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The Ecology of *Vibrio vulnificus, Vibrio cholerae*, and *Vibrio parahaemolyticus* in North Carolina Estuaries

Karen Dyer Blackwell and James D. Oliver*

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223, USA

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While numerous studies have characterized the distribution and/or ecology of various pathogenic *Vibrio* spp., here we have simultaneously examined several estuarine sites for *Vibrio vulnificus, V. cholerae*, and *V. parahaemolyticus*. For a one year period, waters and sediment were monitored for the presence of these three pathogens at six different sites on the east coast of North Carolina in the United States. All three pathogens, identified using colony hybridization and PCR methods, occurred in these estuarine environments, although *V. cholerae* occurred only infrequently and at very low levels. Seventeen chemical, physical, and biological parameters were investigated, including salinity, water temperature, turbidity, dissolved oxygen, levels of various inorganic nutrients and dissolved organic carbon, as well as total vibrios, total coliforms, and *E. coli*. We found each of the *Vibrio* spp. in water and sediment to correlate to several of these environmental measurements, with water temperature and total *Vibrio* levels correlating highly ($P<0.0001$) with occurrence of the three pathogens. Thus, these two parameters may represent simple assays for characterizing the potential public health hazard of estuarine waters.

***Keywords*:** pathogenic vibrios, USA, ecology, environmental parameters

Vibrio vulnificus occurs in coastal marine environments worldwide, being isolated from estuarine water, sediment, oysters, plankton, and several types of fish (Oliver, 2006). This bacterium causes two significant syndromes: septicemia and wound infections. Primary septicemia due to *V. vulnificus* is the most deadly food-borne disease in the United States, accounting for 95% of all seafood related deaths with a mortality rate of approximately 50%. Wound infections occur when *V. vulnificus* enters through a pre-existing wound or a wound incurred while in contact with contaminated water. These infections may lead to skin ulceration and severe necrosis of the surrounding tissue. While mortality is not as high (25%), tissue debridement or amputation of the affected areas is often required (Oliver, 2005).

V. cholerae is the causative agent of Asiatic or epidemic cholera. In 2005 alone, the WHO reported nearly 132,000 cases in 52 countries, with over 2,000 deaths (World Health Organization, 2007). While toxigenic strains are only rarely isolated from the environment, non-toxigenic strains are a normal inhabitant of aquatic ecosystems, including seawater, freshwater, and sewage waters (Oliver and Kaper, 2007). In marine environments they have been reported to attach to surfaces provided by plants, filamentous green algae, copepods, crustaceans, and insects (Colwell *et al*., 1977; Huq *et al*., 1983).

V. parahaemolyticus is the causative agent of two syndromes, gastroenteritis and wound infections. Gastrointestinal symptoms are generally self-limiting, usually lasting one week or less, with no need for antibiotic treatment (Oliver and Kaper, 2007). Major outbreaks typically occur during the warmer months of the year, and during May-June of 1998, the largest documented (416 cases) in North America occurred following ingestion of oysters harvested from Galveston Bay, TX (Daniels *et al*., 2000).

The goals of our study were to characterize the role of 17 chemical, physical, and bacteriological parameters in the occurrence of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in estuarine water and sediment at six sites along the North Carolina coast of the United States. To our knowledge such a multiple-species, multiple-parameter study has not previously been reported.

Materials and Methods

Estuarine sites

Water and sediment samples were collected monthly between May, 2002 and March, 2003 from six sites along the Neuse, Pungo, and Pamlico Rivers along the east coast of North Carolina (Fig. 1). These locations were chosen because of the local activities occurring there (a phosphate plant, a nuclear power plant, several hog farms) that would be expected to provide variations in the environmental parameters monitored. Unpublished results from previous studies conducted by this lab have also shown these sites to be positive for all three pathogenic *Vibrio* spp..

Sample collection and environmental measurements

Water and sediment samples were transported on ice from each site to the laboratory, with a maximum delay of four hours before bacteriological analysis. Environmental param-

* To whom correspondence should be addressed.
(Tel) 1-704-687-8516; (Fax) 1-206-350-1385
(E-mail) jdoliver@uncc.edu

