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Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington, United States

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ABSTRACT

Vibrio parahaemolyticus (*Vp*) and *Vibrio vulnificus* (*Vv*), native to estuaries globally, are agents of seafood-borne or wound infections, both potentially fatal. Like all vibrios autochthonous to coastal regions, their abundance varies with changes in environmental parameters. Sea surface temperature (SST), sea surface height (SSH), and chlorophyll have been shown to be predictors of zooplankton and thus factors linked to vibrio populations. The contribution of salinity, conductivity, turbidity, and dissolved organic carbon to the incidence and distribution of *Vibrio* spp. has also been reported. Here, a multi-coastal, 21-month study was conducted to determine relationships between environmental parameters and *Vp* and *Vv* populations in water, oysters, and sediment in three coastal areas of the United States. Because ecologically unique sites were included in the study, it was possible to analyze individual parameters over wide ranges. Molecular methods were used to detect thermolabile hemolysin *tlh,* thermostable direct hemolysin *tdh*, and *tdh*-related hemolysin *trh*, as indicators for *Vp* and hemolysin *vvhA* for *Vv*. SST and suspended particulate matter were found to be strong predictors of total and potentially pathogenic *Vp* and *Vv.* Other predictors included chlorophyll-*a*, salinity, and dissolved organic carbon. For the ecologically unique sites included in the study, SST was confirmed as an effective predictor of annual variation in vibrio abundance, with other parameters explaining a portion of the variation not attributable to SST.

INTRODUCTION

It has long been established that *Vibrio* spp. are autochthonous to the marine, estuarine, and riverine environment. Vibrios cultured from environmental samples commonly lack genes coding for functions associated with pathogenicity for humans and marine animals, e.g., the thermostable direct hemolysin (*tdh*) in *Vibrio parahaemolyticus*. Yet, pathogenic subpopulations of vibrios are potential agents of disease outbreaks and pandemics (7, 19, 23, 37, 44, 50, 65), notably in developing countries where access to safe drinking water is limited (26, 56) and/or in countries where consumption of raw or undercooked shellfish is common (11, 80). *Vibrio parahaemolyticus* is most frequently associated with gastroenteritis and has been linked to annual outbreaks (7, 8, 44). *Vibrio vulnificus* is more frequently associated with wound infections, with a case fatality rate as high as 50% (5, 10, 27). The abundance and distribution of these three human pathogens have been linked to environmental factors most notably temperature and salinity, depending on the pathogen and its habitat, and the geographic location (4, 13, 14, 18, 24, 29, 31, 35, 39, 70, 72, 83). Dissolved oxygen (30, 54, 58), chlorophyll (6, 20, 31, 33), and plankton (2, 31, 41, 59, 74) have also been found to be important in describing the ecology of vibrios. Regulatory authorities responsible for oversight of recreational waters and shellfish harvesting areas employ rainfall, fecal coliform counts, river stages, and, more recently, enterococcus counts to determine opening and closing of specific areas to protect public health (21, 25, 62, 76). Standard microbiological approaches to classification and opening/closing of oyster harvest areas, unfortunately not useful for control of exposure

72 to pathogenic *Vibrio*, spp., continue to be used and are generally accepted for
73 regulating exposure to other pathogens in the U.S. (36).

74 Naturally occurring pathogens, notably vibrios, are ubiquitous in the aquatic
75 environment and contribute to carbon and other nutrient cycling (24, 61). Clearly,
76 human exposure to these pathogens cannot be completely eliminated, but incidence of
77 illness can be reduced if environmental conditions that significantly elevate risk could be
78 identified and monitored. Communication of such conditions to stakeholders (regulatory
79 agencies, the shellfish industry, public health officials, at-risk consumers, etc.) would
80 reduce exposure and subsequent disease. An informative, robust system of
81 identification of conditions associated with high risk requires quantifying the association
82 of environmental factors with abundance of total vibrio populations and potentially
83 pathogenic vibrios. Given proven associations as predictors of vibrio abundance the
84 relevant environmental data can be collected by satellite remote sensing (13, 39, 83).

85 Development of models to predict presence of vibrio populations is facilitated by
86 collecting observations over a range of environmental parameters and recognition that
87 predictive relationships may vary across regions due to differences in ecology (for
88 example, models developed for the Gulf of Mexico may not be applicable to the Pacific
89 Northwest). Furthermore, potentially pathogenic subpopulations of environmental
90 vibrios are not necessarily a constant proportion of the total vibrio population (17, 18,
91 31, 32, 55, 83). Here we describe an analysis of environmental factors providing the
92 potential for improving upon existing predictive models for *V. parahaemolyticus* and *V.*
93 *vulnificus*. Specifically, we determined densities of total *V. parahaemolyticus* (*tlh*), and
94 potentially pathogenic *V. parahaemolyticus*, as indicated by the presence of the

thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin genes (*trh*) (48, 64), and total *V. vulnificus* as indicated by the presence of the *V. vulnificus* hemolysin gene (*vvhA*) that has been used as a marker for the species (45, 81, 82). These were determined for water, oyster, and sediment samples collected at sampling stations located in the Pacific Northwest, northern Gulf of Mexico, and Chesapeake Bay.

MATERIALS AND METHODS

Sample collection and processing. From December, 2008 to August, 2010, water, oyster, and sediment samples were collected in the Pacific Northwest in Hood Canal in Washington (WA), in the northern Gulf of Mexico spanning Louisiana (LA) and Mississippi (MS), and in the Chester River and Tangier Sound of the Chesapeake Bay in Maryland (MD). All samples were collected concurrently in LA, MS, and MD, but in WA, because of logistical problems, oyster and sediment samples were collected inter-tidally and relayed to a dock where the water samples were collected sub-tidally. At all sampling stations, 6 – 12 L of water, 20 – 25 oysters, and 100 g of sediment were collected and transported to the laboratory in coolers containing ice or ice packs. Water, oyster, and sediment samples were processed as described elsewhere (31). Specifically, water samples were shaken as previously described (1), oysters were scrubbed, shucked, and homogenized, and pore water was decanted from sediment then diluted 1:1 and shaken as previously described (31).

