012; Velazquez-Roman *et al.* 2012; West, Klein and Lovell 2013). Interestingly, a recent study of *V. parahaemolyticus* abundance at two sites along the South Carolina coast found approximately half of oyster isolates to harbor *tdh* and/or *trh* virulence-related genes (Klein and Lovell 2016).

Given the low number of virulent isolates detected in the current study, correlation analyses were not performed; however, Table 3 reveals that these isolates were collected from a range of sites at different times of the year (as early as March, and as late as November). This is in agreement with a study by Baker-Austin *et al.* (2008) in which no correlations between virulent strains and site, sample type or season of isolation were identified. While *V. parahaemolyticus* risk estimates assume that pathogenic levels can be reliably inferred from total *V. parahaemolyticus* levels (FAO/WHO 2011), a number of studies have

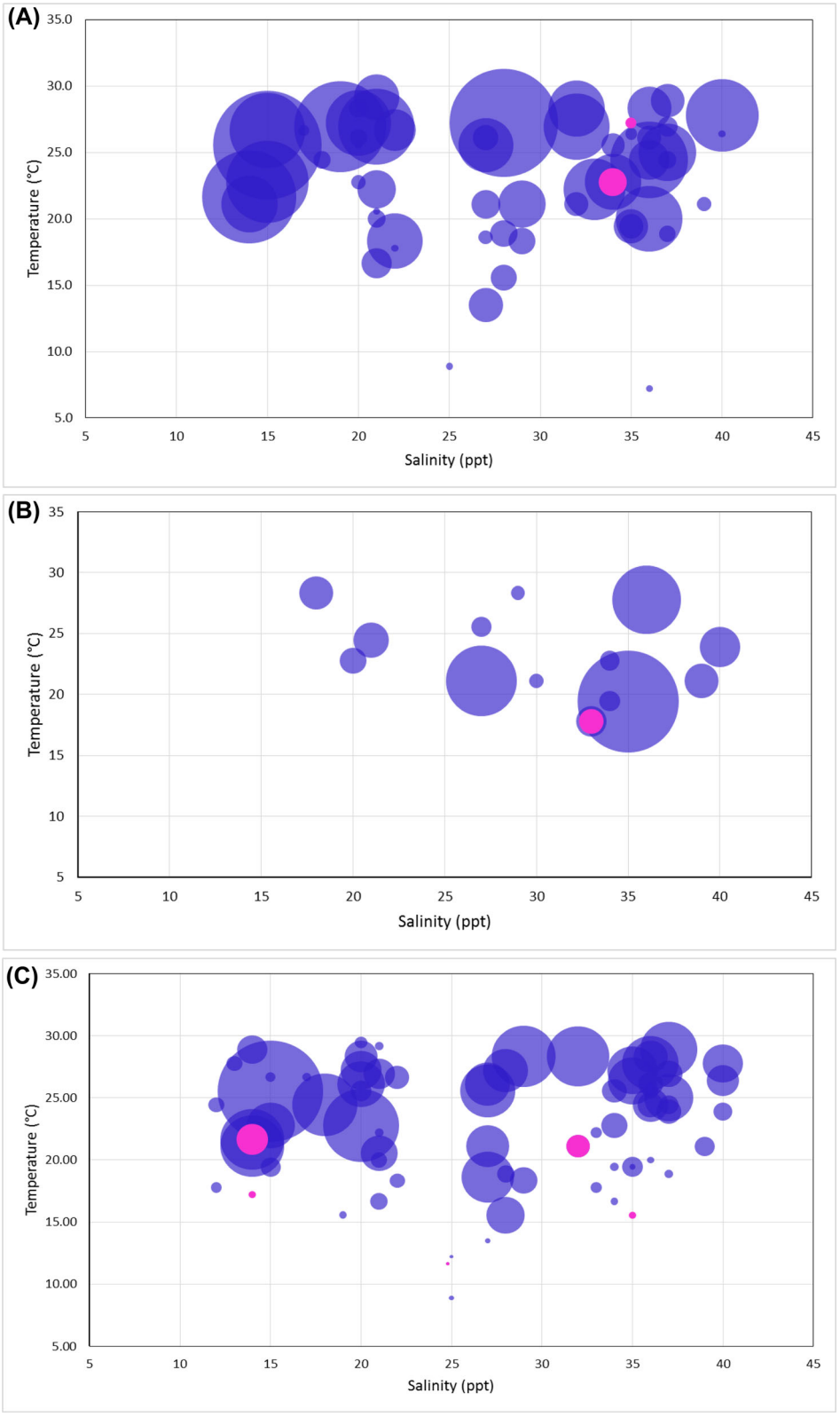

Figure 1. Bubble plots of total *V. parahaemolyticus* abundance (gray disks) and pathogenic *V. parahaemolyticus* abundance (black disks) in oysters (**A**), clams (**B**) and water (**C**). Each disk represents a single sampling event. Disk size is proportional to abundance and is plotted in relation to the temperature and salinity of the site at the time of sample collection. Raw *V. parahaemolyticus* data used to generate bubble plots can be found in Table S1 (Supporting Information).