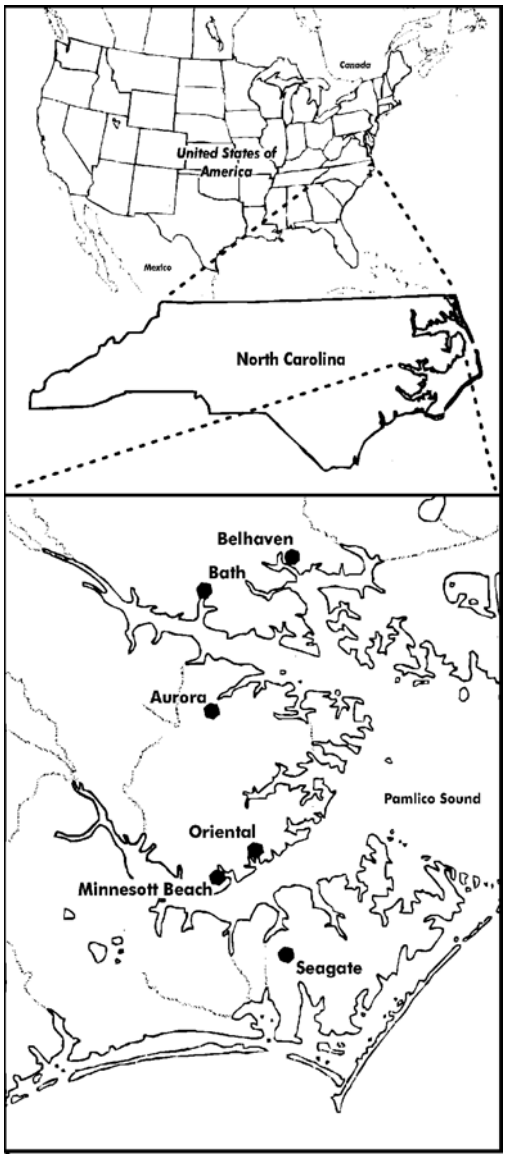

Fig. 1. Locations (●) of the six estuarine sites in eastern North Carolina.

eters measured at each site included water temperature, salinity, turbidity, pH, and levels of ferrous iron, phosphate, ammonium nitrogen, and dissolved oxygen. Water temperature was measured using a waterproof thermometer (Fisher Scientific, USA) and salinity with a handheld refractometer (Schuco, Japan). Turbidity, pH, ferrous iron, phosphate, ammonia nitrogen, and dissolved oxygen were measured using appropriate Hach reagents and a Hach DR/850 portable colorimeter following the manufacturer's instructions (Hach Co., USA). Water samples for DOC analysis were filtered through a 47 mm cellulose nitrate membrane with a 0.2 μm pore size (Nalgene, USA) and were kept at -20°C until measured using a Total Organic Carbon Analyzer with an ASI-5000A autosampler (Shimadzu Instruments, Inc., Japan). Filtered, deionized water was analyzed for DOC to determine any DOC contribution due to the filtration procedure.

Isolation and enumeration of *Vibrio* spp.

Water (0.1 ml) from each site was plated directly onto Estuarine agar [EA; 1 g Proteose peptone (Difco), 1 g yeast extract, 15 g agar per liter of estuarine salts solution (12.36 g NaCl, 0.34 g KCl, 0.68 g $CaCl_2{\cdot}2H_2O$, 2.33 g $MgCl_2{\cdot}6H_2O$, 3.15 g $MgSO_4{\cdot}7H_2O$, 0.09 g $NaHCO_3$ per liter of deionized water), pH adjusted to 7.2∼7.4 and autoclaved] to enumerate total estuarine organisms, Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar (Difco, USA) to enumerate total vibrios, and Colistin-Polymyxin B-Cellobiose agar (CPC) (Massad and Oliver, 1987) for the isolation of *V. vulnificus* and *V. cholerae*. These media were incubated at 22°C, 37°C, and 40°C, respectively. In addition, 10 g of sediment was diluted with 90 ml of estuarine salts solution and plated (0.1 ml) directly onto EA, TCBS, and CPC. When water temperatures dropped below *ca.* 13°C, 10∼50 ml of sample water was filtered through a 0.2 μm cellulose nitrate membrane filter (Nalgene) which was placed directly onto CPC and TCBS agars to increase the detection limit of these *Vibrio*-specific media. For total coliforms and *E. coli*, 1∼10 ml water samples were filtered through a gridded, mixed cellulose ester, 0.45 μm filter (Millipore, USA) and incubated with M-ColiBlue24^{TM} broth (Hach Co.) for 18∼24 h at 37°C. Because samples were directly plated to these selective media, with no enrichment steps employed, the CFU reported here are likely underestimates of actual bacterial levels.