V. parahaemolyticus and *V. vulnificus* were enumerated as follows: 1 mL water, 0.1g and 0.01g oyster, and varying wet weights of sediment (0.0005 – 0.1g) were

spread plated on T_1N_3 agar (1% tryptone, 3% NaCl, pH 7.2) and VVA agar (2% peptone, 3% NaCl, 1% cellobiose, 0.06% bromthymol blue, pH 8.2). Detection of *tdh*+ and *trh*+ *V. parahaemolyticus* was accomplished using 1L, 100mL, and 10mL water enrichments and 10g and 1g oyster enrichments in 10X alkaline peptone water (10X APW, 10% peptone, 1% NaCl, pH 8.5). All samples were incubated at 33 ºC for 16 – 18 hours, as described previously (31).

Enumeration of vibrios. To enumerate *V. parahaemolyticus* and *V. vulnificus* by direct plating / colony hybridization (DP/CH), Whatman 541 ashless filters (Whatman, Kent, ME) were used to lift bacterial colonies from plates, as described elsewhere (31). The filters were probed using alkaline phosphatase-conjugated oligonucleotide probes (DNA Technology A/S, Risskov, Denmark) specific for *vvhA*, *tdh*, *trh*, and *tlh* (31). The DP/CH method is most effective for directly enumerating *tlh* and *vvh*, but *tdh* and *trh* populations are often too sparse to rely on DP/CH for enumeration. Therefore, samples were enriched in APW as described above to increase the *tdh* and *trh* populations to higher levels that could be detected and enumerated using the most probable number (MPN) method (49). For both total (*tlh*+) and potentially pathogenic (*tdh*+ and *trh*+) *V. parahaemolyticus,* serial MPN dilutions were determined in triplicate for water and oyster enrichments using real-time PCR, as previously described (31, 49). For samples collected in Louisiana and in Mississippi, probes and equipment were used that have been described elsewhere (31). For MD samples, probes were used as described previously (49) and reactions were carried out using an AB 7500 thermal cycler (Applied Biosystems, Carlsbad, CA). For WA samples, a Stratagene Mx300Sp Real-Time PCR System (Agilent Technologies, Santa Clara, CA) was used for real-time PCR analysis.

The *tlh* and internal amplification control (IAC) probes were purchased from Integrated DNA Technologies (Coralville, IA), and the *tdh* and *trh* probes were obtained from Applied Biosystems (Foster City, CA). Each 25-µl reaction consisted of 12.5 µl of 2X Brilliant Multiplex QPCR Master Mix (Agilent Technologies, Santa Clara, CA) and the following reaction components (final concentrations): all three probes at 150 nM, all six primers at 75 nM, and BSA (New England Biolabs, Beverly, MA) at 400 ng/µl. The remainder of the reactions consisted of 1 µl of the IAC template at the concentration described above, nuclease-free water, and 5 µl of template. The two-step thermal profile employed throughout the study consisted of an initial 9.5 min denaturation step at 95°C, followed by 40 cycles of 30s denaturation at 95°C and a 45s combined annealing/extension step at 58°C. Fluorescence data were collected at the end of each amplification cycle. The primer and probe sequences employed were the same as described previously (49).

All sediment samples were analyzed by DP/CH alone to enumerate total and pathogenic vibrios in sediment. The PCR/MPN method was not used because sediment has previously been found to contain very high levels of all four gene targets, and they could therefore be effectively enumerated using DP/CH; in addition, PCR analysis of sediment samples has proven unfruitful based on our previous experiences (data not shown). *V. vulnificus* levels were consistently low in the Pacific Northwest; among the 174 water, oyster, and sediment samples collected there during this study, *V. vulnificus* was detected in only one water and in one sediment sample. Thus, determinations of *vvhA* densities in WA samples were excluded from statistical analyses. In addition, during analyses of samples from the Pacific Northwest using the

DP/CH method, cross-reactivity was noted with either another *Vibrio* species or an unknown bacterium. *V. parahaemolyticus tdh* and *trh* data from oyster and water analyses were therefore excluded and only PCR/MPN data from WA were used for water and oyster samples. To correct for possible cross-reactivity at other sites, the DP/CH data were excluded from the analyses for the GC and MD sites when calculating *tdh* and *trh* densities. The resulting data pools are listed in Table 1.

Environmental parameters. At each collection site, water temperature and salinity were measured at the surface and bottom using a digital handheld conductivity meter (model 30-25FT, Yellow Springs Instruments, Yellow Springs, OH). Chlorophyll-*a* was measured by high-performance liquid chromatography at the University of Hawaii as follows. Triplicate volumes of up to 200 mL were filtered using 25 to 47mm diameter GF/F filters (Whatman, Kent, ME). Filters were stored at -20 ºC until shipped overnight on dry ice to the University of Hawaii where concentrations of chlorophyll-*a* (chl-*a*) were measured in methanol extracts on a Cary model 50 UV-visible spectrophotometer, as described previously (38). Suspended particulate matter (SPM) was measured by weighing pre-dried GF/F filters using a high-precision scale and filtering up to 200 mL water; the filters were dried overnight at 65 ºC and re-weighed.

To determine dissolved organic carbon (DOC) concentration, triplicate water samples were pre-filtered using an acrodisc (Pall Acrodisc PSF GHP 0.45 µm) and treated with HCl to convert inorganic carbon to CO_2. The samples were stored at -20 ºC until analysis using the method described as follows. CO_2 from inorganic carbon was manually purged from samples by adding additional HCl followed by overnight incubation at room temperature. Total organic carbon was measured using a Shimadzu

187 TOC-V CSN carbon analyzer equipped with an ASI-V autosampler (Shimadzu Scientific
188 Instruments, Columbia, MD).

189 **Statistical analyses.** Multi-level generalized linear mixed models (GLMM) were
190 used to estimate the distribution of vibrio abundance in oyster, sediment, and water and
191 the relationship between abundance and environmental predictors. Underlying (latent)
192 distributions of vibrio abundance were assumed to be lognormal with mean $\log_{10}$
193 densities generally presumed to be linearly related to environmental parameters being
194 considered as predictors of abundance. However, given the wide range of salinities
195 observed across sampling locations and consequent likelihood of a nonlinear
196 dependence, a quadratic polynomial was used to model the effect of salinity. Estimates
197 of location and scale of latent distributions of abundance for each combination of gene
198 target, sample type, and sampling location were obtained by fitting null (intercept-only)
199 models with no predictor variables. To facilitate identification of associations between
200 abundance and environmental predictors weakly identified when considering each
201 sampling location separately, data were pooled across sampling locations for each
202 combination of gene target and sample type. Raw plate count and real-time PCR-MPN
203 observations for multiple aliquots and dilutions of the same sample were treated as
204 repeated and discrete-valued measurements of the same underlying abundance in the
205 given sample. Raw observations comprise the response variables of GLMM regression
206 with plate counts and PCR-MPN outcomes at each dilution level treated as independent
207 Poisson and binomial outcomes, respectively, conditional on latent distribution of
208 abundance and volume of sample examined in each aliquot or dilution. Given apparent
209 inhibition of the PCR reaction at low dilutions in some samples, the PCR-MPN data