Table 3. Environmental parameters associated with potentially pathogenic *V. parahaemolyticus* isolates.

Source of isolate	Date collected[a]	Site[b]	Salinity (ppt)	Water temp (°C)	Virulence type[c]
Oyster	5/30/2014	HC	34	22.8	*trh*+
	8/12/2014	HPC	35	27.0	*trh*/*tdh*+
Clam	4/25/2014	JNR	33	17.8	*tdh*+
	4/25/2014	JNR	33	17.8	*trh*+
Water	3/12/2014	HC	NA	12.0	*trh*/*tdh*+
	5/23/2014	SR	14	21.7	*trh*/*tdh*+
	10/16/2014	HPC	32	21.0	*trh*/*tdh*+
	11/10/2014	HPC	35	16.0	*trh*/*tdh*+
	11/10/2014	HPC	35	16.0	*trh*/*tdh*+
	3/27/2015	HC	14	17.2	*tdh*+
	3/27/2015	HC	14	17.2	*tdh*+

[a]Month/day/year.
[b]HC—Harlowe Creek, HPC—Hoop Pole Creek, JNR—Jumping Run River, SR—South River.
[c]*tdh*+ = strain positive for the thermostable direct hemolysin (TDH) gene, *trh*+ = strain positive for the TDH-related hemolysin (TRH) gene, *trh*/*tdh*+ = strain positive for both hemolysin genes.

Table 4. Correlations between *Vibrio* spp. and environmental parameters (Spearman's rank correlation coefficient, r).[a]

	Shellfish				Water			
	Total *V.v.*[b]	*V.v.* E+[c]	*V.v.* C+[d]	Total *V.p.*[e]	Total *V.v.*	*V.v.* E+	*V.v.* C+	Total *V.p.*
Temperature	0.389****	0.403****	0.288***	0.528****	0.440****	0.400****	0.307***	0.643****
Salinity	–0.448****	–0.438****	–0.215*	0.153	–0.443****	–0.462****	–0.121	0.058
Total suspended solids	0.294	0.300*	–0.156	0.255	0.415*	0.398*	0.073	0.391
Water depth	0.240**	0.231**	0.052	–0.146	0.148	0.180	–0.021	–0.232*

[a]*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
[b]V.v. = *Vibrio vulnificus*.
[c]V.v. E+ = *Vibrio vulnificus* E-genotype.
[d]V.v. C+ = *Vibrio vulnificus* C-genotype.
[e]V.p. = *Vibrio parahaemolyticus*.

found that pathogenic subpopulations are not necessarily proportional to the total *V. parahaemolyticus* population (McLaughlin *et al.* 2005; Zimmerman *et al.* 2007; Julie *et al.* 2010; Johnson *et al.* 2012). In the current study, 64% (7/11) of confirmed pathogenic *V. parahaemolyticus* isolates were obtained when no other *V. parahaemolyticus* isolates were present (Fig. 1). This indicates that total *V. parahaemolyticus* densities are not sufficient to predict the likelihood of infection. Indeed, this concern was exemplified by the 2005 *V. parahaemolyticus* outbreak in which Alaskan farmed oysters containing very low (<10 MPN/g) levels of *V. parahaemolyticus* harbored 100% *tdh*+ strains (McLaughlin *et al.* 2005).

Vibrio parahaemolyticus concentrations strongly correlated with temperature (Table 4) supporting the well-known seasonality of this bacterium, with the majority of shellfish isolates (87%) and water isolates (96.4%) being collected during the warm season (May through October). Interestingly, 27% of shellfish sampling events yielding no positive isolates came from warmer waters (≥20°C) suggesting that temperature is not the only environmental parameter driving *V. parahaemolyticus* abundance. Current risk estimates depend heavily on water temperature as the primary predictor of *V. parahaemolyticus* presence and abundance in oysters; however, whether pathogenic subpopulations follow this same trend is unclear (FAO/WHO 2011; Johnson *et al.* 2012). For example, DePaola *et al.* (2003) identified an inverse relationship between water temperature and the prevalence of pathogenic isolates. In the current study, 64% (7/11) of pathogenic *V. parahaemolyticus* isolates were collected during the cold season (November through April), when water temperatures were below 18°C. Again, this observed difference between total and virulent *V. parahaemolyticus* presence indicates that this subpopulation does not follow total *V. parahaemolyticus* seasonal trends.

