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Short Communication

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Short-Term Variation in the Abundance of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in a Tidal Estuary

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Abstract

We examined the influence of tide stage and depth on the abundance of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in the Chesapeake Bay. Samples were collected every 3 hours following predicted tides at a fixed location over 3 separate days and *Vibrio* concentrations analyzed by qPCR. Multi-way Analysis of Variance suggest that sampling day explains the vast majority of the variance in abundance for both species ($p<0.0001$) with limited influence of tide and depth. The physio-chemical parameters that define a sampling day were further explored with environmental gradient analysis. Gradients in daily photosynthetic activity and turbidity (PC1) and temperature and salinity (PC2) explained 75% of the environmental variability, and 50% of *Vibrio vulnificus* abundance. However, these same gradients did not explain a significant proportion of variation in the abundance of *Vibrio parahaemolyticus* ($P>0.05$). These results suggest that within day variability is not as important as that associated with environmental changes over time, and further highlight the need for species specific and mechanistic approaches to the study of *Vibrio* ecology.

Keywords

Vibrio spp.; Ecology; Monitoring; qPCR

Introduction

Vibrio spp. are gram negative, flagellated, heterotrophic bacteria indigenous to the estuarine environment. Several species, including *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* are capable of causing severe and occasionally life threatening infections in humans both through water contact and consumption [1,2]. For example, *V. vulnificus* is responsible for 95% of all seafood related mortalities, and carries a 50% mortality rate with primary septicemia [3]. Over the past three years of data availability (2006-2008), an average of over 600 cases have been reported annually through the Center for Disease Control's COVIS system [4]. Further, there is evidence that the incidence has increased [5]. Accordingly, significant effort has been devoted to monitoring *Vibrio* abundance, enhancing detection and differentiation methods, and developing predictive models [6-15]. Many studies have examined the relationship between *Vibrio* abundance and environmental factors [7-9,12,13,16-23]. It is clear from these efforts that temperature is the major temporal driver, while salinity, chlorophyll, and zooplankton govern spatial variability and may differ in importance among species. For example, *Vibrio vulnificus* is strongly governed by preferred salinity in Chesapeake Bay [8,23], while *Vibrio parahaemolyticus* occurs in a wider salinity range with attempts to examine correlation equivocal [23-25]. However, what is less clear is the influence of short term variability perhaps influenced by factors such as tidal stage and depth of sample collection. These may be of particular significance to large scale, coastal water monitoring efforts which routinely collect surface samples and are unable to control for tide. Previous research found tide, depth, and day to all significantly influence *Vibrio*'s in a Florida estuary, however no attempt was made to identify individual species [26]. Thus the aim of this study was to determine what factors influence the concentrations of *Vibrio vulnificus* and *Vibrio parahaemolyticus* over short time scales.

Materials and Methods

Field sampling was conducted on the Tred Avon River at the Cooperative Oxford Lab (Oxford, MD). Water samples were collected at the surface, 0.1 meters above the bottom, and the mid depth of the water column (3 meter total depth) over two complete tidal cycles (Low AM, Flood, High, Ebb, Low PM) and was replicated over the course of three days in July and August of 2010 (n=45). Surface water samples were collected by submerging autoclaved Nalgene bottes and rinsing 2x times prior to sample collection. Sub-surface samples were collected with a Niskin bottle following the same rinsing procedure at depth. The samples were thoroughly mixed and 200 ml of water was filtered through 0.22um sterivex filters, all water removed, and stored at -80°C, and DNA extracted as previously described [27]. Physical water quality parameters were measured concurrently at each depth in-*situ* with a YSI datasonde (YSI Incorporated, Yellow Springs, Ohio, USA). Parameters measured are listed in Table 1.

Primers *tlh* F (5'-ACTCAACACAAGA AGAGATCGACAA-3') and *tlh*_R (5'-GATGAGCGGTTGATGTCCAA-3') were used in conjunction with the probe *tlh*_TXRD (5'- /TxRED/CGCTCGC-GTTCACGAAA CCGT/3BHQ_2/-3') for the detection of *V. parahaemolyticus*. A unique internal control (IC) was incorporated simultaneously into the assay to test for the presence and influence of inhibitors [28]. Primers *vvh*_F (5'-TTCCAACTTCA AACCGAAC-TATGA-3') and *vvh*_R (5'-TTCCAGTCGATGCGAATACGTTG-3') were used in conjunction with the probe *vvh*874 (5'-/56- FAM/ AAC-TATCGTGCAC GCTTTGGTACCGT /3BHQ_1/-3') for the detection of *V. vulnificus* [29]. A unique internal control was also incorporated into this assay [28].