were truncated to one dilution, as described elsewhere (31). In regression analyses, temperature and salinity parameters were expressed in units of degrees Celsius and parts per thousand, respectively, while chl-a, DOC and SPM where expressed in base 10 logarithms of their respective measurement units. GLMM regression parameter estimates were determined by Markov Chain Monte Carlo (MCMC) sampling of posterior Bayesian distributions, conditional on the observed data and noninformative prior distributions. Associations between vibrio abundance and environmental parameters were summarized using McKelvey and Zavoina's pseudo-R^2 (28, 67), as a measure of the proportion of variation in latent distributions of abundance attributable to variation in the environmental parameter. Statistical analyses were conducted using WinBUGS (40) and the R2WinBUGS package of R (57, 71). Statistical significance of associations was assessed by identifying Bayesian 95% credible intervals for regression parameters that were exclusive of zero (51).

For graphical presentation of data, the number of *vvhA*+, *tlh*+, *tdh*+, and *trh*+ vibrios was determined by dividing the total number of colony-forming units (CFU) on one or more plates by the corresponding total volume of water or weight of oyster and sediment examined. Only CFU counts between 1 and 250 CFU per plate were plotted. Therefore, the limit of detection (LOD) ranges for *V. vulnificus* in water, oysters, and sediment were 1–250 CFU/mL, 10–25,000 CFU/g, and 100–83,333 CFU/g, respectively, because 1 mL water, 0.1 - 0.01 g oyster, and 0.01 to 0.003 g sediment were tested per *V. vulnificus* plate and only 1-250 CFU were counted per plate. The LODs for *V. parahaemolyticus* in water, oysters, and sediment were 1–250 CFU/mL,

232 10–25,000 CFU/g, and 20–83,333 CFU/g, respectively, because 1 mL water, 0.1 - 0.01
233 g oyster, and 0.05 to 0.003 g sediment were tested per *V. parahaemolyticus* plate.
234
235 **RESULTS**
236
237 **Environmental parameters.** Sea surface temperature ranges across the four
238 sample sites were relatively similar (Fig. 1). The lowest temperatures were measured in
239 WA, with LA and MS highest, and MD samples showing the widest temperature range.
240 Based on their similar geography and climate, the two Gulf Coast sites, LA and MS,
241 were combined (GC) for analytical and reporting purposes. Salinities were highest for
242 WA samples and lowest for MD samples; GC samples had the widest salinity range.
243 WA samples contained the lowest median chl-*a* concentration, median DOC, and SPM,
244 and GC samples had the highest medians for the three parameters.
245 **Model-based estimates of abundance.** A large number of negative results
246 were obtained using the DP/CH method for quantitation of *tdh* and *trh* in water samples
247 and in oysters. The DP/CH approach, therefore, was concluded not to be as
248 informative as the MPN approach for *tdh* and *trh* enumeration, and these data were
249 excluded for this reason. Results for the WA samples showed *vvhA* to be very low for
250 all three sample types, an indication that *V. vulnificus*, if present, was below the limit of
251 detection levels in agreement with previous studies (34). At all sampling sites, the
252 largest numbers of vibrios (*tlh*, *tdh*, *trh*, and *vvhA*) were determined when the water
253 temperature was high (Fig. 2). All GC samples had high *tlh* and *vvhA* numbers and
254 temperatures. The GC samples also had the highest *tdh* and *trh* densities in oysters,

255 but the WA samples had the highest *tdh* and *trh* densities in sediment. Overall, trends
256 in vibrio population numbers in water were similar for the all sample types, but WA
257 samples exhibited low *V. vulnificus* densities. MD samples exhibited the lowest mean
258 vibrio densities, with respect to the *tdh* and *trh* gene targets, with relatively large
259 standard deviations.

260 **Intra-sample comparisons of *V. parahaemolyticus* and *V. vulnificus*.**
261 Densities of *V. parahaemolyticus* (*tlh*) were compared to *V. vulnificus* (*vvhA*) on a
262 sample-by-sample basis, i.e., each *tlh* abundance was compared to *vvh* abundance in
263 the same sample. Comparisons summarizing relative abundance in CFU/mL water or
264 CFU/g of oyster or sediment were used to infer prevalence of one species over the
265 other across sample type and temperature range (Fig. 3). Data are presented in this
266 fashion due to the relatively high rate of non-detection, making calculation of
267 percentages problematic on a sample-by-sample basis. For LA samples, when *tlh* and
268 *vvhA* were detectable by DP/CH, *tlh* outnumbered *vvhA* in most samples (Fig. 3).
269 Specifically, *tlh* outnumbered *vvhA* about 2/3 of the time in water and sediment and
270 about 4/5 of the time in oysters. The reverse was observed for MS and MD samples,
271 where *vvhA* typically outnumbered *tlh.* Thus, overall, in LA samples, *V.*
272 *parahaemolyticus* was dominant more frequently in all sample types than *V. vulnificus*,
273 whereas MS and MD samples were more often dominated by *V. vulnificus* than by *V.*
274 *parahaemolyticus*.