Species identification

Following purification, cells from cellobiose-positive and negative colonies on CPC (presumptive *V. vulnificus* and *V. cholerae*, respectively), sucrose-positive colonies on TCBS (presumptive *V. cholerae*), and sucrose-negative colonies on TCBS (presumptive *V. vulnificus* and *V. parahaemolyticus*) were confirmed as *V. vulnificus, V. cholerae,* or *V. parahaemolyticus* using colony hybridization or PCR (Kaysner and DePaola, 2001). Colony hybridization for *V. vulnificus* employed an alkaline phosphatase conjugated probe (DNA Technology A/s, Denmark) with the sequence 5'-XCG GCT GTC ACG GCA GTT GGA ACC A-3', which detects the hemolysin gene unique to this species (Wright *et al*., 1993). PCR using the oligonucleotide primer 5'-GC STT TTC RCT GAG AAT G-3' was used to detect the *V. cholerae* 16S rRNA intergenic spacer (Chun *et al*., 1999), and the primer 5'-AA AGC GGA TTA TGC AGA AGC ACT G-3' was used to detect the *tlh* hemolysin gene of *V. parahaemolyticus* (Bej *et al*., 1999). PCR conditions in both cases were as described by the cited authors. *V. cholerae* isolates were not further analyzed as to serotype or *V. parahaemolyticus* isolates for virulence potential. The great bulk of *V. cholerae* and *V. parahaemolyticus* estuarine isolates have been reported by other investigations, however, to be non-01 and *tdh*/*trh*-negative strains, respectively (Bauer *et al*., 2006; Oliver and Kaper, 2007; Zimmerman *et al*., 2007).

Statistical analysis

Averages of the environmental parameters were examined using Spearman's coefficient rank correlation (Sokal and Rohlf, 1985), utilizing the SAS statistical program to compare with previously published research. When arrows are present on figures they represent culturability below the

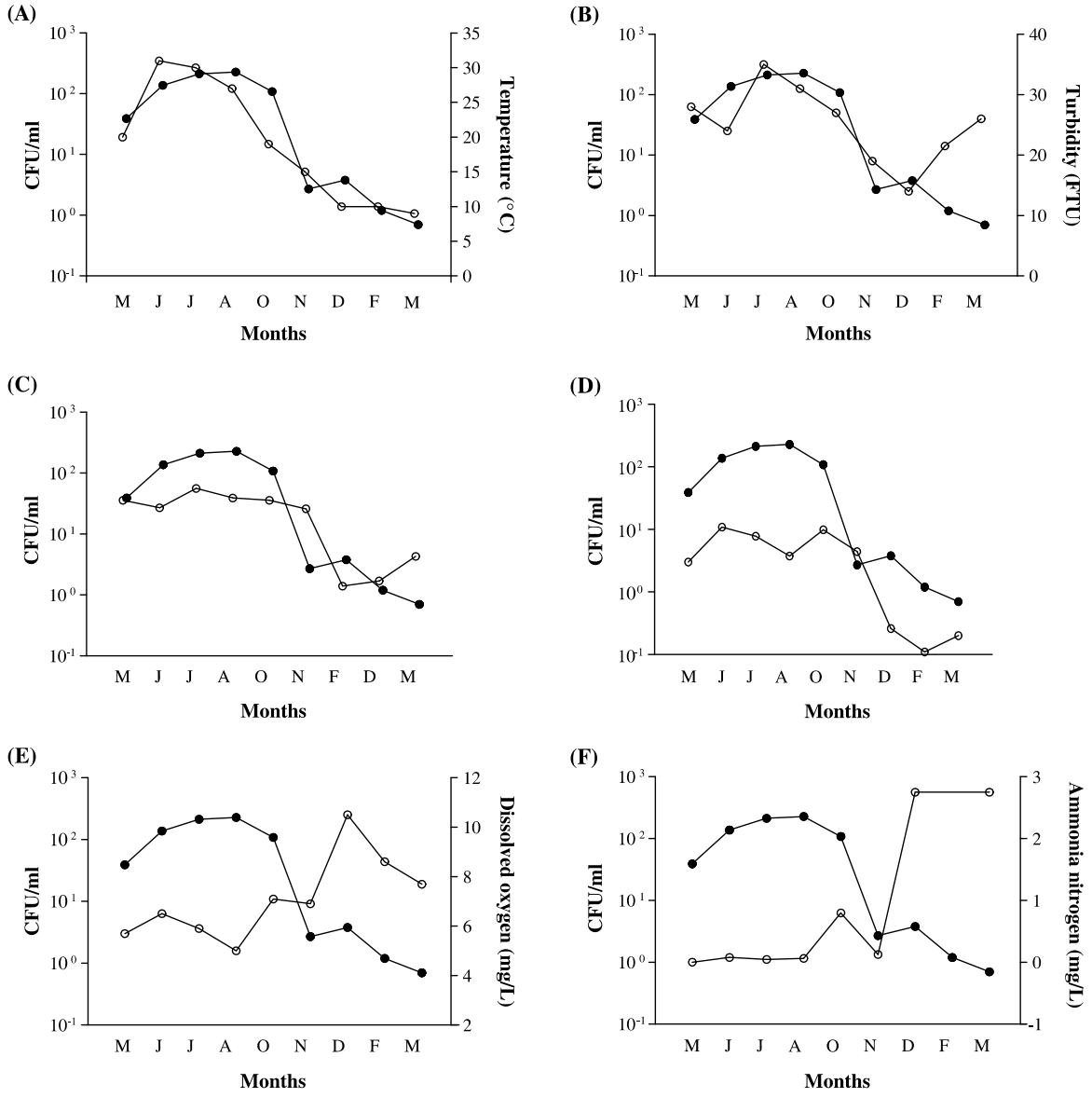

Fig. 2. Correlation between total *Vibrio* isolates in water (●) and physical/chemical water parameters (○). (A) water temperature, (B) turbidity, (C) total coliforms, (D) *E. coli*, (E) dissolved oxygen, and (F) ammonia nitrogen.

limit of detection. Due to space limitations, not all results with significant correlations are presented here, although most of those for *V. vulnificus* are shown as representative examples.