Vibrio vulnificus C- and E-genotype abundance and distribution

Of 1621 presumptive isolates, 43.2% were determined to be *V. vulnificus* using molecular confirmation. Based on the virulence-correlated gene (*vcg*), 89.0% of *V. vulnificus* isolates were determined to be environmental (E-) genotypes, 5.3% were clinical (C-) genotypes and 5.7% were untypeable (i.e. did not amplify either allele). Thus, the current multiyear study shows that E-genotypes still predominate in oysters and waters relative to C-genotypes, with E-genotypes representing 89.7% and 86.2% of the total *V. vulnificus* in shellfish and waters, respectively (Fig. 2). Overall, there was a significant positive correlation between C- and E-genotypes ($r = 0.473$, $P < 0.0001$) suggesting a tight relationship between total *V. vulnificus* and pathogenic subpopulations, and this information may be useful for the development of future risk assessments.

Of the clam isolates collected, only 1 C-genotype was isolated and 77% of clam sampling events yielded no *V. vulnificus* (Fig. 2B). This highlights our previous finding that clams typically harbor much lower levels of *V. vulnificus* (Froelich *et al.* 2016) relative to oysters, and the current study reveals that C-genotypes are a minority in this mollusk, as is seen in oysters. Despite the low abundance of *V. vulnificus* isolated in clams reported here, there is increasing concern regarding the number of *Vibrio* infections

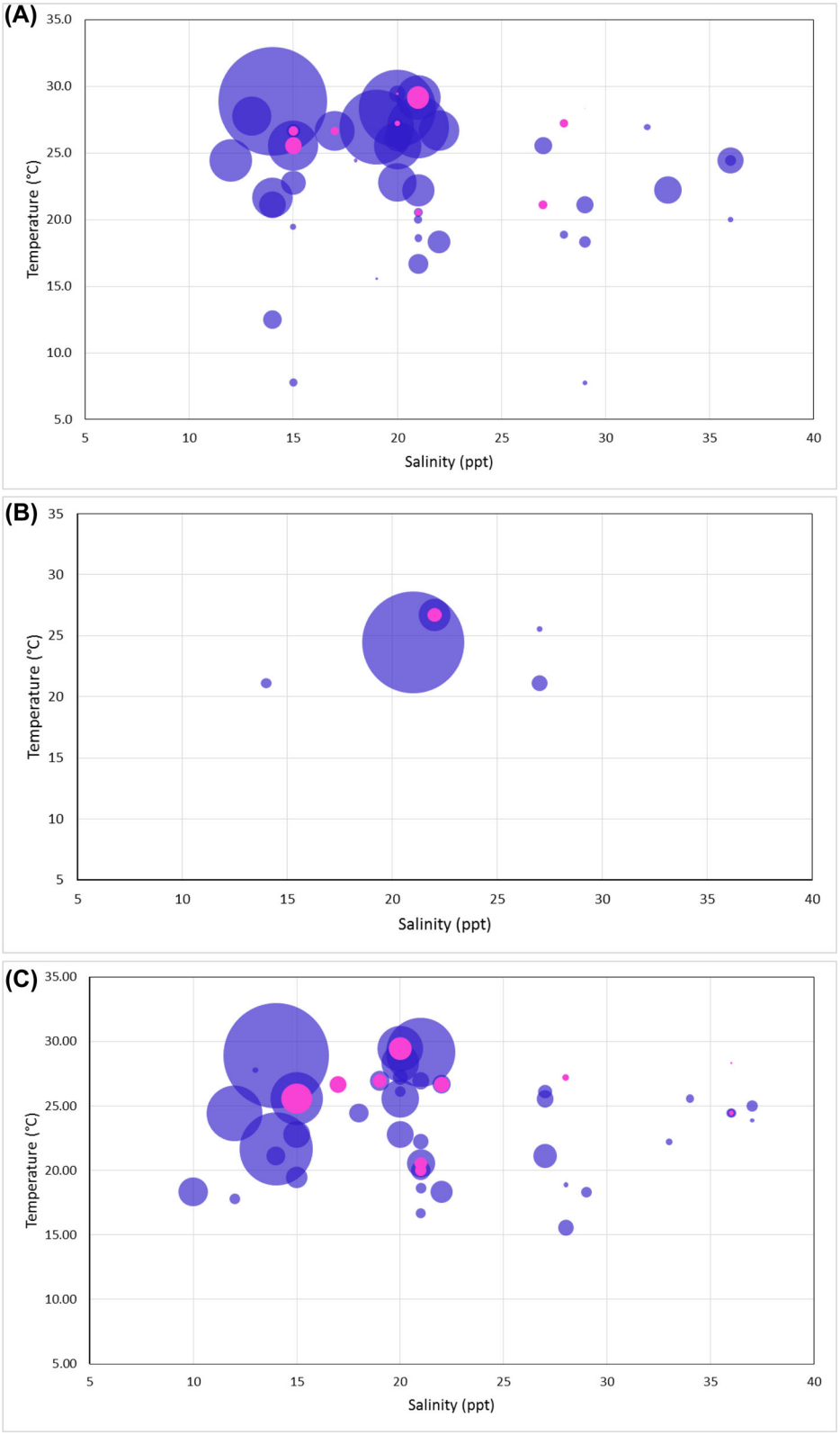

Figure 2. Bubble plots of *V. vulnificus* E-genotype abundance (gray disks) and C-genotype abundance (black disks) in oysters (**A**), clams (**B**) and water (**C**). Each disk represents a single sampling event. Disk size is proportional to abundance and is plotted in relation to the temperature and salinity of the site at the time of sample collection. Raw *V. vulnificus* data used to generate bubble plots can be found in Table S1 (Supporting Information).

that have been reported as a result of consuming raw or undercooked clams. Further studies on the abundance and prevalence of pathogenic *Vibrios* in clams are warranted, as has been previously advised to understand the level of risk associated with this bivalve (CDC 1999; Slayton *et al*. 2014; Froelich *et al*. 2016).