275 **Environmental predictors of abundance.** Multi-level (GLMM) regression
276 models and associated measures of relative importance of predictor variables (pseudo-
277 R^2 values) were applied to data pooled across sampling locations to assess the

278 proportion of variation in vibrio abundance attributable to variation in each
279 environmental parameter. Where identified as statistically significant, DOC accounted
280 for 13% of *tlh* variability in oysters (Table 2), 15% of *tdh* variability in water, and 12% of
281 *trh* variability in sediment; its impact on the other factors were insignificant. Where
282 identified as statistically significant, chl-*a* accounted for 5% of *tlh* variability in sediment,
283 22% of *tdh* variability in sediment,13% of *trh* variability in oysters, and 9.8% of *trh*
284 variability in sediment. Similarly, where identified as statistically significant, SPM
285 accounted for 6 - 29% of variability in vibrio abundance, depending on sample type, with
286 *tdh* in oysters being the highest. Salinity accounted for 9% of *tlh* variability in oysters
287 and 3.7% of *tlh* variability in sediment. Although the pseudo-R^2 value for salinity in
288 Table 2 was high for *tdh* in the water column (31%), this value was not statistically
289 significant and most probably due to chance. SST accounted for 7.1 – 34% of *V.*
290 *vulnificus* and *V. parahaemolyticus* and was a strong predictor in all samples except for
291 *tdh* in water and oysters and *trh* in oysters. In most sample types for which SST was a
292 significant predictor, SST explained a larger percentage of variability than any of the
293 other parameters measured. In instances where SST was not significant, SPM was the
294 strongest predictor (Table 2). DP/CH detection rates were highest in sediment samples,
295 followed by oysters and water, and the highest *tdh* or *trh* non-detect rates were in water
296 and in oysters (Table 3).

297 Estimates determined by analysis of data pooled across sampling locations were
298 further evaluated by comparing to results of analyses of un-pooled data, considering
299 each site separately. Analysis of un-pooled data was conducted to assess consistency
300 of identified associations in the pooled analyses across each sampling location. Similar

patterns of association were observed in analysis of un-pooled data as in the analysis of pooled data, but uncertainty of the identified relationships was much greater. For parameters SST and SPM, which exhibited a relatively strong association with vibrio abundance, the estimated effect size (magnitude and sign of regression coefficients) across sampling locations was more consistent with that of the pooled analysis than was the case with other parameters.

DISCUSSION

A major goal of this group is to develop ecological models that can be used in conjunction with remotely sensed data collected from and applicable to different geographic regions of the world (i.e., algorithms such as http://www.eol.ucar.edu/projects/ohhi/vibrio/). Development of ecological models for bacteria is strengthened by collection and analysis of samples from diverse geographic locations. Inclusion of geographically distinct study sites to maximize understanding of the role of environmental parameters is a unique contribution of this study. An additional strength is the length and intensity of sampling, which included 594 water, oyster, and sediment samples collected weekly to biweekly over 21 months and a range of environmental parameters measured; this was one of the longest and most intensive sampling programs associated with vibrio abundance and distribution (15, 18, 22, 43, 46, 52, 54, 73, 78, 83). Furthermore, our sampling was carried out year-round to examine seasonal variations in vibrio densities. Environmental factors associated with incidence and geographic distribution of *V. parahaemolyticus*, potentially pathogenic *V.*

parahaemolyticus, and *V. vulnificus* at four sampling locations in three U.S. coastal areas were analyzed.

Ranges in vibrio densities were wider and detection rates were higher in this study than in our previous study, as were ranges of environmental parameters (31). The current study identified highest SPM levels on the Gulf Coast, a result that was not surprising since the Mississippi River plume contributes to turbidity and eutrophy (http://earthobservatory.nasa.gov/IOTD/view.php?id=4982, accessed July 4, 2012), and southerly wind events frequently resuspend sediment in the shallow waters of the northern Gulf of Mexico (75). There appeared to be a degree of niche-specific sequestering, as evidenced by the fact that *vvhA* exhibited the highest detection rates in oysters while *tlh* exhibited the highest detection rates in sediment. Thus, *V. parahaemolyticus* and *V. vulnificus* differed in their niches. In addition, the intra-sample dominance of *tlh* in LA as compared to that of *vvhA* in MS and MD samples indicated some state-to-state variability that may merit consideration as model-based risk assessments are further developed; i.e., *vvhA* did not consistently outnumber *tlh* on a within-sample basis, and in the current study this ratio varied by geographic location.

By extending previous work on both vibrio ecology and ecological models for prediction of *Vibrio* spp. abundance in the aquatic environment, it was reaffirmed that temperature is a strong predictor of abundance and distribution of total vibrios (3, 4, 30, 31, 33, 42, 53, 54, 63, 74, 77), and this is particularly useful in the warmer Gulf Coast states included in this study (LA and MS). Though it is clear that temperature is dominant, there is no specific hierarchy among the parameters; environmental factors

346 interact to influence vibrio abundance, but precise details of all such variables and how
347 they interact have yet to be fully described.

348 Despite its dominance with respect to *tlh* and *vvhA*, SST was not a strong
349 predictor for densities of vibrios with the pathogenicity genes *tdh* and *trh* in this study.
350 This finding suggests that environmental factors may differentially affect the abundance
351 of pathogenic subpopulations. This is particularly relevant given previous observations
352 that the percentage of total *V. parahaemolyticus* containing these *tdh* and/or *trh* genes
353 appears to be variable and inversely related to temperature (18, 19, 83).

354 Observed associations between abundance and salinity were minimal despite
355 the relatively wide salinity range of this study. Salinity correlated significantly only with
356 *tlh* in oysters and sediment but with no other measurements in the analysis of data
357 pooled over sampling locations. This was unexpected, given previous observations of
358 significant correlations between salinity and vibrios in samples from Mississippi and
359 Alabama (31). This finding did not appear to an artifact of the pooled data analysis, as
360 analyses of un-pooled data, by sampling location separately, were generally consistent.
361 Specifically, although effects of salinity did not follow the same nonlinear (quadratic)
362 relationship at each sampling location (e.g., due to narrow range of salinity above or
363 below an optimum), the apparent effects at each sampling location were consistent with
364 that of the pooled data, even when the effect overall was identified as not statistically
365 significant. Regression models incorporating an interaction between temperature and
366 salinity were explored but did not significantly improve overall goodness-of-fit or
367 otherwise provide an interpretation for the unexpected findings.

Some studies have identified a significant relationship between vibrios and salinity (6, 12, 30, 31, 60, 69, 79), while others did not (47, 60, 66, 68), so the relationship with salinity may be variable and complex. For example, Griffitt and Grimes (manuscript in preparation) report that large salinity shifts, as seen during the opening of the Louisiana Bonnet Carré Spillway following the Mississippi River floods of 2011, can cause detectible and significant change in the relative numbers of pathogenic vibrios. *In vitro* growth rates of *V. vulnificus* biotypes 1, 2, and 3 (9) have been related to distance from shore (47) with respect to salinity and analyzed to determine the salinity of the coastal ocean and estuaries with respect to vibrio abundance and distribution. Observed differences between studies may also be attributable to different salinity ranges or other factors such as the nutrients sparing the salinity requirement for growth (66).