Assay performance testing was carried out in a manner similar to that as previously described [27]. Primers and probe were tested against strains of *Vibrio vulnificus, V. parahaemolyticus, Enterococcus faecium, Hematodinium* spp. and 17 species of the genus *Mycobacterium*. In both cases the primers and probes were specific only to the organism of interest and negative results were obtained for all other species. Recovery and repeatability estimates as well as the effects of freezing are previously published [8]. Standard curves of Ct values versus concentration yielded an assay efficiency of 104.66% ± 0.96 (standard deviation; n=3) and 88.60% ± 5.90 (standard deviation;

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n=4) for *Vp* and *Vv* (respectively). Detection limits of the assays are 48 and 190 CFU/200ml as determined from spiked water samples. No inhibitors were observed in any environmental samples, based on the amplification of the internal control.

Three-way Analysis of Variance was used to examine the influence of sampling day, tidal stage, sampling depth, and interactions on log transformed count data for each of the two pathogens (Proc GLM, Sas Inc., Carey NC). For the purpose of this study the term 'day" is used to describe the physio-chemical variables and their measurements within a given 24 hour period. Where necessary, least square means comparisons were used to determine significance within a factor (LSmeans procedure, pdiff option, Tukey's adjustment). All models were examined for normality and homogeneity of variance. Spearmans rank correlation analysis was employed to evaluate relationships between environmental variables and *Vibrio* abundance as an initial approach to describe variability among sampling days. Because of a high degree of collinearity among water quality variables, Principal Component Analysis (PCA) was employed to describe the environmental gradients present during the study, and scores subsequently used as composite variables in multiple linear regression.

Results and Discussion

The main intent of this effort was to examine the relative importance of within-day variability on *Vibrio* spp. concentration, with the particular hypothesis that they may be influence by tide and depth. Tide did not significantly influence the abundance of *V. vulnificus*, but accounted for 10% of the total variance for *V. parahaemolyticus* (Table 1). However, this finding is somewhat misleading as the interaction of tide and day is most significant (Table 1B, 20% of total variance), particularly the August 16th flood tide event (Figure 1). Similar results have been previously reported for sucrose negative *Vibrio*s with respect to tide [26]. Depth similarly accounted for a minority of the variance but was significant for both species (Table 1). Concentrations obtained from samples at the bottom of the water column were 30% greater on average for *V. vulnificus* than those from the middle and surface (Figure 2) with a similar trend noted for *V. parahaemolyticus* (Figure 1). These results are supported by others [26] and may represent re-suspension from sediments, which are known to act as a reservoir for several species of *Vibrio*s [30].

The vast majority of the total variance in both *V. vulnificus* (p, 0.0001, f=54.02, 2df) and *V. parahaemolyticus* concentrations (p<0.0001, f =6.076, 2df) was explained by the physio-chemical environment that defines a sampling day (Tables 1 and 2, Figures 1 and 2). In this study, a variety of water quality measurements were obtained simultaneously to offer further explanatory variables for use in describing environmental drivers of *Vibrio* abundance and discriminating among sampling days. As is typical with measures of water quality, however, many of these variables are highly correlated with each other. Indeed, multiple regression analysis initially attempted with our data proved futile due to variance inflation

Table 1: Physio-chemical water parameters measured in this study by sampling day. Mean and standard deviation are presented.

Parameter	19-Jul	3-Aug	16-Aug
Temperature (°C)	29 ± 0.16	27.9 ± 0.61	27.4 ± 0.8
Salinity (ppt)	11.5 ± 0.03	12.2 ± 0.08	12.6 ± 0.05
pH	7.8 ± 0.15	7.9 ± 0.12	8 ± 0.14
Dissolved Oxygen (mg/L)	6.9 ± 0.34	6.4 ± 0.94	7.3 ± 1.42
Turbidity (NTU)	9.7 ± 1	8.7 ± 1.56	8.5 ± 3.9
Chlorophyll (mg/L)	4.3 ± 0.92	7.7 ± 1.69	12.2 ± 6.86

Table 2: Analysis of variance for fixed effects of day, depth, tide and interactions for *Vibrio vulnificus* (A) and *Vibrio parahaemolyticus* (B).

A.

Source of Variation	df	SS	MS	*F*	*P*
Factor					
Day	2	54.055	27.028	50.77	< 0.0001
Depth	2	4.252	2.126	3.99	0.0392
Tide	4	2.56	0.64	1.2	0.3483
Interactions					
Day x Depth	4	0.764	0.191	0.36	0.8343
Day x Tide	8	9.823	1.228	2.31	0.0737
Depth x Tide	8	11.465	1.433	2.69	0.0437
Error	16	8.518	0.532		
Total	44	91.436			

B.