Results and Discussion

Total Vibrios

The number of total estuarine organisms ranged from 1.5×10^2 to 1.3×10^5 CFU/ml in water samples and 6.2×10^3 to 1.6×10^6 CFU/g in sediment samples with the highest concentrations being detected during warm weather months. Based on TCBS counts, total vibrios averaged 1.9% of the total estuarine organisms in water from the combined six sites and 1.5% of those in sediment. Previous studies have described several significant relationships between the isolation of total vibrios and various environmental parameters. Analysis of our data using Spearman's correlation coefficient revealed that the isolation of total vibrios from water samples was positively and highly correlated with water temperature (r^2=0.76766; P<0.0001; Fig. 2A), turbidity (r^2=0.27846; P<0.05; Fig. 2B), total estuarine organisms (r^2=0.43541; $P\leq$0.001), total coliforms (r^2=0.61245; P<0.0001; Fig. 2C) and *E. coli* (r^2=0.61515; P<0.0001; Fig. 2D), and was negatively correlated with dissolved oxygen (r^2=-0.47286; P< 0.001; Fig. 2E) and ammonia nitrogen (r^2=-0.40552; P<0.01; Fig. 2F). A positive correlation between the occurrence of vibrios and water temperature has often been described (Oliver, 2006), and our findings are consistent with those reports. While Koh *et al*. (1994) found no correlation between

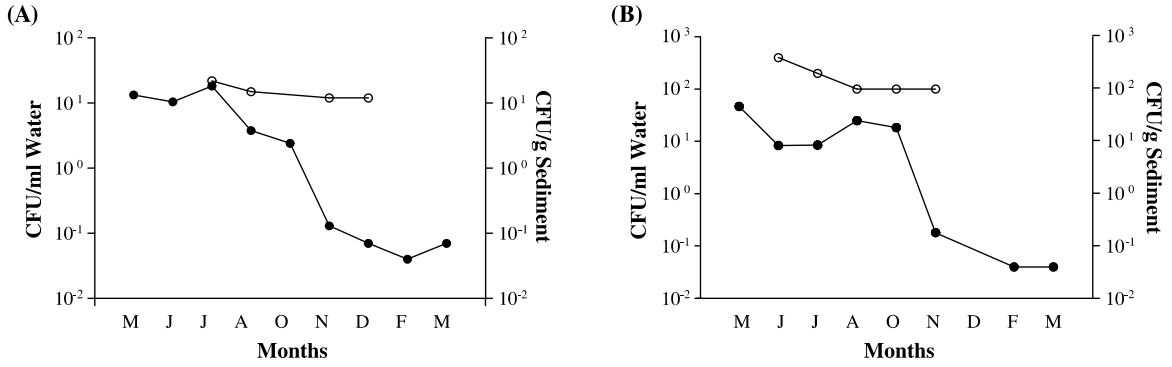

Fig. 3. Levels of pathogenic *Vibrio* spp. in water (●) and sediment (○). (A) *V. vulnificus* and (B) *V. parahaemolyticus*. Extremely low isolation frequency of *V. cholerae* precluded graphical analysis.