Vibrio vulnificus abundance is favored by warm water temperatures causing this organism to exhibit distinct seasonal variations. Throughout the duration of the study, water temperatures ranged from 4.4°C to 29.4°C (average 19.9°C) and both C-genotypes and E-genotypes exhibited a significant positive correlation with temperature (Table 4). As expected, the vast majority of isolates (over 95%) were collected between the months of May and October in both shellfish and the surrounding waters during which the average water temperature was 24.6°C and never dropped below 18.3°C (Fig. 2).

While *V. vulnificus* is capable of tolerating wide salinity ranges from 1 to 35 ppt (Kaspar and Tamplin 1993; Tan *et al*. 2010), this bacterium typically exhibits an optimal salinity range of 15–25ppt (Oliver, Warner and Cleland 1983; Kaspar and Tamplin 1993; Motes *et al*. 1998; Arias *et al*. 1999; Randa, Polz and Lim 2004; Froelich *et al*. 2012). As anticipated, high salinity had a significant negative effect on *V. vulnificus* densities; however, this effect was more prominent in E-genotypes (Table 4). Regardless of isolation source (shellfish or water), E-genotypes exhibited a strong inverse relationship with salinity (Table 4). Conversely, C-genotypes in shellfish correlated with salinity, whereas C-genotypes isolated from water did not. Thus, while salinity may drive C- and E-genotype abundance within shellfish, this bottom–up control does not appear to strongly influence C-genotypes suspended in the water column. Interestingly, nearly half (48.6%) of the C-genotypes isolates were obtained from water samples, whereas the majority (79.9%) of E-genotypes were isolated from shellfish. Of the confirmed C-genotypes, 54% were isolated from Harlowe Creek, a site with a larger salinity range relative to the other sites (Table 1), whereas the majority of E-genotypes were isolated from the South River (a site with a consistently lower salinity). Furthermore, the majority of E-genotypes (72%) were isolated when water salinity was <20 ppt, whereas 57% of C-genotypes were isolated when the water salinity was >20 ppt. These findings suggest that C-genotypes may have a higher salinity optimum relative to E-genotypes and laboratory-based studies comparing the salinity growth range, growth optimum and halotolerance of these two genotypes could potentially substantiate this claim.

DO and pH did not appear to influence *V. vulnificus* C- or E-genotype abundance in shellfish or water. However, E-genotype abundance exhibited a weak but significant positive correlation with water depth (Table 4). Although water depth is a proxy measurement, it is tempting to speculate that this correlation exists due to differences between intertidal oysters and subtidal oysters. Samples were collected from waters ranging from 0 feet (exposed intertidal oysters) to 7 feet in depth (permanently submerged subtidal oysters). Subtidal oysters submerged throughout the day are privy to longer feeding periods providing greater exposure to *Vibrios* in the water column. At low tide, intertidal oysters experience enhanced stress due to heating from direct sunlight and an inability to feed or depurate during this period of exposure (Zhang *et al*. 2016), and this change in oyster physiology could negatively influence *V. vulnificus* abundance. While further studies would be needed to substantiate this hypothesis, a recent study by Jones *et al*. (2016) found that intertidal oysters re-immersed by the incoming tide depurated *V. vulnificus* to >1 log MPN/g below the initial levels.

Vibrio vulnificus E-genotype abundance also exhibited a significant positive correlation with TSS in both shellfish and water, whereas this relationship was not observed with C-genotype abundance. TSS is a measure of particulates (i.e. plankton, silt, wastes) suspended in the water column, and is related to turbidity. Some environmental investigations have identified a relationship between suspended particulate matter and *Vibrio* spp. abundance (Zimmerman *et al*. 2007; Julie *et al*. 2010; Johnson *et al*. 2012), and a number of studies have established the importance of plankton on the ecology of *Vibrios* (Pruzzo, Vezzulli and Colwell 2008; Turner *et al*. 2009; Johnson *et al*. 2010; Rehnstam-Holm *et al*. 2010; Asplund *et al*. 2011; Martinez-Urtaza *et al*. 2012). Moreover, we have previously demonstrated the higher propensity of E-genotypes to attachment to chitin and marine aggregates relative to C-genotypes (Froelich, Ayrapetyan and Oliver 2013; Williams, Ayrapetyan and Oliver 2014, 2015). The correlational patterns observed between E-genotype abundance and water depth and TSS could be insightful in discerning the niche preferences of C- and E-genotypes, and therefore deserves further investigation.

