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Investigation of Reservoirs of Fecal Indicator Bacteria and Water Quality on the Presence of Allochthonous Pathogens and the Ecology and Virulence of Vibrio vulnificus

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Investigation of Reservoirs of Fecal Indicator Bacteria and Water Quality on the Presence of Allochthonous Pathogens and the Ecology and Virulence of *Vibrio vulnificus*

by

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DEDICATION

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ABSTRACT

The quality of recreational and shellfishing waters has historically been monitored using commensal, allochthonous bacteria shed in feces (fecal indicator bacteria, FIB). The fate of FIB in the environment should mimic that of bacterial, protozoan, and viral human pathogens, which may also be allochthonous (*e.g. Salmonella, Cryptosporidium,* or enteric viruses) or autochthonous (*e.g. Vibrio* spp.) to aquatic environments. FIB are contributed to water from human and animal sources; however, pollution source cannot be determined by conventional FIB measurements. Because fecal source determination is important for pollution remediation and assessment of human health risks, microbial source tracking (MST) methods are increasingly used in water quality studies.

The host-specific genes (markers) used for MST include the 16S rRNA of *Bacteroides* HF183 and the T-antigen of human polyomaviruses (HPyVs). In my work, correlations among FIB, MST markers, and autochthonous pathogens were explored in the context of factors that may influence these relationships. Specifically, the effects of stormwater runoff, sediment resuspension, and survival/persistence of FIB on submerged aquatic vegetation were investigated in a recreational lake. Furthermore, the relationship between FIB and concentrations of the autochthonous pathogen, *V. vulnificus*, was investigated at water bodies surrounding Tampa Bay. I hypothesized that degraded water quality would influence the concentration and/or population structure of *V. vulnificus,* a potentially lethal human pathogen. Finally, I hypothesized that the gene encoding a

sodium-phosphate transporter (*nptA*) would be differentially expressed in *V. vulnificus* strains under varying conditions of salinity and phosphate concentration.

I hypothesized that stormwater infrastructure/runoff, SAV, and sediments would serve as reservoirs for FIB, human-associated microbes (HF183 and HPyVs), and allochthonous pathogens (*Salmonella*, *Cryptosporidium*, *Giardia*, and enteric viruses). FIB concentrations in the water were positively associated with those in the sediment, SAV, and with 24hr antecedent rainfall. At least one MST marker or pathogen was found in 35% of samples following rain events. These data were incorporated into a Bayesian model, which predicted pathogen absence when fecal coliform concentrations were low. Stormwater was also shown to be an important reservoir/conveyance system for FIB, human-associated microbes, and pathogens.

I hypothesized that polluted estuarine waters in Tampa Bay, and oysters harvested from them, would contain higher *V. vulnificus* concentrations, and that the population structure would be altered compared to unpolluted waters. Enumeration included direct plating, enrichment followed by plating, and quantitative PCR (qPCR). *V. vulnificus* colonies isolated directly on mCPC agar were rarely PCR-confirmed, although enrichment and qPCR methods yielded a higher confirmation frequency. Unconfirmed colonies resembling *V. vulnificus* were identified as *V. sinaloensis* via 16S rRNA sequence analysis and were more frequently detected in less polluted waters. Comparison of growth rates among *V. vulnificus* and *V. sinaloensis* strains in enrichment media and seawater showed that *V. vulnificus* had faster growth rates (μ) in enrichment media, but that μ of *V. sinaloensis* strains was greater in seawater. *V. sinaloensis* presence can therefore lead to overestimation of *V. vulnificus* concentrations when

samples are directly plated. These results highlight a need for better understanding of the ecology and virulence potential of this newly-described species.

Finally, I hypothesized that *V. vulnificus* strains with varying virulence potential would differentially express the *nptA* gene in response to changes in environmental conditions. Expression studies were performed on biotype 1, 2, and 3 strains, and strains more closely associated with environmental reservoirs (water or oysters) showed up to 100-fold greater *nptA* expression than strains isolated from clinical cases. Gene expression in environmentally-associated, but not clinically-isolated, strains was highest in media at pH 6.0 vs. those at pH ≥ 7.0 and at 10‰ salinity. In contrast, expression was highest among clinical strains at 10‰ salinity, pH 8.0 media. Sequence analysis of the *nptA* gene also divided strains into environmentally- and clinically-isolated groups. These results suggest that differences in gene expression may be related to host preference and may be associated with differential virulence of strains in humans.

These studies demonstrate a relationship between water quality (determined by FIB concentrations) and the prevalence of allochthonous and autochthonous human pathogens, and reveal that many environmental habitats may serve as reservoirs for FIB and pathogens. Differences in water quality were further demonstrated to impact the community structure of *Vibrio* spp. and may affect the relative abundance of strains with greater virulence potential.

CHAPTER 1: BACKGROUND AND RESEARCH OVERVIEW

Water Quality and Risk to Human Health

Fecal contamination of waters poses a significant health risk due to the possible contribution of human pathogens from human and animal sources (29, 37, 163). Furthermore, fecal contamination as well as stormwater runoff may also increase nutrient loads, allowing autochthonous pathogens such as *Vibrio* spp. to grow to higher concentrations than they would in unimpacted waters (159). Fecal indicator bacteria and human pathogens, including bacteria, protozoa and viruses in the water may also be concentrated in shellfish, creating a foodborne as well as a waterborne risk to public health (21, 41, 129). This issue can be particularly serious when *V. vulnificus* is involved due to the high mortality rate (75). Monitoring for all bacterial, protozoan, and viral pathogens, however, is impractical due to the multitude of potential targets as well as the prohibitive cost and labor which would be involved. Fecal indicator bacteria (FIB) including fecal coliforms, *Escherichia coli*, and enterococci have therefore historically been used as a measure of ambient water quality (2, 78, 153, 156, 157).

FIB are commensal organisms that are shed in high concentrations in feces, and elevated concentrations of these bacteria have been associated with elevated risk for waterborne disease as well as the presence of pathogens (78, 162, 163, 179). In a meta-analysis of previous epidemiological studies, Zmirou, *et al.* found that elevated concentrations of fecal coliforms, *E. coli*, and enterococci were all associated with elevated risk for gastrointestinal (GI) illness, and that enterococci served as a better

indicator for health risk than fecal coliforms (179). Wade, *et al.* also found, among previous epidemiological studies, that enterococci concentrations were predictive of GI illness in marine environments, but that *E. coli* concentrations were more consistent indicators of health risk in fresh water (163). In a study at Lakes Michigan and Erie, enterococci concentrations, determined by quantitative PCR (qPCR) rather than standard culture-based enumeration methods, showed a significant positive association with increased GI illness among bathers, and a trend linking elevated *Bacteroidales* concentrations and GI illness was also noticed (162). A drawback to a majority of these studies, however, is that contamination was known to be from human sources, so the effectiveness of FIB at predicting health risk from non-point source contamination is still uncertain. In an epidemiological study at Mission Bay, CA, where pollution was contributed from non-point sources, concentrations of enterococci, fecal coliforms, and total coliforms were not linked to increased risk of GI illness (40). While these studies did not investigate the particular, presumably allochthonous, etiological agents causing GI illness, studies which have focused on the relationship between FIB concentrations and specific pathogen presence have reported an imperfect association between the two (3, 19, 66, 97, 103).

Other groups have focused on the relationship between FIB and autochthonous pathogens, specifically, *V. vulnificus* (76, 160). In Danish waters when the water temperature was frequently < 20° C, *V. vulnificus* occurrence was shown to be significantly associated with the presence of coliform bacteria as well as enterococci (P = 0.0015 and 0.022, respectively), but not with *E. coli* (76). Similarly, a study of *V. vulnificus* in Hawaiian coastal streams revealed positive correlations between *V.*

vulnificus densities and indicators of fecal contamination including enterococci (P = 0.001), *E. coli* (P = 0.037), *Clostridium perfringens* (P < 0.001), and F^+ coliphage (P = 0.026) (160). Unfortunately, studies linking *V. vulnificus* and FIB concentrations are relatively rare in the literature, with a majority conducted in United States finding no correlation between *V. vulnificus* and either fecal or total coliforms (84, 120, 123, 145).

Reasons for the lack of correlation between FIB and allochthonous or autochthonous pathogen presence may be due to the fact that FIB are also contributed from animal sources (52, 70, 93, 151, 169) or, in the case of autochthonous pathogens such as vibrios, factors such as temperature or salinity may have more significant effects on concentrations (82, 110, 120, 123). FIB may also persist and grow in sediments and submerged aquatic vegetation (3, 6, 27, 72, 137), or be contributed from stormwater runoff (23, 80). Several non-point sources including stormwater and agricultural runoff are of particular concern for recreational and commercial water quality due to the contribution of not only FIB and pathogens (23, 57, 80, 114), but also to nutrient loading of nitrogen and phosphorous leading to the eutrophication of water bodies (126, 132). Increased nitrogen and phosphorous concentrations in both fresh and marine waters have been shown to have positive effects on bacterial growth (50). Furthermore, higher nitrogen and phosphorous levels have been suggested to increase the replication rate of aquatic viruses as well, and may influence the virulence and survival of other autochthonous pathogens (136, 168).

To address the issue of fecal source identification when fecal contamination is present, methodologies for microbial source tracking (MST) have been developed to determine sources of fecal pollution [reviewed in (10, 68)]. MST methods identify

contamination source via identification of i) specific phenotypes or genotypes of bacteria which are closely associated with particular host species or ii) specific genetic targets for microbes known to be closely associated with a particular host (141). These methods may be library-dependent or library-independent. Library-dependent methods require collection of a large number of isolates from a variety of sources and characterization of these isolates for some discriminatory attribute such as antibiotic resistance (68) or genotype (122). Due to the labor-intensive nature and high cost of library-dependent methods, recent focus has been on the development of library-independent methods, which generally target specific genes associated with host-associated microbes (10, 106, 107). These methods have been successful at identifying specific sources of fecal contamination, which can inform remediation strategies for impaired water bodies (88, 89).

Vibrio vulnificus in the Environment

Vibrio vulnificus is a Gram-negative, halophilic, opportunistic pathogen of humans. The bacterium is autochthonous in estuarine and marine waters and shellfish (24, 79, 171). In water, concentrations ranging from 3×10^1 to 2×10^2 CFU $\times$ ml^{-1} have been reported in Chesapeake Bay (171), concentrations $> 2.5 \times 10^2$ CFU $\times$ ml^{-1} were reported in the Northern Gulf of Mexico (82), and concentrations in Florida estuaries have ranged from undetectable to 4.6×10^2 MPN $\times$ ml^{-1} (145). In oysters, concentrations were reported over a larger range - < 10 to approximately $10^3 - 10^4$ MPN $\times$ g^{-1} among oysters harvested in southeastern states (110), up to 2.8×10^3 CFU $\times$ g^{-1} in the Northern Gulf of Mexico (82), and between < 1 to 10^5 MPN $\times$ g^{-1} in oysters harvested from Apalachicola Bay (79). Furthermore, diversity in individual oysters has been reported to

be extremely high with > 100 strains isolated from a single oyster (25, 140). Importantly, temperature (> 15° C) has been nearly universally reported to be a key factor controlling *V. vulnificus* densities (82, 123, 124, 145), with unculturable concentrations reported at colder temperatures (82, 110, 145). Salinity is also an important variable affecting the recovery of culturable *V. vulnificus*, where a majority of studies report isolation of the species from the water at salinities between 8 and 23 ‰ (76, 82, 110, 145). Previous studies also have shown a positive correlation between *V. vulnificus* concentrations and those of FIB (76, 160); however, these results are not consistent in the literature (84, 120, 123, 145).

Vibrio vulnificus exhibits a high degree of genetic diversity and was originally divided into two biotypes based on pathogenicity to specific hosts, where biotype 1 was pathogenic to humans while biotype 2 was associated with fish or eel pathogenesis (150). Phenotypically, biotype 2 strains were originally distinguished from biotype 1 strains via negative reactions for indole production, ornithine decarboxylase activity, acid production from mannitol and sorbitol, and growth at 42º C (12, 150). While predominantly associated with eel pathogenesis, biotype 2 strains have also been implicated in human infection (1). Biotype 1 strains are known to be globally distributed while biotype 2 strains are generally isolated from European waters where fish aquaculture is more prevalent, although isolates have been recovered from Asia (130). More recently, a third biotype that is phenotypically distinct from biotypes 1 and 2, but believed to be a genetic hybrid of these biotypes, was implicated in wound infections associated with handling *Tilapia* (14, 15). Biotype 3 strains are presently believed to be geographically limited to Israel (14).

Vibrio vulnificus Pathogenicity

Vibrio vulnificus is the leading cause of death following consumption of raw or undercooked shellfish in the United States (47, 75), but infection may also occur through exposure to open wounds (30, 33). From 2003 to 2004, 142 cases of vibrio-related waterborne disease cases were reported. *Vibrio vulnificus* cases had higher rates of hospitalization (87.2%) and mortality (12.8%) than other *Vibrio* spp. including *V. parahaemolyticus*. From 1998 to 2007, 276 cases of *V. vulnificus* infections were reported in Florida resulting in 76 deaths (166). Immunocompromised individuals and those with liver diseases are at the highest risk of infection (16, 166). Symptoms due to consumption of *V. vulnificus* range from gastroenteritis to primary septicemia, and mortality rates greater than 50% have been reported for patients with septicemia (83). These individuals face the possibility of death within 24 hours of exposure to *V. vulnificus* (74). Wound infections may result in skin lesions or muscle damage which may lead to amputation (16). Antibiotic treatment has been shown to be an effective treatment for infection (75).

Despite high diversity among *V. vulnificus* strains, only single strains have been isolated from clinical cases, suggesting that strains exhibit differing virulence potential (79). Several groups have attempted to establish methods to distinguish more highly virulent strains. These methods are based on differences in the 16S rRNA sequence (5, 112), heterogeneity in a virulence correlated gene (*vcg*) (128), lineage as determined by multilocus sequence typing (39), and genotypic comparison (35). Exploitation of heterogeneity in a conserved hypothetical protein (*hypB*) has also been well correlated with previously established typing methodologies (139, 140). In fact, all typing strategies

are highly correlated with each other (*i.e.* 16S rRNA typing, *vcg* typing, typing by lineage, and *hypB* typing generally all result in assignment of a strain to an environmentally- or clinically-associated strain type with few exceptions) (39, 140). While the origin of strains (*e.g.* clinical isolates vs. those recovered from environmental reservoirs such as water or oysters) are generally highly correlated with strain type by these methods, associations between strain type and virulence are imperfect. Water quality, temperature, and salinity have been shown to affect the relative distribution of environmentally- vs. clinically-associated strain types, but studies assessing these relationships are limited (60, 86, 99). One of the main hypotheses of the current work is that poorer water quality may harbor higher proportions of clinically-associated strains than environmentally-associated strains, which has been previously suggested (60). Nutrient loading, in the form of fecal contamination, may increase availability of nitrogen and phosphorous, which have been previously suggested to enhance virulence and abundance of native bacteria, especially pathogens (136). Furthermore, anthropogenic impacts to water chemistry including sewage contamination have been suggested to impact the abundance of hosts such as copepods for *V. cholerae*, as well as other *Vibrio* spp., which may contribute to their survival or increase in contaminated waters (42).

Virulence Factors of *Vibrio vulnificus*

Despite strain-specific differences in virulence potential among *V.* vulnificus, the virulence mechanism(s) is not well understood. Several literature reviews have been conducted to investigate the relative importance of putative virulence factors investigated; however, a clear consensus regarding those genes which are essential for strain pathogenicity and elevated virulence potential is still lacking (62, 83, 100, 142).

The capsular polysaccharide (CPS) is regarded as the primary virulence factor required for infection (175, 177); however, all encapsulated strains are not equally virulent (109). Strains may also undergo phase variation from encapsulated to un-encapsulated phenotypes where the un-encapsulated types show decreased virulence in mice (135, 175).