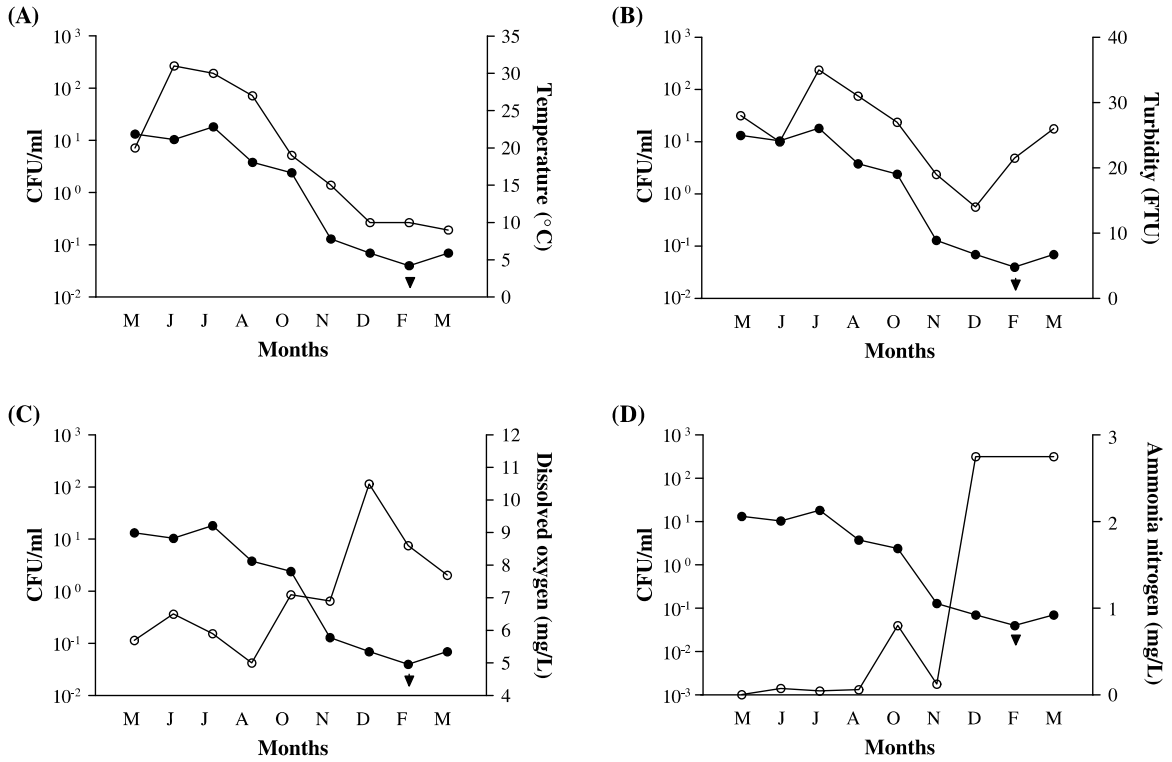

Fig. 4. Correlation between *V. vulnificus* water isolates (●) and physical/chemical water parameters (○). (A) water temperature, (B) turbidity, (C) dissolved oxygen, and (D) ammonia nitrogen.

total or fecal coliforms with vibrios, Oliver *et al.* (1983) reported positive correlations between total vibrios and fecal coliforms, pH and turbidity. The positive correlation in our study between *E. coli*, total coliforms, and turbidity may be related to fecal pollution in estuaries (hog waste spills from holding ponds are frequent occurrences in these waters); such conditions, along with increases in nutrient and temperature, are likely to promote growth of various *Vibrio* spp. According to our data, monitoring total coliform counts could be useful as an indicator of the levels of total vibrios.

V. vulnificus

Of the total vibrios from all sites, an average of 1.9% from water and 11.7% from sediment were identified as *V. vulnificus*. This corresponds well to data from a previous study from our lab in which total vibrios averaged 2.0% of total estuarine organisms, with 7.7% of the total vibrios being identified as *V. vulnificus* (Pfeffer *et al*., 2003). Numbers of *V. vulnificus* ranged from $<4.0\times10^{-2}$ to 3.0×10^{1} CFU/ml in water and from $1\sim2\times10^{1}$ CFU/g in sediment, with the highest concentrations detected during the warmer months of May through October (Fig. 3A). These data agree with a previous study by Pfeffer *et al*. (2003) of these waters, which reported numbers of *V. vulnificus* ranging from to $<1.0\times10^{-2}$ to 2.3×10^{1} CFU/ml in the water samples examined. The presence of *V. vulnificus* in water was positively correlated