Sporadic *Vibrio vulnificus* abundance in shellfish

Throughout the duration of this 33-month study, the average *V. vulnificus* abundance was 5.1e2 CFU/g and one oyster sampling period (South River in June 2015) resulted in considerably high *V. vulnificus* levels (1.2e4 CFU/g) relative to all other sampling events, with the second highest concentration being nearly 2-fold lower (6.2e3 CFU/g, South River, August 2016). Additionally, with the exception of one clam sampling event (Harlowe Creek, October 2016) which yielded 6.1e3 CFU/g *V. vulnificus*, all clams sampled harbored < 600 CFU/g *V. vulnificus,* with 77% of samples having undetectable levels. Given our method of sample pooling, we cannot ascertain whether all of the shellfish tested on the aforementioned days contained high levels of *V. vulnificus,* or if this spike in numbers was attributable to a single mollusk. Previous studies have documented similar events in which individual oysters harbored substantially high levels of *V. parahaemolyticus* relative to neighboring oysters (Kaufman *et al*. 2003; Klein and Lovell 2016). These 'hot' shellfish signify an important concern for shellfish safety management as they may be the cause of sporadic *Vibrio* spp. infections.

The environmental and/or physiological explanations for the observed sporadic increase in *V. vulnificus* levels within shellfish are unclear and should be monitored more closely in future studies. Interestingly, our recent *in vitro* studies found reproductively ripe female clams to retain up to 2.6e4 CFU/g *V. vulnificus* relative to clams not actively producing gametes, which typically retained ca. 800 CFU/g (B.L. Phippen and J.D. Oliver, unpublished). Our findings suggest that the reproductive stage of shellfish may influence *V. vulnificus* abundance which is supported by De Decker *et al*. (2011), who found reproductively mature oysters to be more susceptible to vibriosis by two oyster pathogens. While it is commonly assumed that *V. vulnificus* is a commensal bacterium in shellfish such as clams and oysters, it may be that *Vibrios* tend to have a high affinity for gonadic tissues regardless of whether colonization results in vibriosis. Contrarily, it is quite possible that commensal *Vibrio*–shellfish relationships can turn pathogenic based on the physiological status of the host (indeed, this is the opportunistic nature of *V. vulnificus* in the human host). Spawning is energetically expensive and studies have shown that this process compromises the oyster immune system increasing susceptibility to pathogens (Li *et al*. 2009); thus, our lab is currently in the process of further investigating

the relationship between shellfish gametogenesis and *Vibrio* spp. colonization, with a particular focus on *V. vulnificus*.

The results of this multiyear study revealed that the abundance and correlations of pathogenic subpopulations did not always reflect that of total *Vibrio* spp., a finding that is of clinical relevance. Furthermore, the observed transient spikes in *V. vulnificus* abundance within shellfish presents a concern for the shellfish industry as these 'hot' shellfish could be the source for sporadic outbreaks of disease. While long-term monitoring of *Vibrio* spp. abundance is critical to understand how temporal environmental fluctuations influence *Vibrio* populations, short-term studies with intense sampling periods could provide greater resolution to our understanding of *Vibrio* dynamics in the environment.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGEMENTS

We thank everyone at the North Carolina Division of Marine Fisheries, including Shannon Jenkins, Paul Moore, Mike Millard, Timmy Moore, Phil Piner and Steve Murphy, for assistance in collecting oysters and for scientific input. We would like to thank Leigh Robertson for help with molecular testing and Justin Hart and Simone Razal for oyster processing assistance.

FUNDING

This work was funded by the Saltonstall-Kennedy Grant Program, National Oceanic and Atmospheric Administration (NOAA) Fisheries, Grant No. NA14NMF4270041 and by an Agriculture and Food Research Initiative Competitive Grant (No. 11352692) from the United States Department of Agriculture (USDA) National Institute of Food and Agriculture.

Conflict of interest. None declare.

REFERENCES

Arias CR, Macián MC, Aznar R *et al.* Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. *J Appl Microbiol* 1999;**86**:125–34.

Asplund ME, Rehnstam-Holm A-S, Atnur V *et al.* Water column dynamics of *Vibrio* in relation to phytoplankton community composition and environmental conditions in a tropical coastal area. *Environ Microbiol* 2011;**13**:2738–51.

Baker-Austin C, McArthur JV, Lindell AH *et al.* Multi-site analysis reveals widespread antibiotic resistance in the marine pathogen *Vibrio vulnificus*. *Microb Ecol* 2009;**57**:151–9.

Baker-Austin C, McArthur JV, Tuckfield RC *et al.* Antibiotic resistance in the shellfish pathogen *Vibrio parahaemolyticus* isolated from the coastal water and sediment of Georgia and South Carolina, USA. *J Food Protect* 2008;**71**:2552–8.