The statistically significant contributions of chlorophyll and DOC to the vibrios in this study were minimal, but findings for SPM were suggestive of a stronger effect. This is consistent with the significant relationships previously identified between turbidity and *V. parahaemolyticus* abundance (30, 31, 33, 47, 54). A positive association with turbidity is consistent with expectations because vibrios, like many bacteria, are frequently attached when in the aquatic environment (16). A higher density of particulate matter suspended in the water column logically provides habitat for a greater density of vibrios. The current study represents initial efforts to quantify that relationship.

It was surprising that SST was the only factor that was a statistically significant predictor of *vvhA* density in any sample type, even when accounting for the paucity of

391 *vvhA* in Washington. We and others have previously demonstrated relationships
392 between *vvhA* and environmental parameters including temperature, salinity, and
393 chlorophyll (31). Also interesting, WA samples exhibited the lowest median chl-*a*, DOC,
394 and SPM levels, as well as the highest salinities.
395 The proportions of *tdh* and *trh* in the Pacific Northwest as measured by DP/CH
396 were high compared to total *V. parahaemolyticus* (i.e., *tlh*+), suggesting that *tdh*+ and/or
397 *trh*+ *V. parahaemolyticus* are present in very large numbers. The relatively high
398 salinities in the Pacific Northwest were concluded to be unrelated to the high *tdh*/*trh*
399 rates because similar salinities observed at the other sampling sites in this study were
400 not associated with high rates of *tdh*/*trh* detection (data not shown). To investigate the
401 possibility of cross-reactivity with other vibrios in the Pacific Northwest, a small subset of
402 vibrios in the GC collection was queried. Of the 23 vibrios containing *trh*, only two were
403 identified as *V. alginolyticus*, with the remaining 21 identified as *V. parahaemolyticus*;
404 *tdh* was only found in *V. parahaemolyticus* (Rachel Clostio, personal communication).
405 Other studies of WA *tdh*+ and *trh*+ strains, including strain genotyping
406 (Paranjpye, et al., manuscript in preparation) and both multilocus sequence typing and
407 complete genomic sequencing (Turner, et al., manuscripts in preparation), demonstrate
408 that strains in the Pacific Northwest carrying *tlh*, *tdh*, and *trh* are indeed *V.*
409 *parahaemolyticus*. Thus, the explanation for the high *tdh*/*trh* rates in the Pacific
410 Northwest as measured by DP/CH remains unknown.
411 The unforeseen need for the asymmetrical treatment of DP/CH results from
412 Washington due to unexpectedly high rates of *tdh* and *trh* DP/CH results was deemed
413 acceptable and as contributing minimal artifacts because both DP/CH and PCR/MPN

methods target the same genes (49). The PCR/MPN method does include additional regions of specificity by its nature because it includes two oligonucleotide primers and a fluorescent probe while DP/CH only includes an alkaline phosphatase-conjugated probe that binds to the region targeted by the forward PCR primer. However, potential variability and artifacts were minimized by treating all three sites in the same manner where possible, i.e., only including PCR/MPN results for *tdh* and *trh* gene targets.

Future studies will address the impact of individual parameters on vibrio abundance, for which microcosm studies have been initiated at the University of Maryland that address molecular genetic determination of the vibrios indigenous to the respective geographic regions of this study, and these results will be presented elsewhere. Additional data will also be analyzed as a result of a recently concluded concurrent sampling regime in the four sampling states. A focus of analysis of these data will be exploring possible differences in relationships between vibrio abundance and predictor variables across sampling locations, and this will provide further insight about the initial assessment based on pooling of data. Findings from microcosm study will be evaluated to better inform model selection in the analysis of field study observations. Zooplankton and phytoplankton densities and relationships with additional pigments indicative of phytoplankton will also be analyzed. A sufficiently large complement of data will facilitate identification of statistical models that are both interpretable and provide for the best possible predictive value.

In conclusion, the microbial ecology of selected *Vibrio* spp. has been extensively studied to determine the importance of specific environmental parameters influencing the incidence, distribution, and abundance of total and pathogenic vibrios. This study

437 builds upon existing data sets and findings by including an exceptionally wide range of
438 geographic regions, vibrio densities, seasons, and environmental parameters not
439 studied previously. Maximizing the size of the study made it possible to study
440 parameter ranges that cannot be investigated by studying only a single study site. This
441 study confirmed some previously reported findings (e.g., the impact of temperature) but
442 also identified some new findings (e.g., the differences in the strength of correlation of
443 *V. parahaemolyticus* and *V. vulnificus* densities to environmental parameters).
444 Diversifying the geographic niches included in this study improves the chances of
445 identifying environmental signatures that can be used to predict and possibly prevent
446 vibrio outbreaks in a wide and possibly global range of geographic locations.
447
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449
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744 Appl Environ Microbiol **73:**7589-96.
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747 **Tables.**

Table 1. Pooled data as pooled for WA, GC, and MD according to gene target.

Gene	Sample	PCR/MPN method	DP/CH method
tlh	water	WA, GC, MD[1]	GC, MD
	oyster	WA, GC, MD	GC, MD
	sediment		WA, GC, MD
tdh	water	WA, GC, MD	
	oyster	WA, GC, MD	
	sediment		WA, GC, MD
trh	water	WA, GC, MD	
	oyster	WA, GC, MD	
	sediment		WA, GC, MD
vvhA[2]	water		GC, MD
	oyster		GC, MD
	sediment		GC, MD

[1]WA = Washington; GC = Gulf Coast; MD = Maryland; MPN = most probable number; DP/CH = direct plating/colony hybridization; [2] *vvhA* data for all WA sample sources for WA were omitted.