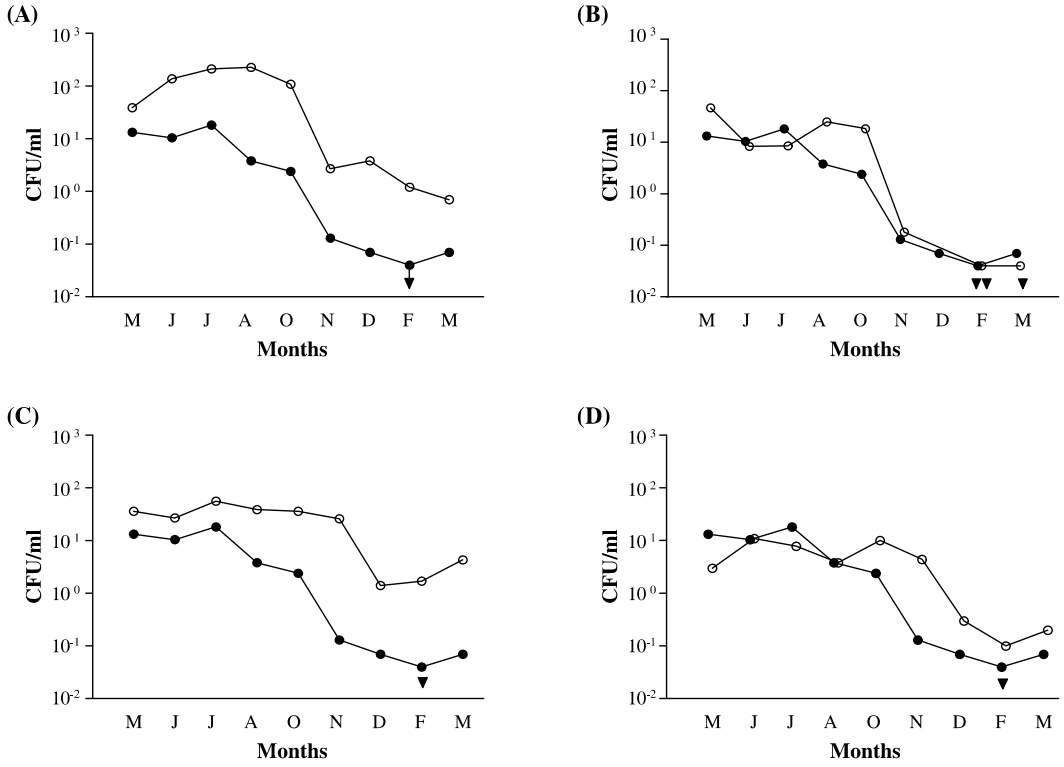

Fig. 5. Correlation between *V. vulnificus* water isolates (●) and biological parameters in water (○). (A) total vibrios, (B) *V. parahaemolyticus*, (C) total coliforms, and (D) *E. coli*.

to water temperature (r^2=0.76453; P<0.0001; Fig. 4A). In the present studies water temperatures ranged from 7 to 32°C and *V. vulnificus* was more prevalent when water temperatures ranged from 19~32°C. These data are in accordance with numerous other researchers who have studied this correlation (Kelly, 1982; Tamplin *et al*., 1982; Kaysner *et al*., 1987; O'Neill *et al*., 1992). Indeed, seasonal changes in water temperature have previously been reported to explain *ca*. 50~60% of the variations in *V. vulnificus* abundance (Pfeffer *et al*., 2003; Randa *et al*., 2004). However, correlations with temperature appear to be salinity dependent (Motes *et al*., 1998; Fukushima and Seki, 2004), and Randa *et al.* (2004) documented a positive temperature correlation with salinity when ranges were 20~25 ppt, but not when salinity ranged from 5~10 ppt. In the present study, salinities ranged from 2.5~20 ppt, with the great majority of sites being between 5~12 ppt. As the optimal salinity range for *V. vulnificus* is 5 to 25 ppt, it is likely that the salinity conditions at all of our study sites were optimal, and thus no correlation was observed between *V. vulnificus* isolation and salinity.

A likely explanation for the difficulty in isolating *V. vulnificus* during the cold weather months (Fig. 3A) is due to its entry into a viable but nonculturable (VBNC) state. Oliver *et al*. (1995) demonstrated this phenomenon *in situ* in an estuarine environment through the use of membrane diffusion chambers. When water temperatures averaged <15°C, a temperature similar to those in this study in November, the cells no longer grew on routine media but were viable, as indicated by direct viability assays. That study also demonstrated *V. vulnificus* was capable of repopulating the waters following resuscitation from the VBNC state when water temperatures averaged 22~24°C, temperatures similar to those in the summer months of the present study.

Similar to other studies (Oliver, 2006), we also found a high correlation (r^2=0.26724; $P\leq$0.05; Fig. 4B) between the isolation of *V. vulnificus* and turbidity. Indeed, Jones and Summer-Brason (1998) reported that turbidity was a major factor affecting the concentrations of *V. vulnificus* isolated from northeastern United States estuarine waters. In agreement with the study of Pfeffer *et al.* (2003), we found a negative correlation for *V. vulnificus* and dissolved oxygen (r^2=-0.44954; P<0.005; Fig. 4C). This might be expected due to the inverse relationship of dissolved oxygen and water temperature. An inverse correlation with ammonia nitrogen levels was also observed (r^2=-0.64429; P<0.0001; Fig. 4D).

V. vulnificus from water was positively correlated to all of the seven bacteriological parameters measured: total estuarine organisms (r^2=0.34156; P<0.05), total vibrios (r^2=0.73749; P<0.0001; Fig. 5A), *V. cholerae* (r^2=0.71437; P<0.0001), *V. parahaemolyticus* (r^2=0.71993; P<0.0001; Fig. 5B), total coliforms (r^2=0.68535; P<0.0001; Fig. 5C), and *E. coli* (r^2= 0.54276; P<0.0001; Fig. 5D). *V. vulnificus* cells present in water were also positively correlated to two of the seven bacteriological parameters in sediment: total vibrios (r^2= 0.52364; P<0.0005) and *V. parahaemolyticus* (r^2=0.31620; P<0.05). A positive correlation between *V. vulnificus* and total vibrios and for *V. vulnificus* and *V. parahaemolyticus*

has been reported (Pfeffer *et al*., 2003; Fukushima and Seki, 2004), likely due to the fact that these organisms are found ubiquitously in the same environment and have similar optimal environmental ranges.