Baker-Austin C, Stockley L, Rangdale R *et al.* Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. *Environ Microbiol Rep* 2010;**2**:7–18.

Baker-Austin C, Trinanes J, Gonzalez-Escalona N *et al.* Non-Cholera *Vibrios*: The microbial barometer of climate change. *Trends Microbiol* 2017. DOI 10.1016/j.tim.2016.09.008.

Baker-Austin C, Trinanes JA, Taylor NGH *et al.* Emerging *Vibrio* risk at high latitudes in response to ocean warming. *Nat Clim Change* 2013;**3**:73–7.

Bier N, Bechlars S, Diescher S *et al.* Genotypic diversity and virulence characteristics of clinical and environmental *Vibrio vulnificus* isolates from the Baltic Sea region. *Appl Environ Microb* 2013;**79**:3570–81.

Bross MH, Soch K, Morales R *et al.* *Vibrio vulnificus* infection: diagnosis and treatment. *Am Fam Physician* 2007;**76**:539–44.

Caburlotto G, Suffredini E, Toson M *et al.* Occurrence and molecular characterisation of *Vibrio parahaemolyticus* in crustaceans commercialised in Venice area, Italy. *Int J Food Microbiol* 2016;**220**:39–49.

CDC. *Vibrio vulnificus* infections associated with eating raw oysters – Los Angeles, 1996.*MMWR-Morbid Mortal W* 1996;**45**:621–4.

CDC. Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound–Connecticut, New Jersey, and New York, 1998. *MMWR Morbid Mortal W* 1999;**48**:48–51.

CDC. *FoodNet 2015 Annual Report. Foodborne Diseases Active Surveillance Network*. Atlanta, GA: Center for Disease Control and Prevention, 2016.

Ceccarelli D, Hasan NA, Huq A *et al.* Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Front Cell Infect Microbiol* 2013;**3**:97.

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

Crim SM, Iwamoto M, Huang JY *et al.* Incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 U.S. Sites, 2006–2013. *MMWR Morbid Mortal W* 2014;**63**:382–32.

Cruz CD, Hedderley D, Fletcher GC. Long-term study of *Vibrio parahaemolyticus* prevalence and distribution in New Zealand shellfish. *Appl Environ Microb* 2015;**81**:2320–7.

Daniels NA, Shafaie M. A review of pathogenic *Vibrio* infections for clinicians. *Infect Med* 2000;**17**:665–85.

De Decker S, Normand J, Saulnier D *et al.* Responses of diploid and triploid Pacific oysters *Crassostrea gigas* to *Vibrio* infection in relation to their reproductive status. *J Invertebr Pathol* 2011;**106**:179–91.

Deepanjali A, Kumar HS, Karunasagar I *et al.* seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. *Appl Environ Microb* 2005;**71**:3575–80.

DePaola A, Nordstrom JL, Bowers JC *et al.* Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Appl Environ Microb* 2003;**69**:1521–6.

FAO/WHO. Risk assessment of *Vibrio parahaemolyticus* in seafood: Interpretative summary and Technical report. *Microbiological Risk Assessment Series No 16*. Rome, 2011, 193.

FDA. *Fish and Fishery Products Hazards and Controls Guidance APPENDIX 7: Bacterial and Viral Pathogens of Greatest Concern in Seafood Processing - Public Health Impacts*. Spring, MD: Food and Drug Administration, 2011.

Froelich B, Ayrapetyan M, Oliver JD. Integration of *Vibrio vulnificus* into marine aggregates and its subsequent uptake by *Crassostrea virginica* oysters. *Appl Environ Microb* 2013;**79**:1454–8.

Froelich B, Oliver JD. The interactions of *Vibrio vulnificus* and the oyster *Crassostrea virginica*. *Microb Ecol* 2013;**65**:807–16.

Froelich BA, Ayrapetyan M, Fowler P *et al.* Development of a matrix tool for the prediction of *Vibrio* species in oysters harvested from North Carolina. *Appl Environ Microb* 2015;**81**:1111–9.

Froelich BA, Phippen B, Fowler P *et al*. Differences in total *Vibrio* spp., *V. vulnificus*, and *V. parahaemolyticus* abundance between clams and oysters in North Carolina. *Appl Environ Microb* 2016, DOI 10.1128/aem.02265-16.

Froelich BA, Williams TC, Noble RT *et al*. Apparent loss of *Vibrio vulnificus* from North carolina oysters coincides with a drought-induced increase in salinity. *Appl Environ Microb* 2012;**78**:3885–9.

Gonzalez DJ, Gonzalez RA, Froelich BA *et al*. Non-native macroalga may increase concentrations of *Vibrio* bacteria on intertidal mudflats. *Mar Ecol Prog Ser* 2014;**505**:29–36.

Hlady WG, Mullen RC, Hopkin RS. *Vibrio vulnificus* from raw oysters. Leading cause of reported deaths from foodborne illness in Florida. *J Fla Med Assoc* 1993;**80**:536–8.