Iron availability has also been related to strain virulence (174). Siderophores (encoded by *venB* and *viuB* genes), which scavenge iron from host cells, have also been implicated in strain virulence. A mutant lacking *venB* showed reduced virulence in an infant mouse model, and virulence was restored following complement mutation (101). Similarly, the *viuB* gene (encoding the vulnibactin siderophore) was identified preferentially in *vcg*C (clinically-associated) strains, and strains carrying this gene were more resistant to lysis by complement-active host serum (18). In this study, *vcg*E (environmentally-associated) strains lacking *viuB* survived comparably to *vcg*C strains in complement-inactivated serum, and, following the addition of iron to complement-active serum, no significant difference in survival was observed between strain types (18).

A hemolysin/cytolysin and metalloprotease (encoded by *vvhA* and *vvpE*, respectively) have been identified as potential virulence factors; however, disruption of these genes did not result in a significant decline in virulence (173). Knockout of another hemolysin (encoded by *hlyIII*) resulted in attentuation of virulence in a mouse model (36). Disruption of the hemolysin/cytolysin and metalloprotease genes (*vvhA* and *vvpE*) did not result in a decline in virulence, but further mutation to disrupt the *flgC* gene, encoding a portion of the flagellar basal body, resulted in attenuation of virulence, presumably due to inhibition of the bacterium's ability to adhere to host cells (87).

The RtxA toxin's potential as a virulence factor has also been recently investigated, and *rtxA*$^{+}$ strains have been shown *in vitro* to damage various cell types; however, disruption of the gene did not result in significant attenuation of virulence *in vivo* in the mouse model (20, 96). The *rtxE* gene, which is part of a separate *rtxBDE* operon whose expression is believed to be induced by exposure to epithelial cells, has been identified as essential to the secretion of the RtxA toxin. Mutant strains with disrupted *rtxE* genes show a significantly lower 50% lethal dose (LD_{50}) in mice as well as a significant reduction in cytotoxicity on epithelial cells *in vitro* (95). Furthermore, INT-407 human intestinal epithelial cells infected with a Δ*rtxE*-mutant strain exhibited a decreased immune response (lower levels of interleukin-8 production and nuclear factor-κB activation and binding) compared to cells infected with the wild-type strain (94).

Specific aims

Research presented here investigates the relationship between water quality, as determined by FIB concentrations, to the prevalence and concentration of both allochthonous and autochthonous human pathogens. Further emphasis is placed on the effect of water quality on the community structure of *Vibrio* spp. as well as genotypic differences between *V. vulnificus* strains which may be associated with differential strain virulence.

Studies described in this dissertation have addressed hypotheses relating to potential reservoirs of FIB, MST markers, and allochthonous pathogens. The stormwater infrastructure and runoff connected to Lake Carroll, a suburban freshwater lake used extensively for recreation, were hypothesized to be important reservoirs/conveyance systems for FIB, MST markers, and allochthonous pathogens into the lake. Furthermore,

sediment resuspension as a result of recreational bathing was suspected of elevating waterborne concentrations of FIB, and SAV, which frequently washes ashore, was also suspected of providing a reservoir for a native enterococci population in this lake. The impacts of temperature, salinity, and water quality surrounding the Tampa Bay area were also hypothesized to influence densities of *V. vulnificus* in water and oyster samples, with high concentrations supported in more contaminated waters. The discovery of *V. sinaloensis* led to further conjecture that this species may be a more dominant member of the *Vibrio* community in water and oysters around Tampa Bay due its ability to grow more rapidly than *V. vulnificus* in certain environmental reservoirs. Finally, differential expression of the *nptA* gene, encoding a sodium-phosphate cotransporter, among varying strains of *V. vulnificus* was hypothesized to play a role in strain virulence or host colonization, a role previously suggested for this gene in *V. cholerae* (92). Factors such as salinity, phosphate, and pH were expected to influence expression of *nptA* differently among strain types.

Specific aims of these studies include:

- Assessment of the effects of sediment resuspension, SAV, and stormwater runoff as sources of FIB, MST markers, and allochthonous pathogens (*e.g. Salmonella, Cryptosporidium, Giardia*, and enteric viruses) to Lake Carroll.
- Determination of the correlation of fecal contamination (determined using FIB) with densities of *V. vulnificus* in tidally-influenced brackish, estuarine, and marine waters and oysters harvested from them.

- Characterization of other *Vibrio* spp. which may confound traditional phenotypic and genotypic methods for enumeration of *V. vulnificus* in water and oysters.
- Evaluation of strain-dependent differences in expression of a sodium-phosphate transport (encoded by *nptA*) which is hypothesized to influence virulence in *V. cholerae*.
- Assessment of how various factors including salinity, phosphate concentration, and pH affect expression of the *nptA* gene in a variety of *V. vulnificus* strains representing all three biotypes.

Research Significance

The research goals and objectives identified will provide useful insights regarding reservoirs of FIB, MST markers, and allochthonous pathogens in an inland Florida lake. The study present will advance knowledge of fecal contamination as well as human pathogens which are contributed as a result of stormwater runoff. Furthermore, the use of sediments and SAV to act as reservoirs of FIB leading to the persistence of a native population of enterococci in this lake highlights a fault in the FIB paradigm and suggests the usefulness of using a toolbox of MST tools to accurately assess water quality and human health risk. The intended benefit of these findings is to better inform resource managers of tools which may be used, as well as potential limitations of traditional methods, in evaluating and remediating recreational water quality.

Evaluation of water quality and physicochemical parameters of watersheds will also provide useful insights as to how these factors interact to affect the ecology and density of autochthonous pathogens such as *V. vulnificus*. Comparison of two culture-

based methods of *V. vulnificus* enumeration (direct plating and MPN enrichment) in conjunction with a rapid qPCR method has also revealed the presence of other vibrios, most prominently *V. sinaloensis*, which are phenotypically indistinguishable from *V. vulnificus*, but which have not been previously identified in water and oysters. Molecular evaluation of *V. sinaloensis* using primer sets previously thought to be specific to *V. vulnificus* highlights a need for more rigorous species confirmation, as *V. sinaloensis* has shown cross-reactivity to these primer sets and may lead to an overestimate of *V. vulnificus* abundance. Furthermore, growth comparison of the two species suggests that while *V. sinaloensis* may outcompete *V. vulnificus* in certain environmental reservoirs, the traditional enrichment medium used does preferentially favor the growth of *V. vulnificus*.

Assessment of differential *nptA* gene expression among *V. vulnificus* strains has provided data that shows for the first time that strains more commonly associated with environmental reservoirs express and regulate this gene similarly, and that the expression pattern is different from strains isolated from clinical cases. This finding may have important implications in understanding *V. vulnificus* virulence as well as the virulence mechanism of other human pathogens which also possess this gene. The gene was shown to be differentially regulated in response to pH and salinity among strain types and was linked to more rapid growth when expressed at higher levels, suggesting that it may play a role in rapid growth during host colonization.

CHATPER 2: ASSESSMENT OF SOURCES OF HUMAN PATHOGENS AND FECAL CONTAMINATION IN A FLORIDA FRESHWATER LAKE[1]

Introduction

Fecal indicator bacteria (FIB) have been used for more than a century as surrogates for human enteric pathogens and are monitored to assess the likelihood of fecal contamination and elevated health risk in recreational waters. Although correlations between elevated FIB concentrations and waterborne illness have been reported (163, 179), weak or no correlation with specific enteric pathogens such as *Salmonella*, *Giardia*, and *Cryptosporidium*, and enteric viruses have also been reported (66, 97). Poor correlations between FIB and pathogens may be due to extended persistence of FIB in environmental waters (3), sediment (6, 26-28), and submerged aquatic vegetation (SAV) (6, 27, 49). Resuspension of FIB from sediments and SAV (3, 6, 28, 43) could potentially lead to beach closures even though human health risk from such reservoirs is very poorly understood (48). Stormwater run-off has also been implicated as a source of FIB (23, 80, 114) and potentially human pathogens (113).

While environmental reservoirs and extended persistence of FIB in the environment confound the utility of FIB as predictors of waterborne pathogens, these bacteria are also known to be harbored in the digestive tracts of most animals, thus providing no interpretation of fecal contamination sources (70). To overcome this

[1]This chapter has been submitted to Water Research. Co-authors included Kenneth H. Reckhow (RTI International, Research Triangle Park, NC), Jerzy Lukasik (Biological Consulting Services, Gainesville FL), and Valerie J. Harwood (University of South Florida, Tampa, FL).

limitation, microbial source tracking (MST) methods which target genes unique to microbes associated with a particular host species or group have been employed to identify sources of fecal contamination (11, 106, 133).

The presence of fecal contamination from human sources has been reported to represent the highest risk to human health compared to pollution from other sources (163). A wide variety of microbial targets have been exploited as MST markers to identify human sources (11, 106, 133). Specific genes from several bacterial species have been identified as human-associated targets for these assays, including the *esp* gene *Enterococcus faecium* (a gram-positive, aerotolerant species) or a segment of the 16S rRNA gene of human-associated *Bacteroides* (HF183) (11, 133). The *esp* gene encodes a putative virulence factor found in strains associated with human illness (133). *Bacteroides* spp. are gram-negative, strict anaerobes and are generally obligate symbionts of the gastrointestinal tract that tend to co-evolve with host species (77), making them well-suited for identification of fecal contamination sources (10). Similarly, assays to detect viral markers indicative of human contamination have also been developed, such as the one to detect the conserved T-antigen of human polyomaviruses (HPyVs) (106). HPyVs are shed in urine and feces of infected individuals, and up to 60% of adults are asymptomatically infected (17, 107, 144). Inclusion of MST assays from morphologically and physiologically diverse microbes is advantageous in studies to detect human contamination as these markers may be subject to differing fates or transport mechanisms in the environment (8, 81), which may prove more or less representative of pathogen presence.

Many human pathogens can be transmitted during recreational water use, including bacteria such as *Salmonella*, protozoa such as *Cryptosporidium* and *Giardia*, and enteric viruses (32). All of these pathogens with the exception of enteric viruses are known to be contributed from both human and non-human sources (52, 151, 169); however, zoonotic infection of humans represents a serious health concern (61, 131, 149). Unfortunately, testing for a multitude of known human pathogens is problematic due to the high cost associated with these assays as well as diversity of known pathogens; however, representative pathogens may provide evidence as to the effectiveness of surrogate markers (*e.g.* FIB or MST markers) (54).

In this study, FIB (fecal coliforms, *Escherichia coli*, and enterococci) were enumerated from waters, sediments, and SAV at Lake Carroll, a freshwater lake with a permitted swimming beach, in Tampa, FL. This lake was selected for study due to frequent exceedences of both fecal coliform and enterococci regulatory standards for recreational waters (56, 67, 154), as well as the many stormwater inputs into this lake. A previous study investigating potential sources for fecal contamination suggested that stormwater runoff may be a significant contributor of FIB, and potentially MST markers for human fecal contamination, particularly at the north end of the lake (67). Furthermore, the results of the previous study suggested that a naturalized population of enterococci persists in the lake's water, sediments, or SAV, which might be disturbed thus reintroducing these bacteria into the water column (6, 67). The primary goals of this study were to i) determine the extent of fecal contamination in the lake and assess whether contamination was of human origin and was indicative of pathogen presence, ii) evaluate the potential for sediments and SAV to act as reservoirs for FIB, iii) determine

whether stormwater runoff or the stormwater infrastructure may serve as a reservoir for FIB, MST markers, or pathogens, and iv) develop a Bayesian model using concentrations of FIB, occurrence of MST markers, frequency of pathogen detection, and physicochemical parameters (*e.g.* temperature and salinity) to determine which factors may be predictive of elevated FIB densities and elevated human health risk, as determined by the presence of human pathogens.

Materials and Methods

Sample Collection. Seven sites at a freshwater lake surrounded by a suburban residential neighborhood in Tampa, FL (28°03'05.63" N, 82°29'14.85" W), were sampled on a monthly basis from April 2008 to March 2009 (Figure 2.1). Sites were selected around the circumference of the lake. A primary criterion for site selection was that the site was located either directly at a stormwater outfall to the lake or in proximity of a stormwater outfall. The stormwater infrastructure upstream of outfalls varied and consisted of a baffle box (site 3), a continuous deflection system (CDS) (site 4), or no treatment (sites 5, 6, 7). Site 6 was located on the southeast of the lake and was expected to be least impacted by stormwater runoff (stormwater outfall was on the opposite bank of the lake, approximately 0.6 km away). Two sites were located on a permitted swimming beach (sites 1 and 2), which was not directly impacted by stormwater, and these sites were sampled to assess potential health risk based on FIB concentrations, MST marker presence, and detection of human pathogens. In addition, the effect of sediment resuspension by bathers on FIB concentrations as well as the presence of MST markers and pathogens was assessed at the beach (site 1).

Ten sampling events were conducted at all sites, with an additional sample event at stormwater outfalls (sites 3, 4, and 5) during a major rain event in December (0.75 inches rain immediately prior to sampling). Because stormwater runoff was hypothesized to be a major source of human fecal contamination and pathogens, an opportunistic sampling strategy was employed to capture peak-flow conditions, where possible, at sites located directly at stormwater outfalls to the lake. Grab samples of water (1 L) were collected in duplicate at each site for FIB and MST analyses. For pathogen analysis, larger volumes were collected or filtered in the field (see below).

Sediment and SAV samples (40 g wet weight) were collected by hand at five and three sites, respectively, for FIB enumeration, and sites were rotated at each sampling event. Site rotation was performed because funding limitations precluded sampling each matrix at each site. Sediment and SAV samples were collected at all sites at least once; however, sampling sites which had high SAV cover were sampled more frequently for SAV while those predominantly uncovered were sampled more frequently for sediments. Rotation allowed for sampling of most sites several times regardless of bottom cover to capture temporal variability in these matrices. All samples were collected in sterile containers, transported in a cooler on ice, and processed within 6 hours of sample collection. Physicochemical parameters (temperature, salinity, dissolved oxygen, pH, and specific conductivity) were also measured at each site. In addition, SAV was collected via a modified rake toss methodology (4) from the shore in order to speciate plants. SAV coverage was determined by visual inspection as described previously (9).

Enumeration of FIB. Fecal indicator bacteria were enumerated from water, sediment, and SAV samples via standard membrane filtration (47 mm nitrocellulose filters, 0.45

µm pore size) for *Escherichia coli* (157), fecal coliforms (2), and enterococci (156). Volumes filtered for FIB, MST, and pathogen analysis were varied based on standard and previously published methods (2, 64, 107, 133, 155-158). Volumes filtered included 1 ml, 10 ml, and 100 ml for water samples. Sediment and SAV samples were diluted 1:10 (wt/vol) in sterile buffered water (2) and sonicated to release bacteria adhering to particle surfaces (3) prior to filtration. Volumes filtered for these matrices included 1 ml, 10 ml, and 25 ml, filtered in duplicate. FIB concentrations were reported as CFU × 100 ml^{-1} for water or CFU × 100 g^{-1} (wet weight) for sediment and SAV.

Sediments were disturbed at the beach by simulating recreational activity, i.e. by walking at approximately calf-depth at the beach site (site 2) during two sampling events in July 2008 and March 2009. This activity followed collection of the initial water sample in order to avoid biasing the monthly sampling results. Water samples (1 L) were collected immediately following disturbance of the sediment, just below the surface of the water, and these samples were processed in duplicate in the same way as water samples.

MST Assays. Water samples were processed using the previously published culture/PCR method for detection of the *esp* gene of *Enterococcus faecium* (88, 89, 133). Detection of HPyVs and HF183 were also performed using previously described, endpoint PCR methods (64, 89). Briefly, water samples were acidified to pH 3.5 with HCl, and 500 ml of sample was concentrated via filtration (nitrocellulose filter, 0.45 µm pore size, 47 mm). Filters were placed in PowerBead tubes of the PowerSoil™ DNA kit (Mobio, Carlsbad, CA) and either processed immediately or frozen at -20°C for up to five days prior to DNA extraction. DNA extraction was carried out following the manufacturer's

instructions for the PowerSoil™ DNA kit (Mobio, Carlsbad, CA). DNA was then used as template using previously described primer sets and PCR reaction conditions (11, 107).