Total and fecal coliforms (*E. coli*) are often used as indicators of water contamination and it is not surprising that a correlation was found. A similar result has been reported by Tamplin *et al.* (1982), Ruple and Cook (1992) and Høi *et al*. (1998) between *V. vulnificus* and total and/or fecal coliforms. However, Pfeffer *et al*. (2003), in agreement with some other studies (Oliver *et al*., 1982; Oliver *et al*., 1983; O'Neill *et al*., 1992), reported no such correlation with total or fecal coliforms.

We found higher concentrations of *V. vulnificus* in sediment than water samples (Fig. 3A). Because our limit of detection for sediment samples was 1.0×10^{2} CFU/g, while the limit of detection for water samples was 1.0×10^{-1} CFU/ml, sediment samples were only positive for this species between July and December. Nevertheless, it may be significant that the levels remained at a fairly constant level in sediment while those in water declined dramatically as water temperature decreased. Previous studies have suggested that sediment may allow *V. vulnificus* to over-winter, with subsequent re-introduction into the water column in the spring (DePaola *et al*., 1994; Fukushima and Seki, 2004; Randa *et al*., 2004). *V. vulnificus* has been shown to survive well in many types of sediment and has been detected at high levels in the sediment of other estuaries (DePaola *et al*., 1994; Wright *et al*., 1996; Høi *et al*., 1998). Of the ten chemical and physical parameters measured, *V. vulnificus* from sediment samples was positively correlated only to pH (r^2=0.31; P<0.05) in agreement with previous studies from our lab (Oliver *et al*., 1995; Oliver, 2006). Surprisingly, *V. vulnificus* from sediment samples was not correlated to any of the biological parameters, suggesting that *V. vulnificus* cells in sediment are affected differently by these parameters than are cells present in water.

V. parahaemolyticus

Numbers of *V. parahaemolyticus* ranged from $<4.0\times10^{-2}$ to 1.0×10^{2} CFU/ml in water (25% of the total vibrios from all water sites) and $<1.0\times10^{1}$ to 5.0×10^{2} CFU/g in sediment, with the highest concentrations detected during the warm weather months of May through October (Fig. 3B). Isolation of this species paralleled that of *V. vulnificus* (Fig. 3A) and like that species, was difficult to isolate during the cold weather months.

V. parahaemolyticus in water was positively correlated to two of the ten chemical and physical parameters measured: water temperature (r^2=0.60881; P<0.0001) and turbidity (r^2= 0.31899; P<0.05). The correlation with water temperature has been reported by many researchers (Barbieri *et al*., 1999; Oliver, 2006). Indeed, our finding is very similar to that of Depaola *et al*. (2003) who reported a strong positive correlation between *V. parahaemolyticus* isolation and water temperature (r^2=0.51; P<0.0001). Further, studies performed by Jiang and Chai (1996) and Mizunoe *et al.* (2000) reported not only a seasonal difference in *V. parahaemolyticus* but suggested that the inability to recover this species during the cold water months may be due to the bacterium entering into a VBNC state, a finding supported by Bates and Oliver (2004). The positive correlation with turbidity was not surprising as this has also been previously reported (Jones and Summer-Brason, 1998). There were strong negative correlations found between levels of *V. parahaemolyticus* and dissolved oxygen (r^2=-0.41607; P<0.005) and ammonia nitrogen levels (r^2=-0.41661; P<0.01). While other studies have found a correlation between *V. parahaemolyticus*, salinity and DOC., our *in situ* studies found no such correlation, likely because neither of these varied sufficiently to elicit a response.

V. parahaemolyticus from water samples was positively correlated to all but one (total estuarine organisms) of the biological parameters measured: total vibrios (r^2=0.62203; P<0.0001; Fig. 6A), *V. vulnificus* (r^2=0.71993; P<0.0001; Fig. 5B), *V. cholerae* (r^2=0.57683; P<0.0005), total coliforms (r^2=0.62927; P<0.0001) and *E. coli* (r^2=0.46744; P<0.001; Fig. 6B).