Honda T, Abad-Lapuebla MA, Ni YX *et al*. Characterization of a new thermostable direct haemolysin produced by a Kanagawa-phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*. *J Gen Microbiol* 1991;**137**:253–9.

Honda T, Iida T. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. *Rev Med Microbiol* 1993;**4**:106–13.

Hunt DE, Gevers D, Vahora NM *et al*. Conservation of the chitin utilization pathway in the *Vibrionaceae*. *Appl Environ Microb* 2008;**74**:44–51.

Johnson CN, Bowers JC, Griffitt KJ *et al*. Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). *Appl Environ Microb* 2012;**78**:7249–57.

Johnson CN, Flowers AR, Noriea NF *et al*. Relationships between environmental factors and pathogenic *Vibrios* in the Northern Gulf of Mexico. *Appl Environ Microb* 2010;**76**:7076–84.

Jones JL, Kinsey TP, Johnson LW *et al*. Effects of intertidal harvest practices on *Vibrio parahaemolyticus* and *Vibrio vulnificus* Levels in Oysters. *Appl Environ Microb* 2016, DOI 10.1128/aem.00721-16.

Jones JL, Lüdeke CHM, Bowers JC *et al*. Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. *J Clin Microbiol* 2012;**50**:2343–52.

Jones MK, Oliver JD. *Vibrio vulnificus*: disease and pathogenesis. *Infect Immun* 2009;**77**:1723–33.

Julie D, Solen L, Antoine V *et al*. Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. *Environ Microbiol* 2010;**12**:929–37.

Kaspar CW, Tamplin ML. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Appl Environ Microb* 1993;**59**:2425–9.

Kaufman GE, Bej AK, Bowers J *et al*. Oyster-to-oyster variability in levels of *Vibrio parahaemolyticus*. *J Food Protect* 2003;**66**:125–9.

Kirs M, DePaola A, Fyfe R *et al*. A survey of oysters (Crassostrea gigas) in New Zealand for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Int J Food Microbiol* 2011;**147**:149–53.

Klein S, Lovell CR. The hot oyster: levels of virulent *Vibrio parahaemolyticus* strains in individual oysters. *FEMS Microbiol Ecol* 2016, DOI 10.1093/femsec/fiw232.

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

Li Y, Qin JG, Li X *et al*. Spawning-dependent stress responses in pacific oysters *Crassostrea gigas*: A simulated bacterial challenge in oysters. *Aquaculture* 2009;**293**:164–71.

McLaughlin JB, DePaola A, Bopp CA *et al*. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. *N Engl J Med* 2005;**353**:1463–70.

Martinez-Urtaza J, Blanco-Abad V, Rodriguez-Castro A *et al*. Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus* in offshore areas. *ISME J* 2012;**6**:994–1006.

Martinez-Urtaza J, Bowers JC, Trinanes J *et al*. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses. *Food Res Int* 2010;**43**:1780–90.

Martinez-Urtaza J, Lozano-Leon A, Varela-Pet J *et al*. Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the Rias of Galicia, Spain. *Appl Environ Microb* 2008;**74**:265–74.

Motes ML, DePaola A, Cook DW *et al*. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (Crassostrea virginica). *Appl Environ Microb* 1998;**64**:1459–65.

Nishibuchi M, Kaper JB. Nucleotide sequence of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus*. *J. Bacteriol* 1985;**162**:558–64.

Oliver JD. *Vibrio vulnificus*. In: Belkin S, Colwell RR (eds.) *Oceans and Health: Pathogens in the Marine Environment*. New York: Springer Science, 2006a, 253–76.

Oliver JD. *Vibrio vulnificus*. In: Thompson FL, Austin B, Swings J (eds.) *The Biology of Vibrios*. Washington, DC: American Society for Microbiology, 2006b, 349–66.

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

Park KS, Ono T, Rokuda M *et al*. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infect Immun* 2004;**72**:6659–65.

Parveen S, Hettiarachchi KA, Bowers JC *et al*. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *Int J Food Microbiol* 2008;**128**:354–61.

Pazhani GP, Bhowmik SK, Ghosh S *et al*. Trends in the epidemiology of pandemic and non-pandemic strains of *Vibrio parahaemolyticus* isolated from diarrheal patients in Kolkata, India. *PLoS Neglect Trop D* 2014;**8**:e2815.

Pruzzo C, Vezzulli L, Colwell RR. Global impact of *Vibrio cholerae* interactions with chitin. *Environ Microbiol* 2008;**10**:1400–10.

Randa MA, Polz MF, Lim E. Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. *Appl Environ Microb* 2004;**70**:5469–76.

Rehnstam-Holm A-S, Godhe A, Harnstrom K *et al*. Association between phytoplankton and *Vibrio* spp. along the southwest coast of India: a mesocosm experiment. *Aquat Microb Ecol* 2010;**58**:127–39.