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Table 2. Relative importance of environmental parameters for all sampling locations combined.

	tlh			*vvhA*			*tdh*			*trh*		
	wat	**oys**	**sed**	**wat**	**oys**	**sed**	**wat**	**oys**	**sed**	**wat**	**oys**	**sed**
log DOC	1.88	**13.1**	0.96	1.56	2.05	4.36	**15.3**	4.17	5.05	7.77	5.89	**12.4**
log Chla	1.98	0.63	**5.01**	1.29	2.55	1.38	12.1	6.31	**22.0**	3.11	**12.9**	**9.80**
log SPM	**15.6**	**5.99**	**7.40**	2.38	2.80	3.80	**22.9**	**28.9**	1.91	8.64	**17.4**	**10.1**
SalBoth	2.45	**8.98**	**3.70**	3.56	3.72	2.60	30.7	5.24	4.17	5.78	4.72	4.79
SST	**11.0**	**18.0**	**34.3**	**11.5**	**27.6**	**9.00**	4.65	3.65	**11.2**	**14.2**	7.72	**7.05**

Relative importance of environmental parameters based on pseudo-R^2 statistics derived from GLMM analysis. SalBoth = both salinity and quadratic salinity combined; bold = $P < 0.05$.

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Table 3. Summary statistics for *V. parahaemolyticus* and *V. vulnificus* densities

	Probe	Range in CFU/mL or CFU/g for respective DP/CH probe (Median)	DP/CH Detects (%)
Water	*vvh*	<1 - >250 (6.0)	79.2
	tlh	<1 - 204 (1.5)	69.5
	tdh	<1 - 66 (<1)	18.1
	trh	<1 - 39 (<1)	19.7
Oysters	*vvh*	<10 - >2.5E4 (673.9)	86.3
	tlh	<10 - 2.2E4 (186)	81.5
	tdh	<10 - 241 (<10)	24.8
	trh	<10 - 982 (<10)	34.9
Sediment	*vvh*	<100 - >8.3E4 (525)	61
	tlh	<20 - >8.3E4 (715)	89.7
	tdh	<20 - 2.4E3 (25)	61.3
	trh	<20 - 3.5E3 (50)	64.2

tlh, thermolabile hemolysin; *tdh,* thermostable direct hemolysin; *trh, tdh*-related hemolysin; *vvh*, *V. vulnificus* hemolysin; DP/CH, direct plating/colony hybridization. CFU/mL and CFU/g data are from DP/CH.

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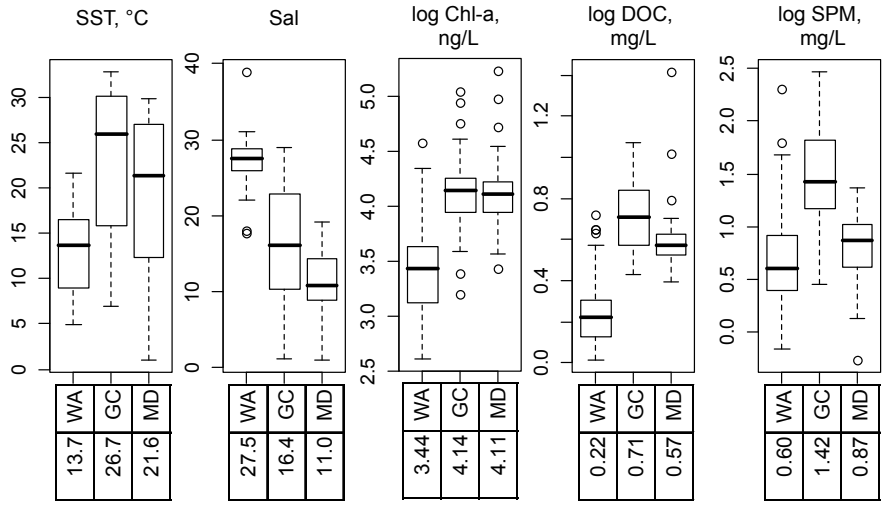

Figure 1.

Figure 1. Box plots of sea surface temperature (SST), salinity (Sal), chlorophyll-*a* (Chl-*a*), dissolved organic carbon (DOC), and SPM (suspended particulate matter) in Washington (WA), Mississippi + Louisiana (Gulf Coast, GC), and Maryland (MD). Box plots summarize distribution by indication of the maximum, 75th percentile, median, 25th percentile, and minimum values. Additional circles indicate outlier values identified by the statistical package R. Points more than 1.5 times the interquartile range above the third quartile or below the first quartile were plotted individually as outliers. Median values are indicated below the graphs.

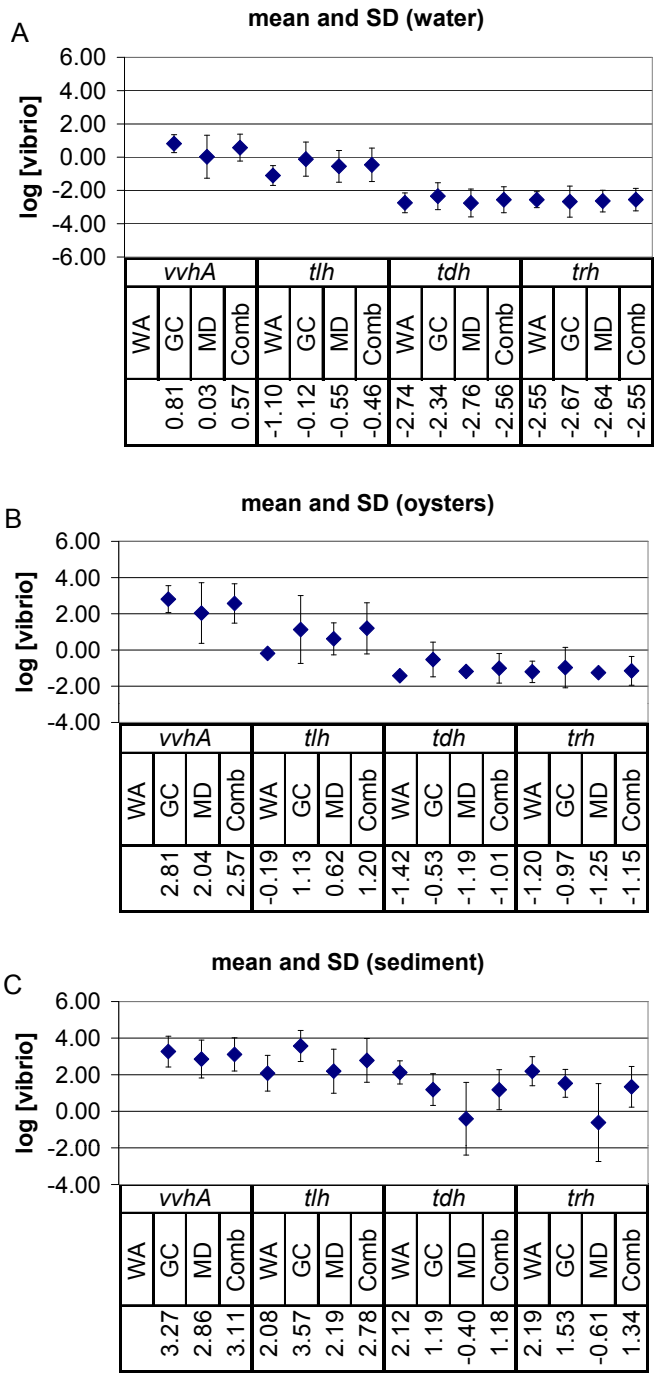

Figure 2.