Source of Variation	df	SS	MS	*F*	*P*
Factor					
Day	2	12.153	6.076	35.76	< 0.0001
Depth	2	1.528	0.765	4.5	0.0124
Tide	4	3.074	0.769	4.52	0.0282
Interactions					
Day x Depth	4	1.413	0.353	2.08	0.1314
Day x Tide	8	6.642	0.830	4.89	0.0034
Depth x Tide	8	2.163	0.270	1.59	0.2041
Error	16	2.720	0.170		
Total	44	29.692			

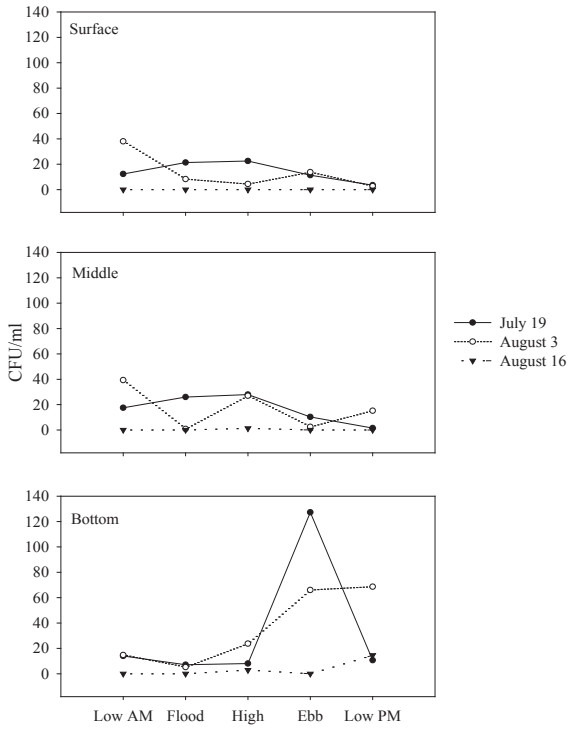

Figure 1: *Vibrio vulnificus* abundance by tide, day and depth.

associated with multicollinearity. Principal component analysis is a particularly valuable data reduction technique in multi-variate analysis where many of the explanatory variables are co-correlated. The orthogonal transformation results in a series of principal components that are linearly uncorrelated and defined in a manner where the majority of the variance is explained by the first component, and less by each successive component. The resulting variables serve as composite explanatory variables for further data exploration.

Ordination clearly demonstrates the difference between sampling days in the physio-chemical environment (Figure 3). The majority of variance in the environment was explained by the first two principal components (75%). PC1 (horizontal gradient, Figure 3) represents 45% of the total variance and describes a gradient in photosynthesis and turbidity as defined by increasing concentration of chlorophyll, dissolved oxygen, and pH. These factors, as well as PC1 in general are increasing during the course of the day in concert with algal production and respiration (PC1 correlation, r=0.49, P=0.001). PC2 (vertical gradient) represents 30% of the environmental variation and represents a gradient in temperature and salinity. As a whole, individual sampling days are clearly differentiated on PC2 by variation in temperature and salinity, and to a lesser extent PC1.

By overlaying *Vibrio* abundance on the environmental gradient analysis, we demonstrate that the two species respond in a dissimilar fashion. The warmer, less saline, and more turbid environments (Figure 3A) nearly always supported *Vibrio vulnificus* at concentrations greater than the median concentration (8 CFU/ml) with a decreasing proportion associated with cooler, more saline days. Regression analysis using PC1 and PC2 as explanatory variables