Controls for inhibition were performed using composite water samples from all sites seeded with approximately 100 cells of *Ent. faecium* (C68) into 300 ml of water for *esp* or 192 viral particles of the BK virus (ATCC VR 837) to 500 ml of water for HPyVs and HF183. In addition, each set of PCR reactions included a positive control consisting of 2µl of DNA extracted from *Ent. faecium* C68 for *esp*, or 2µl of plasmid containing the target sequence for either HF183 or HPyVs. Method blanks consisting of sterile buffered water (300 ml for *esp* and 500 ml for HPyVs and HF183) were also subjected to all methodological steps from filtration through PCR as negative controls for contamination. An extraction blank (empty PowerBead tube – no filter added) was subjected to all steps from DNA extraction through PCR as a negative control.

Pathogen Detection. At each sample event, water samples from two sites were analyzed for pathogens (*Salmonella*, *Cryptosporidium*, *Giardia*, and enteric viruses). Pathogen analysis at more than two sites per sampling was cost-prohibitive. Initially (April to November), pathogen sampling was conducted at both beach sites (sites 1 and 2) as these sites were most frequently utilized for recreational bathing. Due to a heavy rain event resulting in peak-flow conditions, however, pathogen sampling was switched from site 1 to site 4 in December to evaluate the potential for the stormwater infrastructure or stormwater runoff to harbor or contribute pathogens, and continued at site 4 through March.

Culture-based detection of *Salmonella* was performed as described previously (89). Briefly, one liter of water was concentrated via filtration (0.45 µm pore size, 45 mm nitrocellulose filter). The filter was then placed in 100 ml buffered peptone water and enriched at 37°C overnight. Twenty milliliters of the enrichment culture was then diluted 1:1 in selective media (2X Rappaport-Vasiliads RV-10 broth; Difco Laboratories Inc., Defroit, MI), and plated on XLT-4 and *Salmonella-Shigella* agar. Putative *Salmonella* colonies (based on morphology and H_2S production) were confirmed via PCR targeting the *invA* gene (89, 108).

For detection of protozoa and enteric viruses, water samples (up to 100 L) were concentrated in the field using a Diaphragm pump (8000 series, Shurflo Manufacturing USA). *Giardia* and *Cryptosporidium* were concentrated by filtration using Envirochek HV cartridge capsule filters (Pall Corp., Port Washington, NY). Following filtration, samples were shipped on ice overnight to Biological Consulting Services (Gainesville, FL) and processed by immunomagnetic separation and immunofluorescent antibody detection according to U.S. EPA method 1623 (158). Results were reported as cysts or oocysts × 100 L^{-1}.

Enteric viruses were concentrated from up to 400 L water by filtration through a Virusorb 1MDS filters (Cuno, Inc. Meriden, CT), which were shipped on ice overnight to Biological Consulting Services (Gainesville, FL) for processing via standard methods and EPA 600/R95/178 (155). Filters were eluted using 1 L of 1.5% beef extract (BBL V) in 0.05 M glycine (pH 9.5, 25°C). The eluate was concentrated by organic flocculation and assayed for enterovirus by the observation of cytopathic effects (CPE) on recently passed (2-4 days) Buffalo Green Monkey (BGM; Passage 120-180) and MA-104 (ATCC CRL-

2378.1) cell lines. All analysis was conducted as per EPA 600/R95/178. (155). Positive and negative controls along with matrix spikes were conducted as indicated in the method. All analysis was done in accordance to NELAC accreditation standards (ISO17025). For both protozoan and viral pathogens, filtration was stopped once the required volume was filtered or after 45 minutes.

Statistical Analysis. FIB concentrations were $\log_{10}$ transformed to obtain a normally distributed dataset. One-way analysis of variance (ANOVA) was used to compared concentrations of FIB among sites and matrices followed by Tukey's *post hoc* test ($\alpha = 0.05$) using GraphPad Instat software, version 3.0 (San Diego, CA). Disturbance experiment data were analyzed via unpaired t-test using average FIB concentrations for each sampling date (two replicates per date). Linear (Pearson) regression and physical data correlation analysis was also performed using this software ($\alpha = 0.05$). Comparison of the frequency (percentage of positive samples) of MST and human pathogen detection was performed via two-tailed Fisher's exact test using GraphPad Instat software, version 3.0 (San Diego, CA). To compare frequency of MST and pathogen detection in wet vs. dry weather conditions, sites which received ≥ 0.4 inches of rain up to 24 hours prior to sampling were considered to be sampled during wet weather conditions. Relationships between MST marker, pathogen presence, and FIB concentrations were determined via binary logistic regression ($\alpha = 0.05$) calculated using PASW software, version 17 (SPSS, Chicago, IL).

Bayesian Analysis. A Bayesian network structure was developed using Hugin software (www.hugin.com; Aalborg, Denmark) relating FIB concentrations, antecedent rainfall one week prior to sampling, temperature, and pathogen presence; these variables were

selected based on a combination of expert judgment and a machine learning algorithm in Hugin. Only data from sites at which pathogens were analyzed (sites 1, 2, and 4) were used in the model. Data for each variable were sorted into four levels or 'bins' to provide an equal distribution of the data across bins (*i.e.,* 25% of the data were placed in each bin); these data were used to generate a prior probability model of the relationships among nodes (see Figure 2.3) (125). Predictive models were then generated to determine the effect of using fecal coliforms or temperature and rainfall as predictive variables (Figure 2.4 and 2.5). In these models, 100% of the data were assigned to a bin and the resultant distribution of each variable given these conditions was determined (*e.g.* is there a higher probability of pathogen absence when fecal coliform concentrations are < 45 CFU × $100ml^{-1}$?).

Results

Characteristics of SAV. Aquatic macrophytes (SAV) were speciated and the percentage of cover was estimated on each sample date. Benthos vegetation cover varied from < 5% (sites 1 and 2) to nearly 100% (site 5). The native species *Vallisneria americana* was identified at several sites; however, the invasive species *Hydrilla verticillata* was the most commonly collected. Interestingly, at sites where no MST markers were detected, the native species persisted, while *Hydrilla* was more abundant at sites where markers were sporadically detected (see MST, below).

Fecal Indicator Bacteria Concentrations. Exceedences of Florida regulatory standards for FIB concentrations were common among all sites and sample dates. Samples collected immediately after a rain event of 0.75 in were considered separately, due to the high flow conditions and high levels of FIB observed on that date. From 69 samples

collected over the course of the study, enterococci concentrations exceeded regulatory guidelines (61 CFU × 100 ml^{-1}) in 65.2% of samples, fecal coliforms exceeded the standard (400 CFU × 100 ml^{-1}) in 44.9% of samples, and *E. coli* exceeded the standard (235 CFU × 100 ml^{-1}) in 27.5% of samples. At the swimming beach sites (1 and 2), enterococci limits were exceeded in 18 of 20 samples (90%), fecal coliform in 7 of 20 (35%), and *E. coli* in 4 of 20 (20%). Excluding the December rain event, fecal coliforms concentrations (CFU × 100 ml^{-1}) ranged from $3.5 \times 10^0 – 4.0 \times 10^4$, *E. coli* from <1.0 (undetected) to 3.8×10^4, and enterococci from $2.0 \times 10^0 – 2.6 \times 10^4$. Concentrations ($log_{10}$-transformed) of FIB at each site over the entire sampling period (including the December rain event) are shown in Figure 2. FIB concentrations during the December rain event exceeded both bacteriological standards at the three sites sampled (sites 3, 4, and 5), with concentrations ranging from 9.0×10^2 to 4.1×10^4 CFU × 100 ml^{-1} for fecal coliforms, 5.0×10^2 to 4.1×10^4 CFU × 100 ml^{-1} for *E. coli*, and 7.5×10^2 to 1.7×10^4 CFU × 100 ml^{-1} for enterococci). No significant differences in FIB concentrations in the water column were observed among the seven sampling sites ($P = 0.68, 0.48, 0.49$ for fecal coliforms, *E. coli*, and enterococci, respectively, excluding December rain event samples; n = 10 for sites 1-4, 6, and 7; n = 9 for site 5). Mean FIB concentrations in SAV and sediment samples throughout the lake were relatively high (ranging from 2.1×10^3 to 8.7×10^4 CFU × 100 g^{-1} for SAV and 1.6×10^2 to 1.8×10^4 CFU × 100 g^{-1} for sediments).

Linear regression analysis was performed to assess the relationship between FIB concentrations in the water column and on SAV or in sediment. Concentrations of both *E. coli* and enterococci on SAV were significantly positively associated with

concentrations of these bacteria in the water column ($r^2 = 0.28$, $P = 0.0030$ and $r^2 = 0.41$, $P = 0.0002$, respectively; n =29 for both), but not for fecal coliforms (n = 29, $r^2 = 0.02$, $P = 0.5204$). Concentrations of all FIB in sediments were positively related to concentrations in the water column ($r^2 = 0.33$, $P < 0.0001$; $r^2 = 0.24$, $P = 0.0004$; and $r^2 = 0.34$, $P < 0.0001$ for fecal coliforms, *E. coli*, and enterococci, respectively; n = 50 for all three).

A disturbance experiment was conducted in the swimming area of the beach site (site 1) in July 2008 and March 2009 to assess the effects of sediment resuspension on FIB concentrations in the water column. Significant increases in enterococci concentrations were observed post-disturbance in the water column on both dates (n = 2), i.e. from 2.85 to 3.43 and from 2.76 to 3.93 $\log_{10}$ CFU × 100 ml^{-1}, on respective dates, (log ratios of disturbed vs. undisturbed concentrations of 1.2 and 1.4 respectively, $P = 0.0371$). Increases in fecal coliforms and *E. coli* were also observed (log ratios ranging from 1.04 – 1.22) but were not statistically significant. Concentrations increased from 3.39 to 3.53 and 1.73 to 2.11 $\log_{10}$ CFU × 100 ml^{-1} for fecal coliforms and *E. coli*, respectively, in July. In March fecal coliform concentrations increased from 0.74 to 0.81 $\log_{10}$ CFU × 100 ml^{-1}, but *E. coli* was undetectable before and after disturbance (< 4.5×10^{-1} CFU × 100 ml^{-1}).

Among physical parameters measured, water temperature showed positive correlations with all FIB ($r^2 = 0.38$, $P < 0.0001$, $r^2 = 0.15$, $P = 0.001$, $r^2 = 0.13$, $P = 0.002$ for fecal coliforms, *E. coli*, and enterococci, respectively). In addition, pH was weakly positively correlated with fecal coliform concentrations in the water column ($r^2 = 0.06$, P

= 0.038), and dissolved oxygen was weakly positively correlated with enterococci concentrations ($r^2 = 0.09$, $P = 0.011$).

Cumulative rainfall 24 hours, 72 hours, and one week prior to sampling was analyzed for correlation with fecal coliform, *E. coli*, and enterococci concentrations in the water (n = 69) and sediment (n = 50) (Table 2.1). In the water column, weak correlations between 24-hour rainfall and fecal coliforms or enterococci were observed among all sites ($r^2 = 0.10$ and 0.09, $P < 0.05$). Antecedent rainfall accumulated over 72 hours was only weakly correlated with enterococci concentrations in the water column ($r^2 = 0.06$, $P = 0.0479$), but no correlation was observed between rainfall accumulated over one week with FIB concentrations. Correlations between rainfall and water column FIB concentrations at two stormwater outfall sites (sites 3 and 7) were stronger for 24-hour and 72-hour antecedent rainfall for fecal coliforms and enterococci (r^2 ranging from 0.50 to 0.88, $P < 0.05$). Only two sites (sites 2 and 3) showed correlations between weekly rainfall and either fecal coliform or enterococci concentrations (r^2 between 0.73 and 0.82, $P < 0.05$). Although peak flow was measured at three stormwater outfalls during a major rain event in December, and ranged from 0.7 – 3.3 cubic meters per sec, these data were not sufficient to produce a robust statistical analysis.

FIB concentrations in the sediment (n = 50) were weakly to moderately positively correlated with rainfall 72 hours and one week prior to sampling among all sites (r^2 from 0.09 to 0.027, $P < 0.031$; Table 2.1), but were rarely correlated with 24 hour antecedent rainfall. At beach site 1, moderate positive correlations were observed between fecal coliform and *E. coli* concentrations in sediment and 72 hour or weekly antecedent rainfall ($r^2 = 0.47$-0.61, $P < 0.03$). At beach site 2, concentrations of all FIB were strongly

associated with rainfall accumulated during these time periods (r^2 = 0.64-0.95); these relationships were significant for fecal coliforms and enterococci, however, the relationship between *E. coli* concentrations and either 72 hour or weekly antecedent rainfall did not quite reach significance (P = 0.0569 and 0.0529 for 72 hour and weekly antecedent rainfall). At site 6, only fecal coliform concentrations in the sediment were moderately correlated with weekly prior rainfall (r^2 = 0.43, P = 0.0394). In terms of 24 hour antecedent rainfall, only enterococci concentrations in the sediment at site 7 were positively correlated (r^2 = 0.69, P = 0.0106).

MST. Human-associated markers for fecal contamination (*esp*, HPyVs, and *Bacteroides* HF183) were detected sporadically at five sites (Table 2.2). The HF183 marker was detected most frequently (10.1% of samples tested) followed by *esp* (7.6%) and HPyVs (6.8%); however, the difference in frequency of detection of MST markers was not significant (P = 0.7445). Co-occurrence of MST markers in any given sample was observed only during rain events in July and December. Detection of all three markers at a site was observed only once in December (site 5), and this was the only time HF183 was detected in conjunction with other MST markers. The *esp* and HPyVs markers were both detected at 28.6% of sites where at least one of these markers was present (n = 7).

No human markers were detected at site 6 during the study period. MST markers were also not detected at one beach site (site 1), although the HF183 marker was detected once at the other (site 2). Human markers were occasionally detected at the remaining sites (20 to 33% of samples per site, Table 2.1). A high proportion of sites (43%, n = 7) tested positive for at least one human-associated MST marker during a rain event in July. To compare frequency of MST marker detection during wet and dry conditions, data

from sites which received ≥ 4 inches of rainfall 24 hours prior to sampling were pooled. MST markers were detected significantly more frequently (37.5%) at sites sampled during wet conditions (n = 16) compared to those sampled during dry conditions (12.5%, n = 56; $P = 0.0140$). Among the MST markers, only detection of the *esp* gene was correlated with FIB concentrations ($r^2 = 0.464$, $P < 0.0001$; $r^2 = 0.194$, $P = 0.013$; $r^2 = 0.39$, $P < 0.0001$ for fecal coliforms, *E. coli*, and enterococci, respectively). Detection of HPyVs was also weakly positively correlated with *esp* detection ($r^2 = 0.149$, $P = 0.018$).

Pathogens. Testing for bacterial, protozoan, and viral pathogens was conducted at beach sites 1 and 2 through the November sampling event (Table 2.2). Beginning with the rain event sampling in December, pathogen testing was moved from the beach (site 1) to a stormwater outfall downstream of the CDS unit (site 4) to assess pathogen contribution from stormwater run-off. Each of the pathogens was detected at least once at each beach site (sites 1 and 2) throughout the study period. *Salmonella* was detected least frequently (10% of samples), while *Cryptosporidium, Giardia*, and enteric viruses were each detected in 20% of samples; however, the differences in detection frequency were not significant ($P = 0.2949$). Pathogens, like MST markers, were detected significantly more frequently during wet weather sampling events (100%, n = 5) compared to dry weather samplings (40%, n = 15; $P = 0.0004$). It is notable that pathogens were detected at both beach sites on each sampling event from June through November (66.7% of samples), when the beach was likely to be used more frequently by recreational bathers.

At sites where pathogen testing was conducted, pathogens were detected significantly more frequently than MST markers (Fisher's exact test, $P = 0.0007$), and detection of pathogens was not correlated with MST marker detection ($P = 0.43$).