Figure 2. Model-based estimates of mean and standard deviation (SD) of log abundance by location and gene target. Estimates of mean and SD of log CFUs/mL (water) and log CFUs/g (oysters and sediment) are based on measurements from DP/CH (all *vvhA* and sediment data points), from real-time PCR/MPN (*tdh* and *trh* in all water/oyster, and *tlh* in WA water/oyster), or from both (*tlh* in GC water/oyster and MD water/oyster). Means are presented with standard deviation of the distributions and not standard error of means.

Figure 3A. The *tlh:vvhA* densities for water samples.

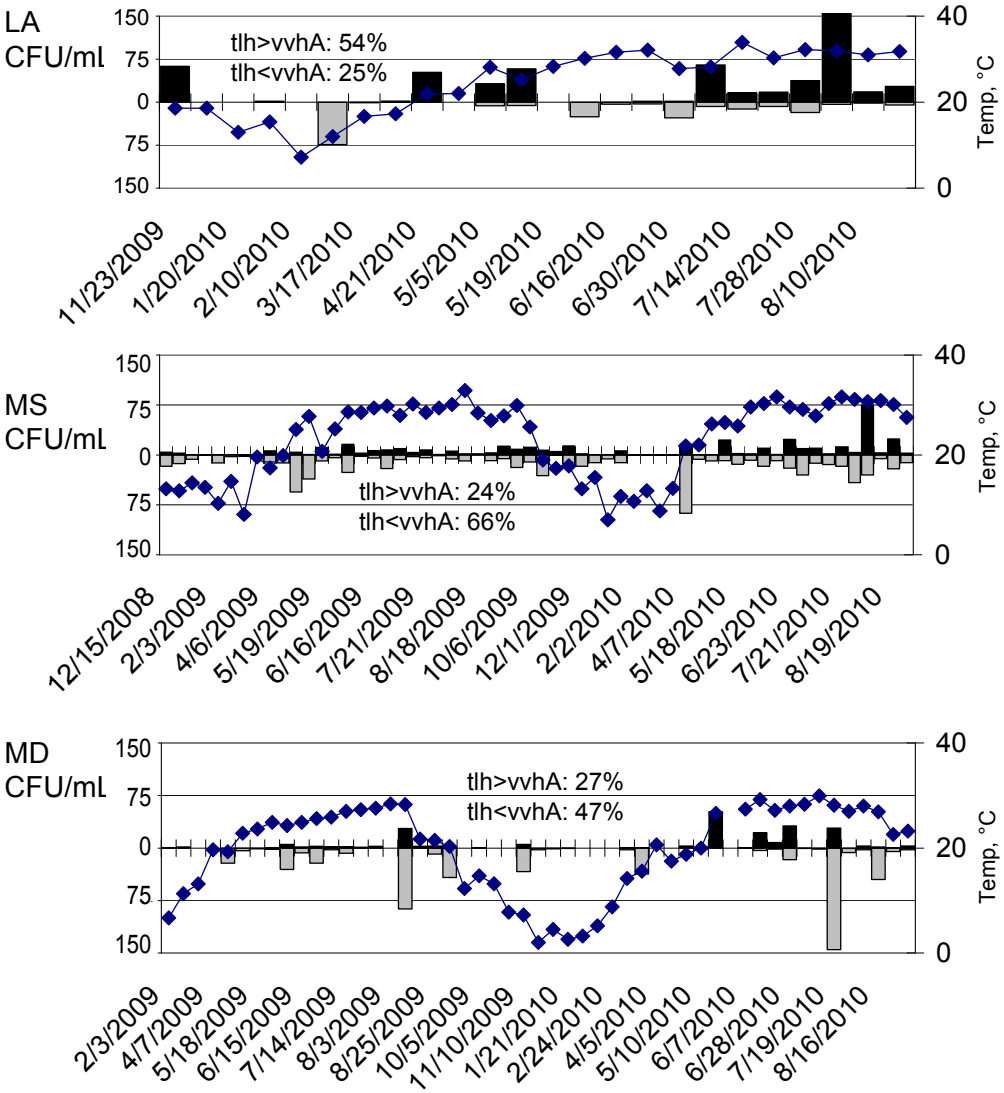

Figure 3B. The *tlh:vvhA* densities in oyster samples.