As in the case with *V. vulnificus*, we found higher concentrations of *V. parahaemolyticus* in sediment samples compared to water (Fig. 3B). However, because sediment could only be evaluated to $>1.0\times10^{2}$ CFU/g, sediment data for *V. parahaemolyticus* were only obtained from June through November. Nevertheless, as with *V. vulnificus*, the levels remained above those of *V. parahaemolyticus* in water during this time, even when numbers in water were falling. *V. parahaemolyticus* levels in sediment samples were positively correlated to water temperature (r^2=0.50547; $P\leq$0.0005) and turbidity (r^2=0.31899; P<0.05). As was observed for this

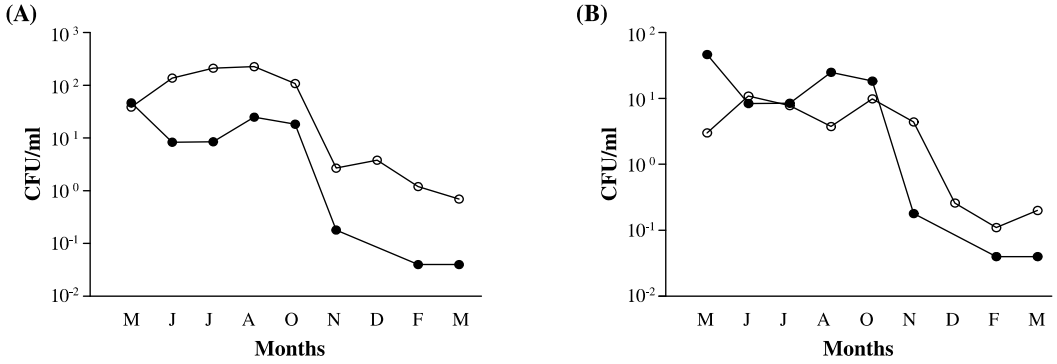

Fig. 6. Correlation between *V. parahaemolyticus* isolates in water (●) and microbiological parameters (○). (A) total vibrios and (B) *E. coli*.

species in the water column, *V. parahaemolyticus* correlated highly with levels of total vibrios (r^2=0.41; P<0.05), *V. vulnificus*, *V. parahaemolyticus* (r^2=0.71993; P<0.0001), *V. cholerae* (r^2=0.34; P<0.05), and *E. coli* (r^2=0.36; P<0.05) in water.

V. cholerae

This species was isolated from water by direct plating on only five occasions (out of a total of 54 sampling events). Numbers were generally undetectable at our limits of detection (<0.04~<0.7 CFU/ml) with the highest being 20 CFU/ml in one sample in October. Numbers in sediments were always below our limit of detection (<100 CFU/ml). Bauer *et al*. (2006) detected *V. cholerae* in only 1% of the Norwegian mussels they examined, despite water temperatures exceeding 19°C during some samplings. The fact that *V. cholerae* was isolated so infrequently and at such low numbers in our study may indicate that the environmental conditions at these sites are not suitable for proliferation of *V. cholerae*. Previous studies have shown that *V. cholerae*, like *V. vulnificus,* enters into a VBNC state in response to low temperatures (Colwell, 1998; Oliver, 2000), and this may also have been the case during periods of low temperature water.

Despite the infrequent isolation of *V. cholerae* from water samples, we were able to show a positive correlated to water temperature (r^2=0.80428; P<0.0001). As was the case with the other vibrios, its isolation was negatively correlated to dissolved oxygen (r^2=-0.43155; P<0.005) and ammonia nitrogen (r^2=-0.38327; P<0.05).

Like *V. vulnificus*, *V. cholerae* in water was positively correlated to all of the biological parameters measured: total estuarine organisms (r^2=0.45698; P<0.005), total vibrios (r^2=0.75285; P<0.0001), *V. vulnificus* from water samples (r^2=0.71437; P<0.0001), *V. parahaemolyticus* from water samples (r^2=0.57683; P<0.0005), total coliforms (r^2=0.61453; P<0.0001), and *E. coli* (r^2=0.53974; P<0.0005).

Conclusions

To our knowledge, this is the first study to simultaneously examine multiple estuarine sites for levels of *Vibrio vulnificus, V. cholerae* and *V. parahaemolyticus* and to correlate their incidence with multiple chemical, physical, and biological parameters. The three *Vibrio* spp. were highly correlated (P<0.0001) to total *Vibrio* levels and to temperature, suggesting that simple measurements of water temperature coupled with levels of total vibrios (as determined by TCBS counts) could be an excellent predictor of the level of these human pathogens. Indeed, water temperature alone may be an adequate predictor, as the correlation was extremely high (P< 0.0001) between this parameter and the three individual pathogenic species (*V. vulnificus*: r^2=0.77; *V. parahaemolyticus*: r^2=0.61; *V. cholerae*: r^2=0.80). Thus water temperature may represent a simple method for characterizing the potential public health hazard of these pathogens in estuarine waters, as has been suggested in a recent FAO/WHO risk assessment study of these pathogens (FAO/WHO, 2002).

Acknowledgements

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