Interestingly, when pathogens were detected at a beach site, human MST markers were not detected, although during all sampling events except November, FIB concentrations exceeded Florida State standards for fecal coliforms, enterococci, or both, at both sites. *Salmonella* was detected during the rain event in December at site 4 in conjunction with HPyVs. Both *Giardia* and enteric viruses were detected in January at site 4, although no human MST markers were detected during this sampling. Importantly, among the three sites tested for pathogens during rain events (sites 1 and 2 in July and site 4 in December), at least one human pathogen was detected at each site.

Bayesian Modeling. A Bayesian net model relating the following measured variables (prior probabilities) at sites 1, 2 and 4 was generated: temperature, cumulative antecedent rainfall one week prior to sampling, enterococci, *E. coli,* fecal coliforms, and pathogens (*Salmonella*, *Cryptosporidium*, *Giardia*, or enteric viruses) (Figure 2.3). MST markers were not incorporated into the model due to the low frequency of detection. Each variable and arrow represents a node, and its relationship with another variable. Observations were divided into discrete bins, each representing 25% of all observations. The data in Figures 2.3-5 should be interpreted following this example: for enterococci, 25% of all observations were between 1.9 and 51 CFU × 100 ml^{-1} (lowest bin), and 25% were between 1168 and 25,600 CFU × 100 ml^{-1} (highest bin), while intermediate values were placed in the bins between lowest and highest. Predictive models for Bayesian nets can be derived from the prior models by setting one variable at a given value, and asking what the predicted distribution of the other variables would be given this circumstance. The strongest association between FIB levels and pathogen detection was predicted when fecal coliforms were set at the lowest bin concentration –< 45 CFU × 100 ml^{-1}. In this

circumstance, the probability of pathogen absence was 93% (Figure 2.4). Interestingly, while *E. coli* levels were also predicted to be low under these conditions (100% between 1 and 60 CFU × 100 ml^{-1}), the predicted probability of enterococci levels ranged fairly evenly from low to high levels (i.e., enterococci levels had little relationship with the probability of pathogen presence).

In contrast, when temperature and cumulative antecedent rainfall were the predictive variables and each was fixed at their highest levels (Figure 2.5), the predicted relationship with pathogen detection was much weaker, i.e. the probability of pathogen presence was 59%. This model was more predictive of enterococci and fecal coliform levels, as the probability of levels in the greater 50^{th} percentile (top two bins) was greater than 90% (corresponding to > 257 CFU × 100 ml^{-1} enterococci and > 157 CFU × 100 ml^{-1} fecal coliforms). Interestingly, *E. coli* did not follow the same pattern, as it had about equal probability of being in any of the top 3 bins (between 15 and 41,000 CFU × 100 ml^{-1} when temperature and rainfall were elevated).

Discussion

In this study, SAV, sediments, and stormwater runoff were evaluated as potential reservoirs and inputs of FIB and pathogens to an inland, freshwater, recreational lake located in a sub-tropical region of the US that is impacted by multiple stormwater outfalls. Compared to coastal waters and the Great Lakes, inland waters are under-studied, and a great deal remains to be learned about the ability of FIB and MST marker presence to predict the presence of human pathogens in such waters (48), particularly when nonpoint source pollution is the major contributor. To provide the most robust and complete assessment of sources of fecal contamination in this lake, it was necessary to

conduct a multi-faceted study which investigated the potential of all of the aforementioned matrices to serve as reservoirs for FIB, MST markers, and human pathogens. The effects of SAV and sediment resuspension (beach sand) on FIB densities were measured to assess their impact as significant reservoirs for FIB. Furthermore, opportunistic sampling during or shortly after rain events was conducted at outfalls that delivered stormwater to the lake in order to assess the potential of stormwater run-off and the stormwater infrastructure to serve as a reservoir for FIB, MST markers, and human pathogens. Due to the high cost associated with the analysis of the pathogens, only selected sites and samples were analyzed for pathogens.

Concentrations of enterococci and *E. coli* isolated from SAV and concentrations of all FIB from sediment were positively correlated with FIB concentrations in the water column. Lack of correlation between fecal coliform concentrations in SAV vs. the water column may indicate that fecal coliforms are predominantly contributed to the water column via sediment resuspension and/or fecal contamination events. These data, as well as the observation of FIB concentrations (normalized to mass) one to two log units higher than those observed in the water (normalized to volume), suggest that both matrices (SAV and sediment) serve as additional reservoirs of FIB, and enterococci in particular, which may contribute to high concentrations throughout the lake (7). Even at sites where growing SAV was not abundant (e.g. beach sites 1 and 2), we observed that mats of vegetation frequently washed onshore in quantities that necessitated physical removal. These vegetation masses may have contributed to elevated FIB concentrations in the water by shedding FIB as they washed onshore. A previous study conducted at Lake Michigan demonstrated the ability of *E. coli* populations to persist on *Cladophora*, and

suggested that the movement of algal mats via wave action may serve to inoculate waters and sediments with FIB (27). Furthermore, a previous study of the enterococci population in this lake demonstrated that mesocosms with vegetation added maintained significantly higher enterococci densities than those without vegetation, and that > 96% of isolates were a single strain of *Enterococcus casseliflavus* (6).

A prior study assessing FIB concentrations at both beach sites and the center of the lake found that concentrations of fecal coliforms were significantly lower at the center of the lake compared to the beach (mean concentration of 2.21 $\log_{10}$ CFU × 100 ml^{-1} at beach sites vs. 1.10 in the center of the lake; $P < 0.0001$); however, no significant difference was observed in enterococci concentrations (mean concentration 2.40 $\log_{10}$ CFU × 100 ml^{-1} at beach sites vs. 1.60 in the center of the lake; $P = 0.0898$) (67). Based on previous work and the findings in the current study, SAV harbors a naturalized population of enterococci and may also serve as a conveyance mechanism and reservoir for this population throughout the lake.

Intentional sediment disturbance was shown to increase concentrations of enterococci in the water column; however, this result should be interpreted cautiously due to the small sample size (n = 2) tested. Sediment was previously shown to be a significant reservoir for *E. coli* in an estuarine setting, increasing concentrations of *E. coli* in the water column due to tidal fluctuation (137). Furthermore, sediments have been previously shown to be reservoirs for pathogens as well as FIB suggesting that resuspension of sediments may result in elevated health risk (53), although evaluation of this finding was beyond the scope of this study. Another study conducted at Lake Carroll and other watersheds extensively investigated the contributions of sediment to FIB

concentrations in water (7). This study found that resuspended sediment may make a significant contribution to FIB concentrations in the water column in shallow waters (as were tested in the current work), but that in deeper waters or other sediment types than beach sand, FIB contribution from sediment is less significant (7). The general practice of normalizing FIB concentrations to mass of habitat (e.g., CFU × g^{-1}) can lead to misconceptions about the relative contribution of water, sediments, and SAV to overall microbial loads in an aquatic habitat, as the relative size of these reservoirs can vary greatly within and between water bodies (7).

Rainfall was correlated to FIB concentrations in water and sediments as well as to detection of MST markers and human pathogens, suggesting that stormwater runoff, which may enter the water body from surface/subsurface flow or stormwater infrastructure, has a significant impact on water quality in this inland water. Stormwater has been previously implicated as a reservoir or conveyance mechanism for human fecal contamination and pathogens (23, 80, 113, 114). Detection of pathogens at site 4, where water was collected less than a meter from the stormwater outfall, suggest that bacterial, protozoan, and/or viral human pathogens are associated with stormwater runoff in this system and highlights a need to further investigate and remediate the stormwater infrastructure to protect surface waters.

Based on the finding that each reservoir investigated showed the potential to harbor and/or contribute FIB to the water column, the association between FIB concentrations, as well as MST marker presence, and pathogens was more closely examined. Exceedences of FIB standards were observed in 82% of samples in which a pathogen was detected (n = 11). The positive predictive value for each FIB (percentage of

samples in which a pathogen was detected when the regulatory limit was exceed) (69) toward pathogens was 67%, 50%, and 47% for fecal coliforms, *E. coli*, and enterococci, respectively, with false-positive rates (exceedence occurred without pathogen detection) of 33%, 50%, and 53%, respectively. This suggests that FIB exceedences, while sometimes indicative of pathogen presence, are often observed in the absence of the pathogens tested and that fecal coliforms are most indicative of pathogen presence in this water body.

Pathogen detection was not significantly correlated with detection of human MST markers ($r^2 = 0.04$, $P = 0.43$). At least two possible reasons for this discrepancy exist: (1) fewer samples and larger water volumes were analyzed for pathogens than for MST, so the samples were not directly comparable; and (2) with the exception of the enteric viruses, all of the pathogens tested can be shed by animals as well as humans (52, 151, 169). The MST markers used here were chosen in part because of their demonstrated ability to detect sewage pollution in ambient water volumes of several hundred mL or less (64, 102, 106, 107, 133); however, improved methods for sample filtration and purification, which could allow processing of larger sample volumes for MST assays without inhibiting the PCR, would increase the sensitivity of these assays and would probably result in a higher frequency of co-detection of pathogens and MST markers when the contamination is from a human source (98). Conversely, the inclusion of markers for fecal contamination from animals such as birds, dogs, and raccoons, all of which are plentiful in the Lake Carroll watershed, may have resulted in better agreement between marker and pathogen detection; however, these methods were not generally available when the study was performed. It is important to note that, regardless of

potential zoonotic sources of pathogens in this watershed, all of the pathogens studied have been shown to represent a risk to human health (61, 131, 149).

The Bayes net model developed from the data collected from Lake Carroll found that increased water temperature and antecedent rainfall were predictive of elevated FIB concentrations in the water, particularly in the cases of enterococci and fecal coliforms. Covariance of temperature and rainfall was observed, which was expected due to the fact that the summer is Tampa's rainy season. The relationship between rainfall and FIB levels was corroborated in some instances by linear Pearson correlations (Table 2.1), and was particularly strong in sediments (antecedent rainfall 72 h or one week prior to sampling). However, conditions of elevated temperature and rainfall were not particularly predictive of pathogen presence (58.9% probability of detection). In this system, fecal coliform concentrations were the single best predictor of pathogen detection, as the lowest level (< 45 CFU × 100 ml^{-1}) corresponded with a low probability of pathogen detection (7%). These conditions corresponded with prediction of low *E. coli* levels (< 60 CFU × 100 ml^{-1}). The same relationship was not found for enterococci. Under conditions of low probability for pathogen detection, when fecal coliform and *E. coli* concentrations all fell into the two lowest bins, the probability of enterococci concentrations being greater than 258 CFU × 100 ml^{-1} was ~37%. In all probability, the naturalized population of enterococci in Lake Carroll leads to consistently elevated enterococci levels, which negates the relationship between this FIB and pathogen presence.

The construction of Bayesian networks has been previously demonstrated to be an effective tool for modeling processes affecting eutrophication of a water body (125). The current study demonstrates that the Bayes net model can also be an extremely helpful

tool for teasing apart the complex relationships among physical-chemical and biological parameters measured for water quality. In particular, the failure of enterococci to predict pathogen presence in this lake is an important finding. One of the hallmarks of Bayes nets is the flexibility to incorporate both data and expert judgment into a probabilistic model, which makes them a good fit for modeling indicator/pathogen relationships in environmental waters.

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Table 2.1 - Correlations of FIB concentrations in the water and sediment with rainfall 24 h, 72 h, and 7 days prior to sampling. Individual sites for which no significant correlation was observed ($\alpha = 0.05$) are not shown. Bolded values are significant positive correlations.

Matrix	Site	Indicator	24-hour Rainfall	72-hour Rainfall	Weekly Rainfall
Water	All	Fecal coliforms	**$r^2 = 0.10$, $P = 0.0076$**	$r^2 = 0.04$, $P = 0.0927$	$r^2 = 0.03$, $P = 0.1608$
		E. coli	$r^2 = 0.03$, $P = 0.1481$	$r^2 < 0.01$, $P = 0.5649$	$r^2 < 0.01$, $P = 0.6628$
		Enterococci	**$r^2 < 0.01$, $P = 0.0122$**	**$r^2 = 0.06$, $P = 0.0479$**	$r^2 = 0.04$, $P = 0.0861$
	2[a]	Fecal coliforms	$r^2 = 0.33$, $P = 0.0851$	**$r^2 = 0.88$, $P < 0.0001$**	**$r^2 = 0.82$, $P = 0.0003$**
		E. coli	**$r^2 = 0.65$, $P = 0.0046$**	**$r^2 = 0.40$, $P = 0.0482$**	$r^2 = 0.32$, $P = 0.0861$
		Enterococci	$r^2 = 0.13$, $P = 0.3054$	$r^2 < 0.01$, $P = 0.9578$	$r^2 < 0.01$, $P = 0.9388$
	3[b]	Fecal coliforms	**$r^2 = 0.52$, $P = 0.0181$**	$r^2 = 0.28$, $P = 0.1156$	$r^2 = 0.21$, $P = 0.1747$
		E. coli	$r^2 = 0.01$, $P = 0.8409$	$r^2 < 0.01$, $P = 0.9375$	$r^2 < 0.01$, $P = 0.9263$
		Enterococci	$r^2 = 0.01$, $P = 0.8301$	**$r^2 = 0.67$, $P = 0.0039$**	**$r^2 = 0.73$, $P = 0.0016$**
	7[c]	Fecal coliforms	**$r^2 = 0.88$, $P < 0.0001$**	$r^2 = 0.31$, $P = 0.0934$	$r^2 = 0.22$, $P = 0.1711$
		E. coli	$r^2 = 0.37$, $P = 0.0641$	$r^2 = 0.06$, $P = 0.4962$	$r^2 = 0.03$, $P = 0.6092$
		Enterococci	**$r^2 = 0.83$, $P = 0.0002$**	**$r^2 = 0.50$, $P = 0.0229$**	$r^2 = 0.39$, $P = 0.0540$
Sediment	All	Fecal coliforms	$r^2 = 0.02$, $P = 0.2760$	**$r^2 = 0.26$, $P = 0.0002$**	**$r^2 = 0.27$, $P = 0.0001$**
		E. coli	$r^2 < 0.01$, $P = 0.7943$	**$r^2 = 0.09$, $P = 0.0309$**	**$r^2 = 0.12$, $P = 0.0146$**
		Enterococci	$r^2 = 0.06$, $P = 0.0961$	**$r^2 = 0.20$, $P = 0.0010$**	**$r^2 = 0.21$, $P = 0.0009$**
	1[a]	Fecal coliforms	$r^2 = 0.10$, $P = 0.3820$	**$r^2 = 0.60$, $P = 0.0087$**	**$r^2 = 0.61$, $P = 0.0074$**
		E. coli	$r^2 < 0.01$, $P = 0.8962$	**$r^2 = 0.47$, $P = 0.0298$**	**$r^2 = 0.52$, $P = 0.0191$**
		Enterococci	$r^2 < 0.01$, $P = 0.8913$	$r^2 = 0.33$, $P = 0.0817$	$r^2 = 0.38$, $P = 0.0571$
	2[a]	Fecal coliforms	$r^2 = 0.24$, $P = 0.3206$	**$r^2 = 0.96$, $P = 0.0007$**	**$r^2 = 0.95$, $P = 0.0010$**
		E. coli	$r^2 = 0.01$, $P = 0.8214$	$r^2 = 0.64$, $P = 0.0569$	$r^2 = 0.65$, $P = 0.0529$
		Enterococci	$r^2 = 0.12$, $P = 0.4967$	**$r^2 = 0.77$, $P = 0.0220$**	**$r^2 = 0.76$, $P = 0.0226$**
	6[c]	Fecal coliforms	$r^2 = 0.05$, $P = 0.5347$	$r^2 = 0.35$, $P = 0.0734$	**$r^2 = 0.43$, $P = 0.0394$**
		E. coli	$r^2 = 0.05$, $P = 0.5181$	$r^2 = 0.25$, $P = 0.1408$	$r^2 = 0.32$, $P = 0.0897$
		Enterococci	$r^2 = 0.09$, $P = 0.4011$	$r^2 = 0.13$, $P = 0.2949$	$r^2 = 0.12$, $P = 0.3214$

	7[c]	**Fecal coliforms**	$r^2 = 0.26$, $P = 0.2009$	$r^2 = 0.30$, $P = 0.1621$	$r^2 = 0.22$, $P = 0.2411$
		E. coli	$r^2 = 0.03$, $P = 0.6643$	$r^2 < 0.01$, $P = 0.8140$	$r^2 < 0.01$, $P = 0.8784$
		Enterococci	**$r^2 = 0.69$, $P = 0.0106$**	$r^2 = 0.41$, $P = 0.0851$	$r^2 = 0.33$, $P = 0.1354$

[a]No stormwater structure

[b]Baffle box stormwater infrastructure

[c]Stormwater outfall, no treatment

Table 2.2 – Results of MST marker and pathogen analysis by site and date. Presence/absence of human polyomavirus (HPyVs), human-associated *Bacteroides* (HF183), and the *esp* gene of *Ent. faecium* were tested at each site on each date unless otherwise noted (NT). Assays for pathogen detection including *Salmonella* (Sal), *Cryptosporidium* (Crypto), *Giardia*, and enteric viruses (EV) were performed at sites 1 and 2 from April through November and at sites 2 and 4 from December through March. Positive tests are bolded and cells containing positive results are heavily outlined.