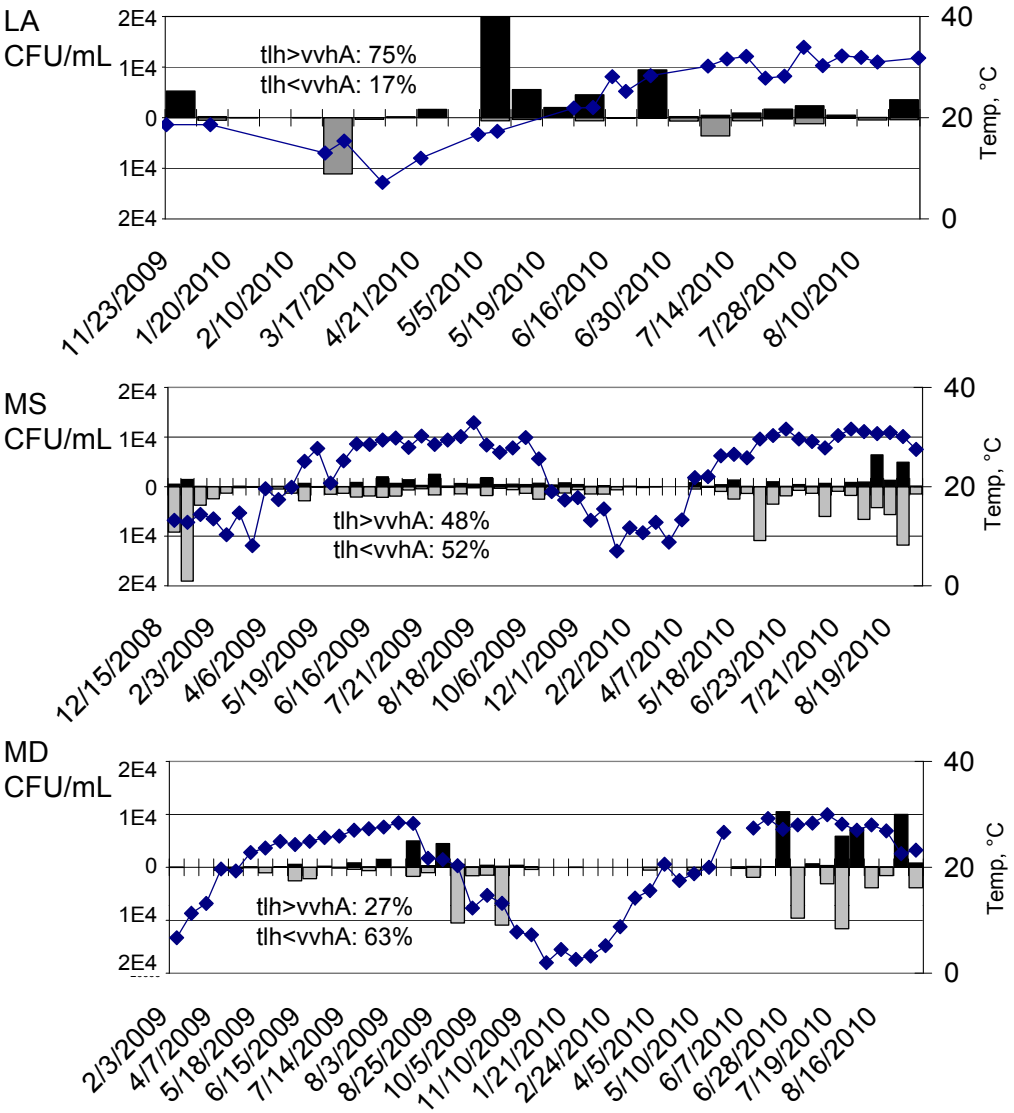

Figure 3C. The *tlh:vvhA* densities in sediment samples.

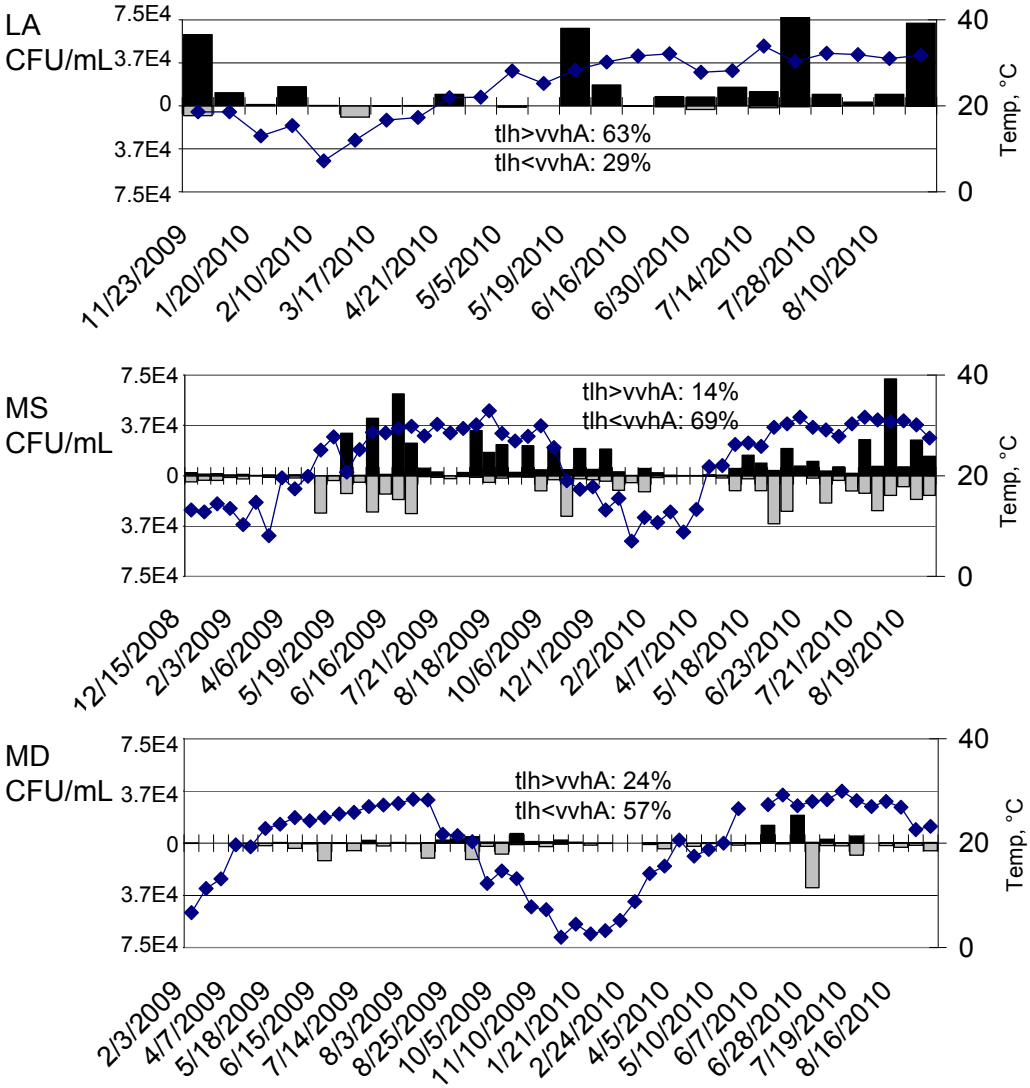

Figure 3. The *tlh*:*vvhA* relative densities by habitat and location. The DP/CH-derived *tlh* densities were compared to DP/CH-derived *vvhA* densities on a sample-to-sample basis for water (A), oysters (B), and sediment (C). Black bars = *tlh* densities; gray bars = *vvhA* densities; diamond lines = sea surface temperature in ºC plotted on secondary (right) y-axis. WA data were excluded from these graphs because of the lack of *vvhA* counts.