Reynaud Y, Pitchford S, De Decker S *et al*. Molecular typing of environmental and clinical strains of *Vibrio vulnificus* isolated in the Northeastern USA. *PLoS One* 2014;**8**:e83357.

Robert-Pillot A, Guenole A, Lesne J *et al*. Occurrence of the tdh and trh genes in Vibrio parahaemolyticus isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. *Int J Food Microbiol* 2004;**91**:319–25.

Rosche TM, Yano Y, Oliver JD. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol Immunol* 2005;**49**:381–9.

Shaw KS, Rosenberg Goldstein RE, He X *et al*. Antimicrobial susceptibility of *Vibrio vulnificus* and vibrio parahaemolyticus recovered from recreational and commercial areas of chesapeake Bay and Maryland Coastal Bays. *PLoS One* 2014;**9**:e89616.

Slayton RB, Newton AE, Depaola A *et al*. Clam-associated vibriosis, USA, 1988–2010. *Epidemiol Infect* 2014;**142**:1083–8.

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

Tarr CL, Patel JS, Puhr ND *et al*. Identification of vibrio isolates by a multiplex PCR assay and rpoB sequence determination. *J Clin Microbiol* 2007;**45**:134–40.

Tey YH, Jong KJ, Fen SY *et al*. Occurrence of Vibrio parahaemolyticus, Vibrio cholerae, and *Vibrio vulnificus* in the aquacultural environments of Taiwan. *J Food Protect* 2015;**78**:969–76.

Thiaville PC, Bourdage KL, Wright AC *et al*. Genotype is correlated with but does not predict virulence of *Vibrio vulnificus* biotype 1 in subcutaneously inoculated, iron dextran-treated mice. *Infect Immun* 2011;**79**:1194–207.

Thompson JR, Polz MF. 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, 2006, 190–203.

Turner JW, Good B, Cole D *et al*. Plankton composition and environmental factors contribute to Vibrio seasonality. *ISME J* 2009;**3**:1082–92.

Velazquez-Roman J, León-Sicairos N, de Jesus Hernández-Díaz L *et al*. Pandemic Vibrio parahaemolyticus O3:K6 on the American continent. *Front Cell Infect Microbiol* 2013;**3**:110.

Velazquez-Roman J, Leon-Sicairos N, Flores-Villasenor H *et al*. 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* 2012;**78**:1794–803.

Vezzulli L, Grande C, Reid PC *et al*. Climate influence on Vibrio and associated human diseases during the past half-century in the coastal North Atlantic. *P Natl Acad Sci USA* 2016;**113**:E5062–71.

Warner E, Oliver JD. Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Appl Environ Microb* 2008a;**74**:80–5.

Warner E, Oliver JD. Multiplex PCR assay for detection and simultaneous differentiation of genotypes of *Vibrio vulnificus* biotype 1. *Foodborne Pathog Dis* 2008b;**5**:691–3.

Warner JM, Oliver JD. Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other vibrio species. *Appl Environ Microb* 1999;**65**:1141–4.

West CKG, Klein SL, Lovell CR. The virulence factor genes tdh, trh and tlh occur at high frequency in V. parahaemolyticus isolated from a pristine estuary. *Appl Environ Microb* 2013, DOI 10.1128/aem.03792-12.

Williams TC, Ayrapetyan M, Oliver JD. Implications of chitin attachment for the environmental persistence and clinical nature of the human pathogen *Vibrio vulnificus*. *Appl Environ Microb* 2014;**80**:1580–7.

Williams TC, Ayrapetyan M, Oliver JD. Molecular and physical factors that influence attachment of Vibrio vulnificus to Chitin. *Appl Environ Microb* 2015;**81**:6158–65.

Williams TC, Froelich B, Oliver JD. A new culture-based method for the improved identification of Vibrio vulnificus from environmental samples, reducing the need for molecular confirmation. *J Microbiol Methods* 2013;**93**:277–83.

Yeung PS, Boor KJ. Epidemiology, pathogenesis, and prevention of foodborne Vibrio parahaemolyticus infections. *Foodborne Pathog Dis* 2004;**1**:74–88.

Yokochi N, Tanaka S, Matsumoto K *et al*. Distribution of Virulence Markers among *Vibrio vulnificus* isolates of clinical and environmental origin and regional characteristics in Japan. *PLoS One* 2013;**8**:e55219.

Yu WT, Jong KJ, Lin YR *et al*. Prevalence of Vibrio parahaemolyticus in oyster and clam culturing environments in Taiwan. *Int J Food Microbiol* 2013;**160**:185–92.

Zhang G, Li L, Meng J *et al*. Molecular basis for adaptation of oysters to stressful marine intertidal environments. *Annu Rev Anim Biosci* 2016;**4**:357–81.

Zimmerman AM, DePaola A, Bowers JC *et al*. Variability of total and pathogenic vibrio parahaemolyticus densities in Northern Gulf of Mexico water and Oysters. *Appl Environ Microb* 2007;**73**:7589–96.