Date	Sampling Sites													
	Site 1		Site 2		Site 3		Site 4		Site 5		Site 6		Site 7	
	MST[a]	Pathogens[b]	MST	Pathogens	MST	Pathogens	MST	Pathogens	MST	Pathogens	MST	Pathogens	MST	Pathogens
4/15	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT
5/27	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), **EV (0.6)**	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT
6/24	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), **EV (0.5)**	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), **Crypto (2.2)**, *Giardia* (-), **EV (1.7)**	*esp* (-), HF183 (-), HPyVs (-)	NT	***esp* (+)**, HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT

NT	NT	NT	NT	NT	**7/29[c]**
***esp* (+),** HF183 (-), **HPyVs (+)**	*esp* (-), HF183 (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), **HPyVs (+)**	
NT	NT	NT	NT	NT	**10/1[c, d]**
esp (-), HF183 (-), HPyVs (-)	*esp* (-), HF183 (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	
NT	NT	NT	NT	NT	**11/18**
***esp* (+),** HF183 (-), **HPyVs (+)**	NT	*esp* (-), HF183 (-), HPyVs (-)	***esp* (+),** **HF183 (+),** **HPyVs (+)**	*esp* (-), HF183 (-), **HPyVs (+)**	
NT	NT	NT	NT	NT	**12/11[c, e]**
NT	NT	NT	**Sal (+),** Crypto (-), *Giardia* (-), EV (-)	NT	
esp (-), HF183 (-), HPyVs (-)	*esp* (-) HF183 (-)	*esp* (+), HF183 (-), HPyVs (-)	*esp* (-), HF183 (-), HPyVs (-)	*esp* (-), HF183 (-), HPyVs (-)	
NT	NT	NT	NT	NT	**12/16**
esp (-), HF183 (-), HPyVs (-)	*esp* (-), HF183 (-)	*esp* (-), HF183 (-), HPyVs (-)	***esp* (+),** HF183 (-), HPyVs (-)	*esp* (-), HF183 (-), **HF183 (+),** HPyVs (-)	
Sal (-), **Crypto (3.2),** ***Giardia* (6.5),** EV (-)	Sal (-), Crypto (-), ***Giardia* (1.9),** EV (-)	**Sal (+),** Crypto (-), *Giardia* (-), EV (-)	NT	NT	
esp (-), HF183 (-), HPyVs (-)	*esp* (-), HF183 (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	NT	
Sal (+), **Crypto (88.2),** *Giardia* (-), EV (-)	Sal (-), Crypto (-), ***Giardia* (5.3),** EV (-)	Sal (-), **Crypto (0.5),** *Giardia* (-), EV (-)	NT	Sal (-), Crypto (-), *Giardia* (-), EV (-)	
esp (-), **HF183 (+)** HPyVs (-)	*esp* (-), HF183 (-)	*esp* (-), HF183 (-), HPyVs (-)	***esp* (+),** HF183 (-), HPyVs (-)	*esp* (-), HF183 (-), HPyVs (-)	

1/13	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (+), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (2.4), EV (0.6)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	NT
2/17	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (+), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	NT
3/24	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (+), HPyVs (-)	Sal (-), Crypto (-), *Giardia* (-), EV (-)	*esp* (-), HF183 (-), HPyVs (-)	NT	*esp* (-), HF183 (-), HPyVs (-)	NT	NT

[a]MST target and PCR result (+ or -)

[b]Pathogen target detected and result: presence/absence (+/-) for *Salmonella* or quantities for *Cryptosporidium, Giardia*, or enteric viruses (oocysts, cysts, or MPN, respectively, per 100 L).

[c]Rain event (at least 0.5 inches of rainfall prior to sampling).

[d]A method blank for HPyVs was contaminated, so results are not reported for this target.

[e]Sampling was conducted during a major rain event (0.75 inches of rainfall < 1 hour prior to sampling).

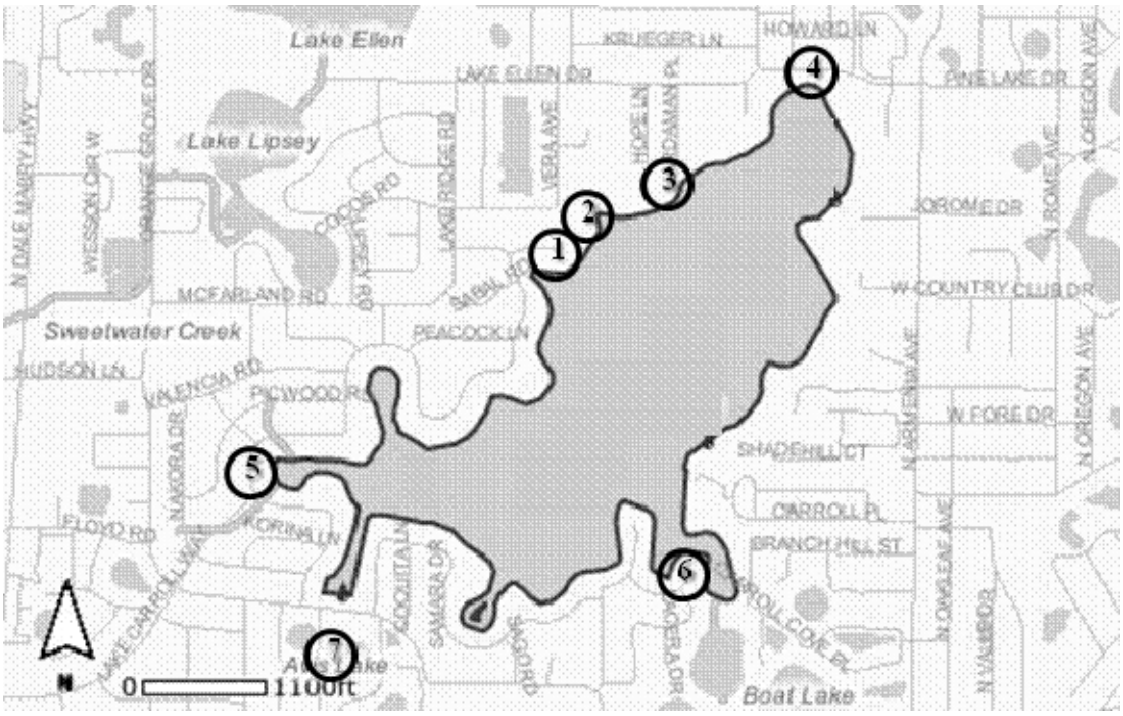

Figure 2.1 – Lake Carroll sites: 1 and 2, White Sands Beach; 3-6, stormwater outfalls draining into the lake; 7. retention pond into which Lake Carroll drains. Site 3 is downstream of a baffle box and site 4 is downstream of a continuous deflection stormwater system.

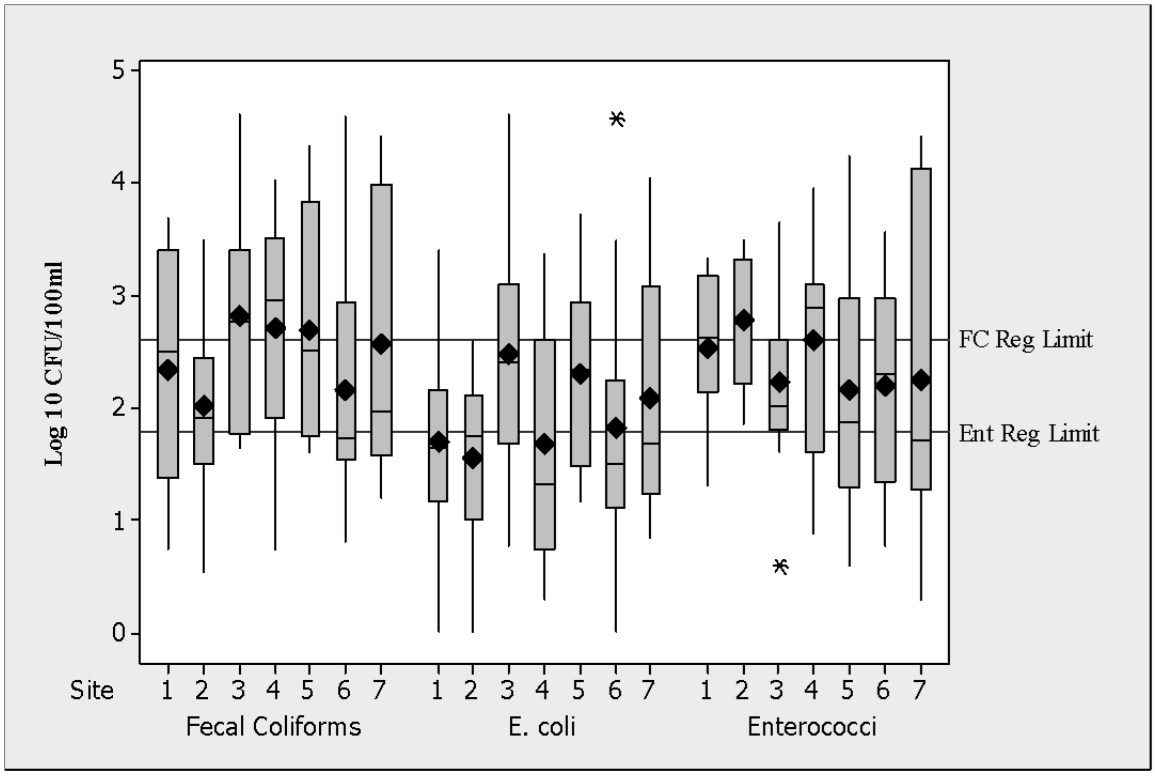

Figure 2.2 – Box plots showing FIB concentrations (fecal coliforms, *E. coli* and enterococci) (log_{10} CFU × 100 ml^{-1}) in the water column at each site over the entire study period. Diamonds reflect mean concentrations. The bar (-) reflects the median concentration and box lengths reflect the upper and lower quartiles of the data set. Whisker lengths represent the maximum and minimum values, excluding outliers. Sites 1, 2, 6, and 7 were sampled 10 times. Data for sites 3 and 4 include an additional sampling during a rain event (sampled 11 times). Site 5 could not be sampled in October, but was also sampled during a rain event (sampled 10 times). Horizontal lines reflect regulatory limits for fecal coliforms (FC, 400 CFU × 100 ml^{-1}) (56), and enterococci in freshwater (Ent, 61 CFU × 100 ml^{-1}) (154).

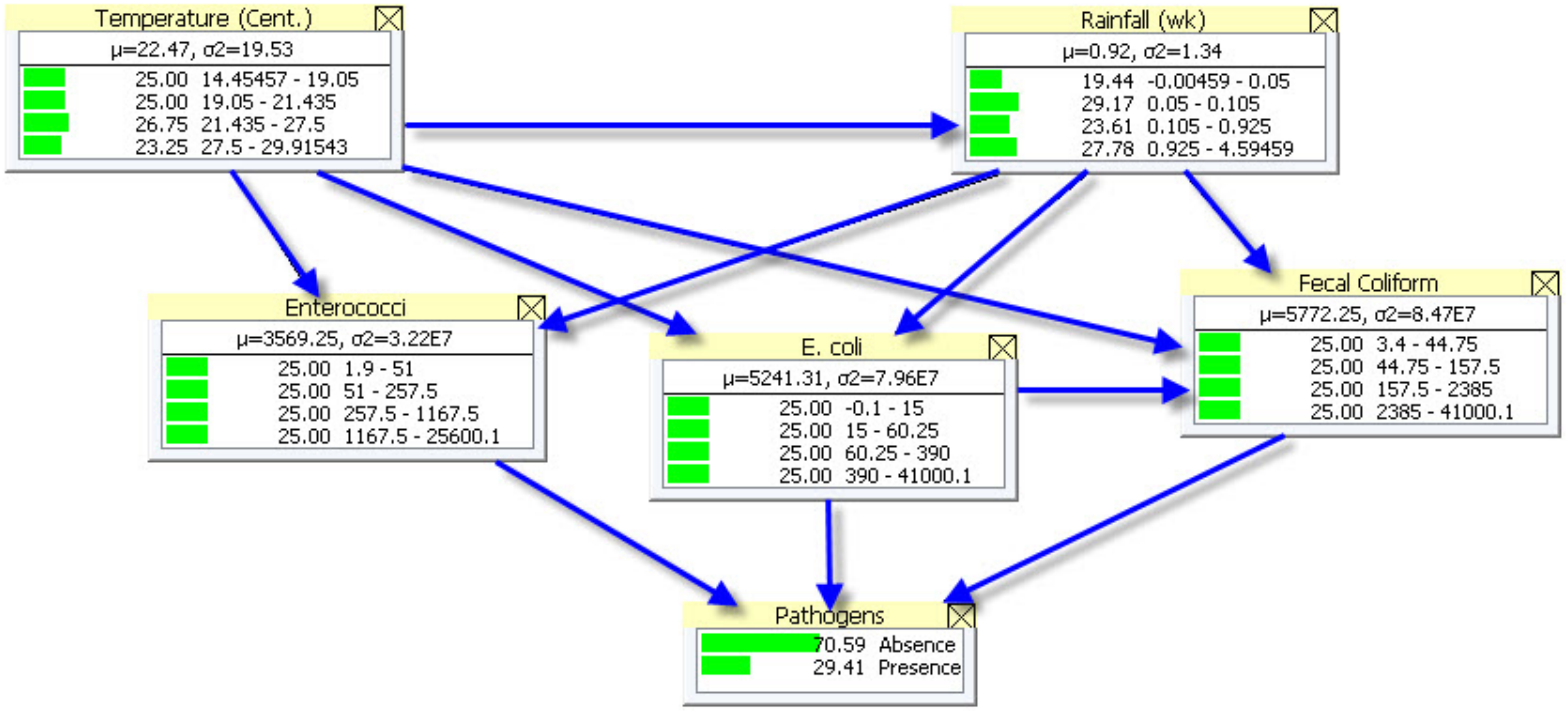

Figure 2.3 – A Bayes net prior probability model for Lake Carroll derived from observations at sites 1, 2 and 4 (where pathogens were measured). Each variable and arrow (arc) represents a node and its interaction with another variable. Bin sizes (ranges) for all parameters were set such that data in the prior model was distributed evenly among the bins; units for FIB are CFU × 100 ml^{-1}.