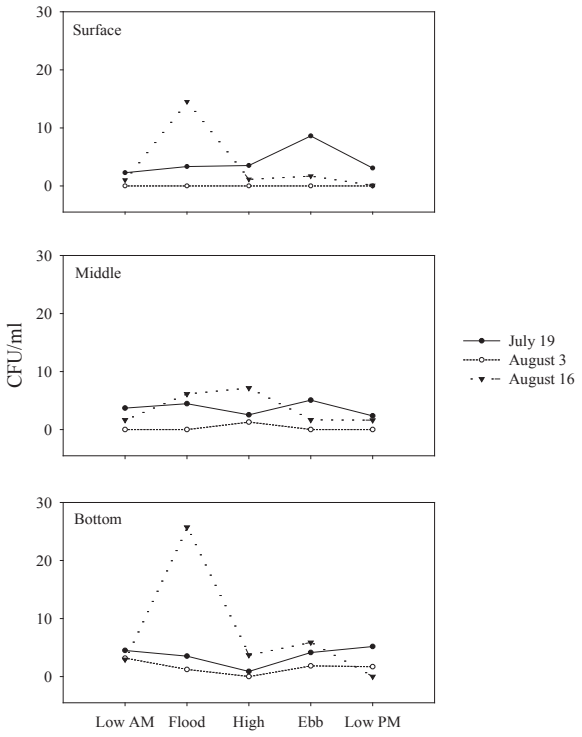

Figure 2: *Vibrio parahaemolyticus* abundance by tide, day, and depth.

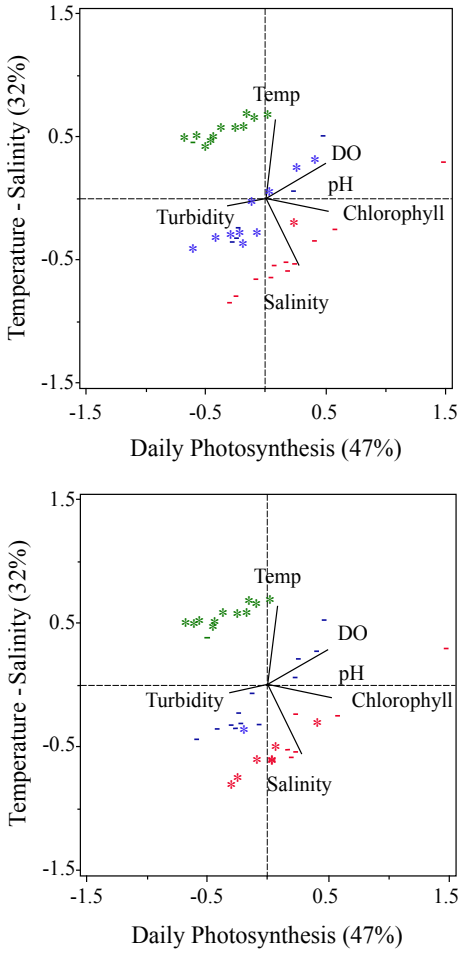

Figure 3: Environmental gradient analysis of the physio-chemical patterns associated with each sampling day. Green = July 19, Blue = August 3, and Red = August 16. Asterisks (*) represent either *Vv* (panel A) or *Vp* (panel B) samples which exceed the median concentration obtained in this study (8 and 2 CFU/ml respectively).

explains 49% of the variation (P <0.0001) in *Vv* abundance as follows: *LnVv=1.90 – (0.45 x PC1) + (0.52 x PC2)*. We have previously demonstrated that the presence of *V. vulnificus* declines rapidly with distance from optimal salinity (11.5 ppt) in Chesapeake Bay [8], and similar results were obtained here even within a relatively narrow salinity range (11.4 – 12.8ppt). This is supported by other work noting a general preference of 5-15 ppt in Chesapeake Bay and other coastal systems [9,23]. Turbidity is also an important component of current models used to forecast the distribution and abundance of *Vv* in the Chesapeake system (Authors unpublished data).

Vibrio parahaemolyticus did not demonstrate the same degree of association with environmental gradients described in this study (Figure 3B). Regression analysis using PC1 and PC2 demonstrates a stronger, negative association with PC1 than PC2, however the model is insignificant and explains a paucity of variance *(LnVp=1.04 –(0.12 x PC1) – (0.02 x PC2), P=0.34, R^2=0.05)*. While temperature is clearly a driver of *Vp* abundance seasonally, it is clear that other factors not directly measured in this effort are influential [31].

While of interest in describing the general ecology of *V. parahaemolyticus* and *V. vulnificus*, the significance of this effort is

in understanding site specific variability on short time frames and its application to monitoring and forecast efforts. This work confirms that changes in physio-chemical variables over time exert a much stronger influence on *V. vulnificus* and *V. parahaemolyticus* abundance than within-day variability associated with tides or sampling depth. This is important information for monitoring programs and modeling efforts in that a daily sample or prediction, regardless of depth or tide taken, is generally representative of overall conditions at that location for the given day. However, the study also points to the need for moving towards a more mechanistic understanding of the ecology of *Vibrio* spp and relationships with the ecosystem as a whole. While general habitat preferences have been well defined for many species and described through empirical relationships, detailed study of the interactions with the microbial community, other biota, and ecological processes over short time scales may greatly improve our understanding of the ecology and dynamics of *Vibrio* spp.

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