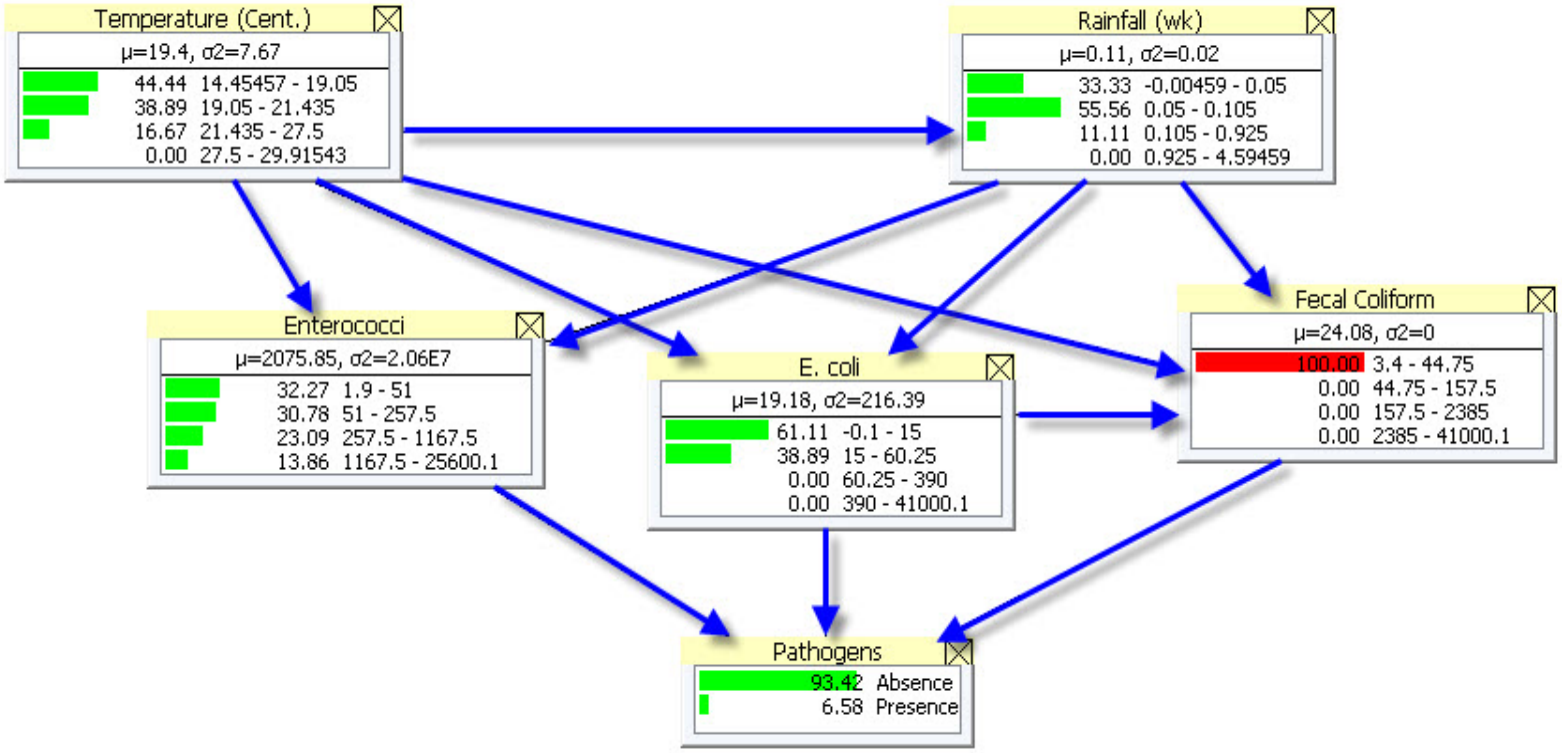

Figure 2.4 – A predictive Bayes net model for the relationship between physical parameters, FIB and pathogens at sites 1, 2 and 4. Fecal coliforms are the predictive variable. Bin sizes (ranges) for fecal coliforms were set such that data in the prior probability model were distributed evenly in each bin. Fecal coliform concentrations were allocated to the smallest bin to determine the effect on pathogen detection and other variables; units for FIB are CFU × 100 ml^{-1}.

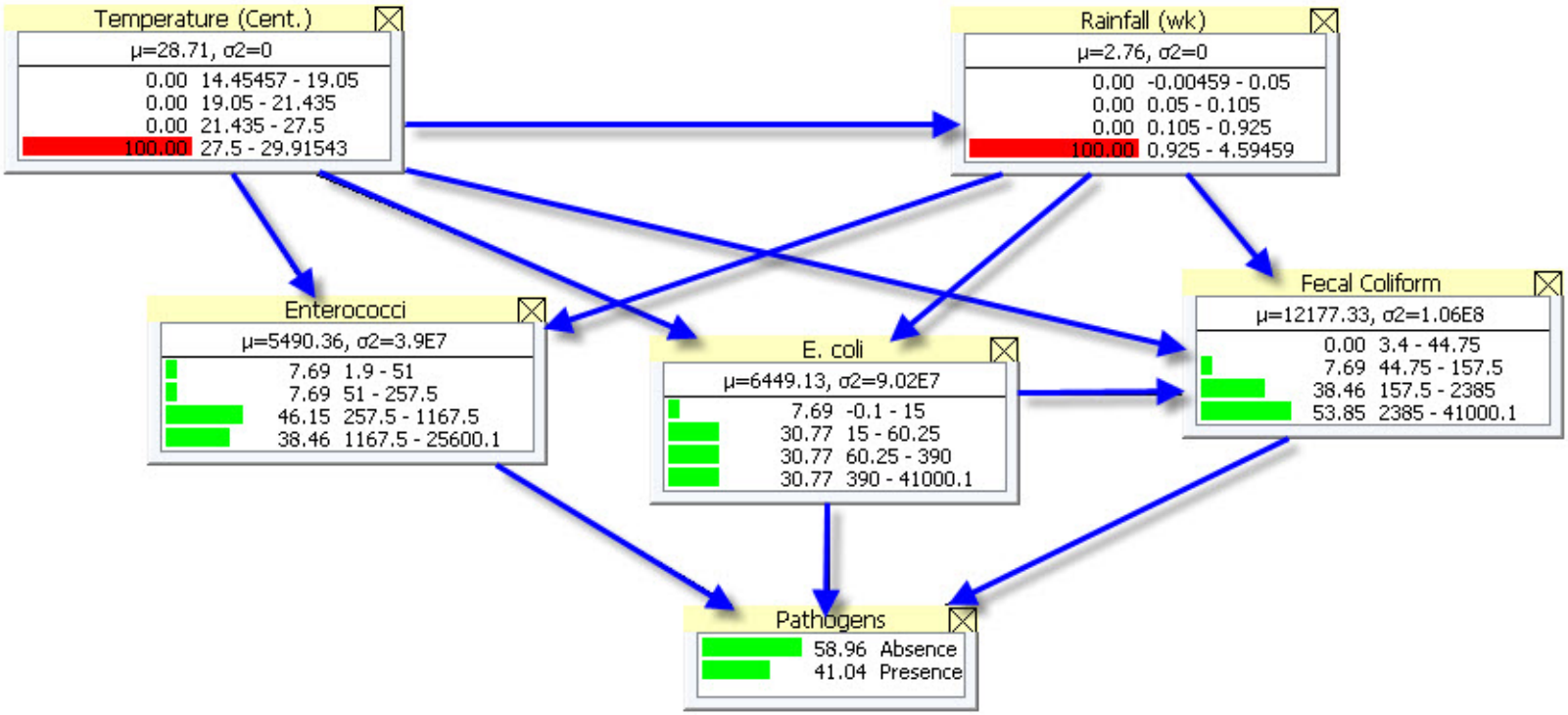

Figure 2.5 – A predictive Bayes net model for the relationship between physical parameters, FIB and pathogens at sites 1, 2 and 4. Temperature and one-week cumulative antecedent rainfall are the predictive variables. Bin sizes (ranges) for predictive variables were set such that data in the prior probability model were distributed evenly in each bin; units for FIB are CFU × 100 ml^{-1}. Temperature and rainfall variables were allocated to the largest bins to determine the effect on pathogen detection and other variables.

CHAPTER 3: DETECTION AND DIFFERENTIATION OF *VIBRIO VULNIFICUS* AND *V. SINALOENSIS* IN WATER AND OYSTERS OF A GULF OF MEXICO ESTUARY[2]

Introduction

Shellfish, particularly oysters, are known to harbor high concentrations of *Vibrio vulnificus*, an autochthonous, gram-negative, opportunistic human pathogen (82, 140). The bacterium exhibits a high degree of genetic diversity and is divided into three biotypes (1, 2, and 3) based on genotypic as well as phenotypic differences. All three biotypes have the ability to infect humans; however, biotype 1 is most frequently implicated in human infections (1, 14, 83). *V. vulnificus* is the leading cause of deaths associated with the consumption of raw or undercooked shellfish; the mortality rate from cases resulting in septicemia is greater than 50%, and approximately 50 cases are reported each year in the United States (117). To reduce the public health risk associated with shellfish consumption, shellfish beds are approved for retail shellfishing on the basis of microbial water quality determined by fecal coliform concentrations (78), although the allochthonous source of fecal indicator bacteria suggests that it would not be a good predictor for autochthonous *Vibrio* pathogens.

V. vulnificus concentrations in harvested from the U.S. Gulf of Mexico have been reported to be highest between May and October (*e.g.*, median monthly concentration of 2300 MPN × g^{-1} (110)), with a reduction to fewer than 10 MPN × g^{-1} from November to

[2]This chapter was co-authored by Eva Chase and Valerie J. Harwood (University of South Florida, Tampa, FL).

March (79, 82, 110). Studies have consistently shown that culturable concentrations of *V. vulnificus* are lower when water temperatures are cooler (110, 120, 123). Water salinity is also an important factor affecting concentrations of the bacterium in oysters; the highest concentrations are typically detected when salinity is between 5 and 25 ‰ (110, 120, 123). Under favorable temperature and salinity conditions, *V. vulnificus* concentrations have been reported to vary from > 10^5 CFU × g^{-1} to undetectable in individual oysters sampled from the same site (13). Similarly, genetic diversity of *V. vulnificus* populations in individual oysters has been reported to be very high, with more than 100 strains present in a single oyster (25, 140).

V. vulnificus concentrations have, in some cases, been shown to correlate with fecal indicator bacteria (FIB) concentrations (76, 160). In Danish waters, the occurrence of *V. vulnificus* was significantly correlated with concentrations of both total coliforms and enterococci (76). Similarly, in Hawaii, *V. vulnificus* concentrations were correlated with *E. coli* and enterococci as well as *Clostridium perfringens* and F^+ coliphage, which are also indicative of fecal contamination (160). Furthermore, one study has suggested that poor water quality increases the relative abundance of type B (clinically-associated) *V. vulnificus* strains compared to type A (environmentally-associated) strains (60). Results of other studies have not found correlation of *V. vulnificus* with FIB concentrations (84, 120, 123, 145); however, many of these studies focused on areas permitted for shellfishing where water quality was good. Fecal contamination of environmental waters results in increased nitrogen, phosphorous, and organic carbon concentrations, leading to the eutrophication of water bodies (126, 132) and positive effects on growth of certain bacteria (50, 165). Furthermore, increased nutrient levels

may influence the virulence and growth of autochthonous pathogens including *V. vulnificus* (136, 168). Therefore, in the present study, sites representing varying water quality were sampled to assess the relationship between FIB levels and *V. vulnificus* concentrations in water and oysters.

Colistin-polymyxin B-cellobiose (CPC) agar (105), or a modification thereof (mCPC), has been frequently used for the isolation of *V. vulnificus* from environmental sources (118) and is recommended in the Food and Drug Administration's (FDA) *Bacteriological Analytical Manual* for preliminary identification of the species, which forms round, flat, yellow colonies, 1 to 2 mm in diameter (152). The FDA methodology includes an enrichment step in alkaline peptone water (APW) and plating on mCPC agar followed by molecular confirmation – typically by either PCR targeting the *vvhA* gene (hemolysin) (85, 152) or colony hybridization with an alkaline phosphatase-labeled probe targeting *vvhA* (172). Direct plating (without enrichment) is more rapid and, when performed using *V. vulnificus* agar followed by confirmation using an alkaline phosphatase-labeled probe, shows less variability in concentration estimates between replicates than the FDA enrichment methodology (46). CPC and mCPC are reportedly superior to media which differentiate among *Vibrio* species based on sucrose fermentation or alkaline sulfatase production, and up to 80% of colonies matching the *V. vulnificus* phenotype were confirmed by molecular methods in other studies (119, 143). These studies employed direct plating of shellfish samples without an enrichment step, suggesting that enrichment may not be necessary for recovery of culturable *V. vulnificus* using CPC.

In the present study, water and oyster samples were collected from estuarine and marine (Gulf of Mexico) sites and from a tidally-influenced creek over a one-year period. We hypothesized that water and oysters sampled from a site with higher FIB levels would harbor greater concentrations of *V. vulnificus* compared to less impacted sites, therefore, we compared culturable concentrations of FIB (enterococci and fecal coliforms) (2, 156) with *V. vulnificus* concentrations determined via direct plating on mCPC agar (146), the FDA enrichment method (152), and qPCR (31). We unexpectedly observed a high percentage of colonies from samples plated directly on mCPC agar which were phenotypically indistinguishable from *V. vulnificus* but which could not be confirmed by PCR for *vvh*A. The identity and growth characteristics of these *Vibrio* spp. were explored by DNA sequencing and determination of growth rates under various nutrient and temperature regimes.

Materials and Methods

Sample Collection and Preparation. Grab samples (2 L) of water and five individual oysters per sample event were collected from a tidally-influenced creek [Bull Frog Creek (BFC); 27°50'17"N, 82°22'55"W], an estuarine beach [Ben T. Davis (BTD); 27°58'14"N, 82°34'44"W], and a marine beach [Fort DeSoto (FD); 27°38'42"N, 82°43'5"W]. Both BFC and BTD sites are prohibited waters for shellfishing due to elevated fecal coliform levels, while FD was conditionally approved during the period of sampling (55). Samples were collected every other month at BFC from July 2010 to July 2011 (n = 10), at BTD from August 2010 to July 2011 (n = 9), and FD from February 2011 to July 2011 (n = 6). FD sampling was added in 2011 as a control (relatively pristine) site. BFC was sampled on a separate, but consecutive day, from BTD and FD

due to the distance between sites and the need for prompt processing of the samples. Physicochemical parameters (temperature, pH, dissolved oxygen, turbidity, and salinity) were measured at each site on each date (Table 3.1). Water samples were collected in sterile, 2 L bottles, and oysters were placed in plastic bags; samples were transported back to the lab in a cooler on ice and processed within six hours of sampling.

Oysters were dissected aseptically and the total tissue mass (wet weight) from each of five individual oysters per sampling location on each sampling event was determined (mean 7.8 ± 4.9 g). Individual oyster tissues were diluted 1:2 to 1:5 (wt/vol) in phosphate buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCL, 0.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and homogenized by blending at high speed for one minute in a 250 ml-capacity Waring blender (Waring Products, Torrington, CT). The extent of dilution was manipulated based on expected bacterial concentrations as well as to ensure that the volume was appropriate to allow for blending (> 10 ml). In addition, using the method described in the FDA's *Bacteriological Analytical Manual* (152), composite oyster homogenates were created for each site for the final six sampling dates (Feb 2011 – July 2011). The composites were made up of tissues from as many oysters as necessary to obtain 50 g were diluted 1:10 in PBS, serially diluted and enriched in alkaline peptone water (APW) at 37° C, overnight.

Culture-Based Enumeration of Bacteria. Fecal coliform and enterococci concentrations were determined via standard membrane filtration (47 mm nitrocellulose filters, 0.45 µm pore size) protocols (2, 156). Water samples were filtered in duplicate at volumes of 1, 10, and 100 ml. One milliliter of individual oyster homogenate was filtered in duplicate for FIB, but greater volumes could not pass through the filter.

For the direct-plating assay (individual oysters), *V. vulnificus* was enumerated by diluting the initial homogenate obtained from an individual oyster (see above) 1:10 (vol/vol) in PBS. One hundred microliters of undiluted water samples and oyster homogenates and 100µl of 1:10 diluted oyster homogenates were spread-plated on mCPC agar plates (100 mm) in duplicate. All putative *V. vulnificus* colonies (those that formed round, flat, yellow colonies, 1 to 2 mm in diameter, according to the FDA description (152)) were subjected to PCR targeting the *vvhA* gene for confirmation (described below).

For the FDA enrichment assay, water or oyster homogenates were serially diluted in PBS, and dilutions were inoculated into 10 ml APW for enrichment. Enrichment cultures were streaked in triplicate onto mCPC agar, and one putative *V. vulnificus* colony from each streak was subjected to PCR confirmation. Concentrations for both direct plating were reported as CFU × 100 ml^{-1} (water) or CFU × g^{-1} wet weight (oysters) and FDA enrichment methods were reported as MPN × $100ml^{-1}$ (water) or MPN × g^{-1} wet weight (oysters).

Conventional PCR. Conventional (presence/absence) PCR for *V. vulnificus* confirmation was performed on all isolates matching the FDA's description for *V. vulnificus* on mCPC using previously described primers (Vv 1 and 3) targeting the *vvhA* gene (22) (Table 3.2). When none of the colonies (n = 168) from the samples collected at BTD and FD in June 2011 were confirmed by PCR using Vv 1 and Vv 3 primers despite favorable temperature (28° C) and salinity (25 ‰) for isolation of culturable *V. vulnificus*, a subset (n = 7) of randomly selected environmental isolates from this sampling event were also tested for *vvhA* using two other primer sets also targeting *vvhA* (31, 85). The 16S rRNA gene of

several of these isolates, as well as one oyster isolate sampled from BTD in October 2010 (Oy2-1), were sequenced (n = 8) (see below).

A primer set specific to *V. sinaloensis* (Vs-2F and Vs-4R, Table 1) was developed based on the alignment of 16S rRNA sequences and tested against three strains of *V. vulnificus* (two biotype 1: ATCC 27562 and CMCP6, and one biotype 2: 33147) as well as 13 environmental samples, two of which were identified as *V. brasiliensis*, and 10 of which were identified as *V. sinaloensis* via 16S rRNA sequence analysis.

All conventional PCR reactions consisted of 12.5 µl 2× GoTaq Green (Promega, Madison, WI), 0.4 µM of each primer (Integrated DNA Technologies, Coralville, IA), and nuclease-free water to bring the reaction to a total volume of 25 µl. Template DNA for each reaction was obtained by picking an isolated colony from an mCPC agar plate with a sterile toothpick, and adding it directly to the assay tube. For each set of PCR reactions, a no template negative control was performed, and DNA extracted from *V. vulnificus* 9067-96 (161) was used as a positive control. PCR products were visualized via gel electrophoresis on a 2% agarose gel.

DNA Extraction. For qPCR analysis, samples were filtered through 47 mm nitrocellulose filters (0.45 µm pore size) at volumes of 500 ml for water and 1 ml for individual oyster homogenates at the original dilution (*e.g.* 1:2 in PBS). Filters were placed in PowerBead tubes from the PowerSoil™ DNA kit (MoBio, Carlsbad, CA) and stored at -20° C until DNA was extracted (up to one week). For each sampling event, a method blank (500 ml sterile buffered water (2)) was also filtered and subjected to DNA extraction. DNA was extracted following the manufacturer's instructions for the PowerSoil™ DNA kit with modifications that included proportional increases in buffers to allow for as much of the

supernatant as possible to be carried forward during the extraction (65). For each set of samples extracted, an extraction blank consisting of an empty PowerBead tube (no filter added) subjected to all methodological steps was also included.

To obtain DNA for sequencing and PCR controls, cultures were grown in 1.8 ml brain heart infusion broth supplemented to a final NaCl concentration of 1% (wt/vol) (BHI + 0.5% NaCl; Becton Dickson, Sparks, MD), and DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA to construct a standard curve for enumeration of *V. vulnificus* by qPCR (described below) was prepared from a 5 ml culture of *V. vulnificus* CMCP6, and DNA was extracted using the QIAamp DNA Blood MIDI kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

qPCR Analyses. *V. vulnificus* concentrations were determined by qPCR by modifying a previously described method targeting the *vvhA* gene (31, 170) from a SYBR green format to a BYRT green format (170). Each reaction consisted of 12.5 µl 2X GoTaq qPCR Master Mix (Promega, Madison, WI), 0.4 µM of each primer (Integrated DNA Technologies, Coralville, IA), and nuclease-free water to bring the volume to 20 µl. The SYBR green assay has been previously shown to retain 100% sensitivity and specificity for *V. vulnificus*, and the limit of detection was observed at a comparable cycle threshold between the TaqMan and SYBR green assays (170). We also found that results obtained using the BYRT green chemistry were comparable to the SYBR green assay (data not shown). Five microliters of sample (see "DNA extraction", above) or standard DNA was added as template to each reaction. Cycling conditions were as previously published (170). All samples were run in duplicate, and duplicate no-template controls were

performed with each run. Standard curves were included with each run and were generated using genomic DNA extracted from *V. vulnificus* CMCP6 diluted to an estimated number of gene copies ranging from 10^1 to 10^6 (10-fold dilutions) determined as the product of the DNA concentration multiplied by Avogadro's number and divided by mass of the genome (178). The mean r^2 values for the *vvhA* standard curve was 0.994 ± 0.006, and mean efficiency was 97.6 ± 3.9 %, respectively. All qPCR reactions were performed using 96-well plates and all reactions were run using the Applied Biosystems 7500 Real-Time PCR System (Carlsbad, CA). The melting temperature for each amplicon was evaluated to determine specificity; only amplicons with a melting temperature of 83.7 ± 0.2° C were considered to represent specific amplification of *vvhA*. This value was a determined from the mean and standard deviation of the melting temperature from standard curve.

Sequence Analysis. The 16S rRNA genes of eighteen colonies that were phenotypically indistinguishable from *V. vulnificus* were sequenced (GenBank accession numbers JN871695 - JN871710, JQ796768, and JQ796769). These colonies were selected randomly from typical colonies isolated from BTD and FD (where *V. vulnificus* was rarely PCR-confirmed) in October 2010 as well as April and June 2011from both water and oysters. In addition, for phylogenetic comparison, the biotype 2 strain ATCC 33147 was also subjected to 16S rRNA sequence analysis (accession number JQ253967), and this sequence is among the first published in GenBank for a known biotype 2 strain. Amplicons (approximately 1480 bp) were produced using the Eco8F-1492RC universal bacterial primer set (90) via conventional PCR (described above) and purified using the QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA) according to the

manufacturer's instructions. Duplicate single-extension sequencing reactions were performed by Macrogen Corp., USA (Rockville, MD). Contigs for isolates sequenced were assembled using DNA Baser software (HeracleBiosoft, Pitesti, Romania). Sequences for *V. vulnificus* CMCP6 and *V. parahaemolyticus* RIMD 2210633 were obtained from GenBank (accession numbers NC_004459 and NC_004603, respectively). Sequence alignment, bootstrap analysis, and maximum likelihood tree generation were performed using MEGA 5.0 software (147).

Growth Comparison Studies. The growth rates of three *V. vulnificus* strains, including two biotype 1 strains (CMCP6 and an environmental isolate designated BFC W1, isolated from water at Bull Frog Creek) and a biotype 2 strain (ATCC 33147), were compared with three confirmed (16S rRNA sequencing) *V. sinaloensis* strains isolated from oysters (designated Oy1-3, Oy2-1, and Oy3-51). Designations refer to the oyster and isolate number for each strain (e.g. Oy1-3 is the third isolate from the first oyster homogenized). Oy1-3 and Oy 3-51 were isolated from the July 2011 sampling at BFC and confirmed

□□□

nd Vs-4R) while Oy 2-1 was isolated from BTD during October 2010 and was confirmed via 16S rRNA sequence analysis. Strains were grown overnight in BHI + 0.5% NaCl at room temperature. Overnight cultures were diluted 1:20 (15 ml total volume) in sterile seawater, APW, or BHI + 0.5% NaCl. Triplicate cultures for each condition were grown at 25°, 30°, and 37° C with agitation (140 rpm). Growth rate (μ) was determined based on the change in absorbance at OD_{600}, measured using the Nanodrop 2000 spectrophotometer (Fisher Scientific, Waltham, MA). OD_{600} values were related to culturable concentrations via

plating in triplicate at three time points during the exponential growth phase on Difco™ typtic soy agar (TSA) (Becton Dickson, Sparks, MD).

Statistical Analyses. To achieve normally distributed data sets, all bacterial concentrations were $\log_{10}$ transformed. Due to frequent non-detects of *V. vulnificus* by both culture and qPCR methods, one-half the limit of detection was substituted for all non-detect values for all analyses (66, 71). All statistical analyses were evaluated at $\alpha = 0.05$. Bacterial concentrations among sampling sites and specific growth rate (μ) for the various strains were compared via one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test using GraphPad Instat software, version 3.0 (San Diego, CA). The frequency of detection of *V. vulnificus* among sampling sites was compared using a Chi-square test for independence (GraphPad Instat). Spearman correlations were calculated to determine relationships between bacterial concentrations and physicochemical parameters using PASW software, version 17 (SPSS, Chicago, IL).

Results

V. vulnificus Detection and Enumeration. The frequency of detection of *V. vulnificus* (confirmed by *vvhA* amplification) varied among the methods of enumeration (direct plating, enrichment, and qPCR) as well as by site (Table 3.3). Detection of *V. vulnificus* in water or individual oyster samples (n = 150) was observed in only 19% of samples by direct plating and in 29% by qPCR. The enrichment method, which was used during the final six months of sampling, detected *V. vulnificus* significantly more frequently than the other methods (in 97% of 36 samples; $P \leq 0.0002$). Concentrations of *V. vulnificus* in oyster samples determined by each method of enumeration among all three sites were not significantly different ($P = 0.18$ to 0.73). There was also no significant difference in *V.*

vulnificus concentrations in the water column among sites determined by direct plating or enrichment methods ($P = 0.08$ and 0.44, respectively); however significantly higher *V. vulnificus* concentrations were found at BFC compared to either BTD or FD via qPCR enumeration ($P = 0.0007$). *V. vulnificus* detection by direct plating and qPCR in water and oysters was also significantly higher at BFC than at other sites ($P = 0.0003$). Detection in the water at BFC by direct plating occurred during many of the warmer months (April through July, Figure 3.1). Few clear temporal trends in *V. vulnificus* detection emerged, with the exception that detection was infrequent at all sites in October, December and February. The recommended fecal coliform standard for shellfishing (43 CFU × 100 ml^{-1}) (78) was exceeded at all sites from which *V. vulnificus* was detected by direct plating.

Among all water and oyster samples (combined), *V. vulnificus* concentrations determined by direct plating were correlated with concentrations determined by the FDA enrichment and qPCR methods ($r = 0.417$, $P = 0.011$ and $r = 0.471$, $P < 0.0001$, respectively). At BFC, where detection was relatively frequent, this relationship was maintained among samples from both matrices, combined ($r = 0.326$, $P = 0.011$). Among all samples collected, no correlations were observed between FIB concentrations and *V. vulnificus* enumerated by any of the three methods. However culturable *V. vulnificus* concentrations (by direct plating among all samples) were negatively correlated with salinity ($r = -0.367$, $P < 0.0001$) and positively correlated with turbidity ($r = 0.289$, $P < 0.0001$); whereas, concentrations determined by qPCR were negatively correlated with salinity ($r = -0.192$, $P = 0.018$) and positively correlated with temperature ($r = 0.281$, $P < 0.0001$).

Identification of V. sinaloensis. Despite a high number of colonies with the characteristic *V. vulnificus* phenotype on mCPC agar, a low proportion (≤ 3%) were confirmed by amplification of *vvhA* from BTD and FD samples over the entire study period (Table 3.4). The unconfirmed isolates produced either no amplicon or amplicons of the wrong size (Table 3.2, Figure 3.2), indicating that other bacteria may mimic the *V. vulnificus* phenotype on mCPC agar. To investigate this possibility, eighteen isolates that were phenotypically indistinguishable from *V. vulnificus* but unconfirmed by *vvhA* PCR were subjected to 16S rRNA sequence analysis. Most isolates (75%) were identified as *V. sinaloensis*; however, other isolates that also resembled *V. vulnificus* on mCPC were identified as *V. brasiliensis* (12.5%), *V. coralliilyticus* (6.25%), and *V. harveyii* (6.25%). Comparison of 16S rRNA sequences of these environmental isolates with those of two *V. vulnificus*, biotype 1 strain CMCP6 and biotype 2 strain 33147, and *V. parahaemolyticus* RIMD 2210633 revealed that all isolates identified as *V. sinaloensis* were 99% identical to each other, between 97.2 to 96.2% identical to *V. vulnificus*, and 96.8 to 95.8% identical to *V. parahaemolyticus* (Figure 3.3). The *V. sinaloensis* group was most closely related (98.3% to 99% identical) to *V. coralliitycus* as well as *V. brasiliensis* (97.7 to 98.3% identical).

Some *V. sinaloensis* isolates produced an amplicon which was approximately 75 bp smaller than that typical of *V. vulnificus* when subjected to species-confirmation PCR using Vv 1 & 3 (Table 3.2, Figure 3.2) (22). Isolates that produced this smaller amplicon accounted for approximately 11% and 3% of putative colonies at BTD and FD. A second PCR method for *vvhA* that is recommended by the FDA and utilizes primers Vvh 785F and 1303R (152) was also applied to *V. sinaloensis* isolates, yielding an amplicon

approximately 200 bp larger than that expected for *V. vulnificus* (Figure 3.2). No strain that was tested with both primer sets (n = 7) produced an amplicon from both PCR assays. The primer set used for qPCR (31) produced an amplicon of approximately the same size as *V. vulnificus* with the same seven *V. sinaloensis* strains. To further verify amplification of *V. sinaloensis* strains using the qPCR primer set, duplicate qPCR reactions were performed using DNA extracted from the three *V. sinaloensis* strains used for growth comparison. All strains showed amplification at late C_T values (> 35). Melting temperatures for two strains were outside the range accepted for positive results (83.7 ± 0.2° C) with means of 82.1 ± 0.1° C and 82.0 ± 0.0° C for strains Oy 1-3 and Oy 2-1, respectively; however, amplicons from the third strain, Oy 3-51, would have been considered positive (mean melting temperature 83.8 ± 0.3° C). DNA from the biotype 2 strain (ATCC 33147) was similarly tested and amplicons had a mean melting temperature of 83.7 ± 0.1° C, as expected for *V. vulnificus*.

A *V. sinaloensis*-specific primer set was also developed (Table 3.2) and tested against ten randomly-selected isolates that were confirmed as *V. sinaloensis* via 16S rRNA sequencing and were collected in June and July 2011. All *V. sinaloensis* isolates, but none of the non-target *Vibrio* spp., including three *V. vulnificus* strains (ATCC 27562, CMCP6, and 33147) and two environmental isolates identified as *V. brasiliensis* via 16S rRNA sequencing, amplified with the *V. sinaloensis*-specific primer set.

Fecal Indicator Bacteria. FIB concentrations in the water column at each site are shown in Figure 3.4. The fecal coliform regulatory limit for a one-time grab sample for shellfishing waters (43 CFU × 100 ml^{-1}) (78) was exceeded in water samples at every sampling event at BFC, at 56% of sampling events at BTD, and during no sampling event

at FD. Culturable concentrations of fecal coliforms and enterococci were significantly higher in BFC water samples compared to BTD or FD ($P < 0.0001$ for both FIB), and no significant difference was observed between FIB concentrations at BTD and FD. Among oyster samples, those collected from BFC harbored higher concentrations of fecal coliforms than those collected from BTD or FD (means of 1.4, 0.8, and 0.8 $\log_{10}$ CFU × g^{-1}, respectively; $P = 0.0021$). Differences in enterococci concentrations from oysters were not significantly different among sites ($P = 0.2596$).

Growth Characteristics of V. vulnificus vs. V. sinaloensis. To test the hypothesis that *V. sinaloensis* and *V. vulnificus* may out-compete one another under conditions relevant to environmental conditions or enrichment culture, their growth rates (μ) were compared in APW (10 ‰ NaCl), BHI + 0.5% NaCl broth (10 ‰ NaCl), and seawater (35 ‰ NaCl) *in vitro* (Figure 3.5). In seawater at 25° C, all strains of *V. sinaloensis* and the biotype 2 *V. vulnificus* (eel pathogen) had significantly higher mean μ than the other *V. vulnificus* strains ($\mu > 0.15$, $P < 0.0001$). Increased temperature in seawater resulted in more rapid growth of *V. vulnificus* strains, which diminished the strength of this relationship. At 30° C only two *V. sinaloensis* strains showed significantly greater μ than *V. vulnificus*, and μ at 37° C was not significantly different between the species. Conversely, in APW at all temperatures, the environmental *V. vulnificus* strain as well as the biotype 1 strain had significantly higher mean μ (> 0.45) than any strain of *V. sinaloensis* or the biotype 2 *V. vulnificus* ($P < 0.0001$). Interestingly, under nearly all conditions, μ of the biotype 2 *V. vulnificus* strain was more similar to *V. sinaloensis* strains than to other *V. vulnificus* strains. The most rapid growth for both species was observed in BHI + 0.5% NaCl.

Discussion

The hypothesis underpinning this study was that *V. vulnificus* concentrations in estuarine waters and oysters samples would co-vary with levels of FIB, and would be significantly different among sites known to be impacted to varying degrees by anthropogenic activities and fecal pollution. As expected, FIB concentrations were significantly greater in BFC waters and oysters than at BTD or FD. The frequency of *V. vulnificus* detection by direct plating and qPCR was also greatest at BFC, as was *V. vulnificus* concentration in the water column determined by qPCR. At least two factors could be contributing to the differences in *V. vulnificus* concentrations: (1) waters affected by fecal contamination may support higher densities of *V. vulnificus* due to greater availability of nitrogen, phosphate, and organic carbon as a result of fecal contamination (126, 132) and/or (2) lower salinity at BFC supported higher concentrations of *V. vulnificus* (110, 120, 123).

An unexpected finding was the failure to isolate *V. vulnificus* from water and oysters at favorable salinity and temperature levels, although colonies mimicking the correct phenotype on mCPC agar were plentiful. No similar phenomenon had been noted in our previous studies in the Tampa Bay estuary (60). Others have reported effective isolation of *V. vulnificus* from water and oysters on mCPC agar without enrichment (164), although at least one study found low detection frequency by PCR using oyster homogenates as template without enrichment (121). A novel *Vibrio* sp., *V. sinaloensis*, was identified from isolates on mCPC agar in many samples, and may be a major component of the *Vibrio* community in Tampa Bay. The presence of *V. sinaloensis* and other vibrios that mimic the *V. vulnificus* phenotype on mCPC agar impaired the ability

of direct plating methodology to assess *V. vulnificus* concentrations. *V. vulnificus* was detected most frequently at BFC, the most polluted and least saline site. The FDA enrichment method yielded greater recovery of *V. vulnificus* than direct plating or qPCR, potentially due to stress on *V. vulnificus* resulting from high salinity at BTD and FD which inhibited growth on mCPC without enrichment. Differences in detection frequency by method may also be a result of method limits of detection (4.5 CFU × 100 ml^{-1}, 4.5 × 10^{-2} cells × 100 ml^{-1}, and 9.1 target copies × 100 ml^{-1}, for the direct-plating, FDA enrichment, and qPCR methods, respectively), with the lowest detection limit for the enrichment method.

V. sinaloensis strains did exhibit faster growth than *V. vulnificus* biotype 1 and environmentally-isolated strains in seawater at temperatures ≤ 30° C, suggesting that *V. sinaloensis* may be able to out-compete *V. vulnificus* and represents a higher relative proportion of vibrios in the environment than *V. vulnificus* under certain conditions. Alternatively, mCPC may have been inhibitory to *V. vulnificus,* but not *V. sinaloensis,* under the particular environmental conditions in which very few *V. vulnificus* were detected (*i.e*. high salinity). The specific growth rate of the biotype 2 *V. vulnificus* strain was generally more similar to that of *V. sinaloensis* strains compared to biotype 1 *V. vulnificus*. Differences in growth characteristics among *V. vulnificus* biotypes that are influenced by temperature, pH, and salinity have been previously noted (34). The growth rate of biotype 1 *V. vulnificus* strains was significantly greater in APW enrichment medium compared to all other types and species, and this may explain why this species (or specifically biotype 1) could be detected using this methodology but not by direct plating. This finding suggests the possibility that biotype 2 strains could be abundant in

U.S. Gulf of Mexico waters and oysters, but are outcompeted by biotype 1 strains in enrichment media and are therefore not detected.

Of note, *V. sinaloensis* as well as unidentified environmental isolates which were phenotypically indistinguishable from *V. vulnificus* produced atypical amplicons when assayed with both conventional PCR primer sets targeting the *vvhA* gene. Furthermore, amplicons with similar melting temperatures to *V. vulnificus* amplicons were produced via the qPCR assay. As a result, the qPCR concentrations reported in this study may be overestimates due to amplification of *V. sinaloensis* as well as *V. vulnificus* DNA. While this possibility was not noticed until completion of sampling, the results suggest that qPCR-based methods relying on the *vvhA* gene target may be over-estimating *V. vulnificus* concentrations in environmental samples, unless a further confirmatory step is performed. This caveat has important implications for the development of new, rapid tools to enumerate *V. vulnificus*.

This study is among the first to identify *V. sinaloensis* in water and oysters, and the ecology, geographic distribution, and virulence potential of this species remains unknown. Previously, this species has only been reported to have been isolated from the liver and kidney of cultured rose snapper on the western coast of Mexico (59). The ecological and public health implications of the presence of *V. sinaloensis* in Tampa Bay waters and oysters require further study; however, the ability of this species to mimic the *V. vulnificus* phenotype as well as cross-reactivity of *vvhA* primers with this species highlights a need for careful molecular confirmation when enumerating *V. vulnificus*. Furthermore, developing rapid methods for *V. vulnificus* enumeration should be carefully validated to ensure assay specificity.

Table 3.1 – Mean values and standard deviations of physicochemical parameters at each sampling site. Values in parentheses show the range for each parameter.

Site (n)	Temperature (°C)	Salinity (ppt)	Dissolved Oxygen ($mg \times L^{-1}$)	pH	Turbidity (NTU)
BFC (10)	22.5 ± 5.7 (10.0 – 28.1)	9.1 ± 8.6 (0.3 – 25.0)	8.9 ± 5.4 (3.2 – 22.6)	7.8 ± 0.3 (7.4 – 8.6)	24.5 ± 48.2 (4.3 – 160.0)
BTD (9)	23.0 ± 5.9 (10.2 – 29.0)	25.2 ± 2.5 (20.0 – 30.0)	9.6 ± 5.1 (4.3 – 12.9)	8.2 ± 0.3 (7.8 – 8.7)	21.3 ± 45.5 (2.2 – 142.0)
FD (6)	24.0 ± 4.0 (18.1 – 28.4)	34.3 ± 1.8 (32.0 – 37.0)	8.2 ± 3.5 (3.7 – 13.9)	8.2 ± 0.2 (7.8 – 8.6)	2.7 ± 1.1 (1.5 – 4.4)

Table 3.2 - Primers used in this study and amplicon sizes observed.

Target Gene	Primer	Sequence (5' – 3')	Expected Amplicon Size (bp)[a]	Atypical Amplicon Size (bp)[b]	Reference
vvhA	Vv 1	CGCCGCTCACTGGGGCAGTGGCTG	387	312	(22)
	Vv 3	CCAGCCGTTAACCGAACCACCCGC			
vvhA	Vvh-785F	CCGCGGTACAGGTTGGCGCA	519	732	(152)
	Vvh-1303R	CGCCACCCACTTTCGGGCC			
vvhA	vvhA-qPCR-F	TGTTTATGGTGAGAACGGTGCA	99	ND[c]	(31)
	vvhA-qPCR-R	TTCTTTATCTAGGCCCCAAACTTG			
16S rRNA (generic)	Eco-8F	AGAGTTTGATCMTGGCTCAG	1484	ND	(90)
	1492RC	GGTTACCTTGTTACGACTT			
16S rRNA (*V. sinaloensis*)	Vs-2F	CACTCGTATCTCTACAAGCTTCTGAG	618	ND	This study
	Vs-4R	AGAAGGCCTTCGGGTTGTAAAG			

[a]Anticipated amplicon sizes for *vvhA* targets were determined *in silico* based on the GenBank sequence for *V. vulnificus* CMCP6 and *Enterococcus faecalis* 62 (accession number CP0024291). Amplicon size for the *V. sinaleonsis*-specific primer set was determined based on an alignment of 16S rRNA sequences.

[b]Approximate amplicon size observed from colonies with the *V. vulnificus* phenotype not confirmed as *V. vulnificus* by PCR.

[c]No difference in amplicon size was observed for *V. vulnificus* vs. other environmental isolates mimicking the *V. vulnificus* phenotype.

Table 3.3 - Detection frequency and mean concentration of confirmed *V. vulnificus* in water and oysters at each site as estimated by the direct-plating, enrichment, and qPCR methods. Mean values ± standard deviation are shown.

		mCPC-PCR (Direct Plating)		Enrichment-mCPC-PCR		qPCR	
Matrix	**Site (number of sample events)**	**Proportion of Samples Positive for *V. vulnificus* (n))**	**Mean Concentration[a]**	**Proportion of Samples Positive for *V. vulnificus* (n)**	**Mean Concentration[b]**	**Proportion of Samples Positive for *V. vulnificus* (n)**	**Mean Concentration[c]**
Water	BFC (10)	0.50 (10)	1.80 ± 1.37	1.00 (6)	1.85 ± 1.00	0.90 (10)	3.27 ± 1.31
	BTD (9)	0.22 (9)	1.05 ± 1.08	1.00 (6)	1.75 ± 1.23	0.67 (9)	1.57 ± 0.85
	FD (6)	0.00 (6)	ND[e]	0.83 (6)	1.37 ± 0.88	0.33 (6)	1.19 ± 0.54
Oysters[d]	BFC (10)	0.34 (50)	1.36 ± 0.54	1.00 (6)	2.27 ± 0.51	30	2.31 ± 0.39
	BTD (9)	0.11 (45)	1.22 ± 0.43	1.00 (6)	2.48 ± 0.84	18	2.27 ± 0.47
	FD (6)	0.00 (30)	ND[e]	1.00 (6)	2.01 ± 0.63	10	2.18 ± 0.26

[a]Units are $\log_{10}$ CFU × 100 ml^{-1} for water and $\log_{10}$ CFU × g^{-1} for oyster samples. For samples with undetectable concentrations, one half the limit of detection was used for the calculation.

[b]FDA enrichment methodology (APW followed by mCPC and PCR confirmation) was used at all sites from Feb to July 2011 and concentrations are given as MPN × $100ml^{-1}$ for water and MPN × g^{-1} for oysters. Oyster concentrations represent composite homogenates rather than individual oysters. For samples with undetectable concentrations, one half the limit of detection was used for the calculation.

[c]Units are $\log_{10}$ target copies × 100 ml^{-1} for water and target copies × g^{-1} for oyster samples. For samples with undetectable concentrations, one half the limit of detection was used for the calculation.

[d]Each individual oyster represents a single sample for direct plating and qPCR methodologies. Composite oyster samples were used for enrichment.

[e]*V. vulnificus* was not detected in these samples; one-half the limit of detection was used for statistical analyses (0.51 for water and 1.00 for oysters).

Table 3.4 - Percentage of putative (cellobiose-fermenting) colonies isolated by direct plating that were identified as *V. vulnificus* by PCR of *vvhA*.

Site	Matrix	Number Colonies[a]	*V. vulnificus*[b]
BFC	Total	292	45.9%
	Water	36	41.7%
	Oysters	256	46.5%
BTD	Total	1827	2.8%
	Water	64	6.2%
	Oysters	1763	2.7%
FD	Total	368	0
	Water	37	0
	Oysters	331	0

[a]Number of colonies matching the phenotypic description of *V. vulnificus* on mCPC agar (all were subjected to PCR confirmation).

[b]Percentage of isolates confirmed to be *V. vulnificus* by PCR using Vv primers 1&3

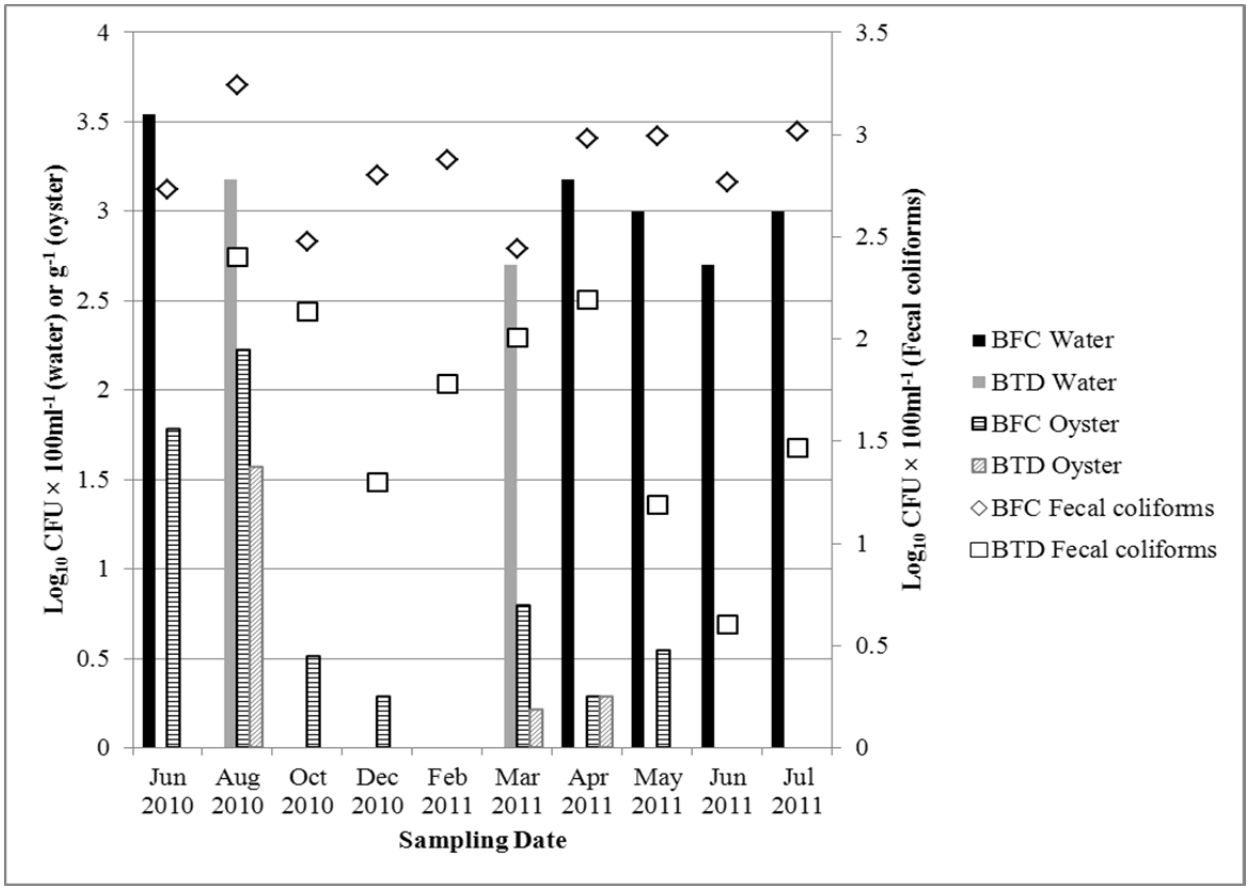

Figure 3.1 - Mean concentrations of confirmed *V. vulnificus* determined by direct plating of water (one sample per site) and oyster samples (samples from five oysters per site) on each date. Fecal coliform concentrations from the water column at BFC (◊) and BTD (□) are also shown. BTD was not sampled in July 2010. The absence of a value for a site on any given dated indicates that *V. vulnificus* was not detected; FD is not shown in the graph as *V. vulnificus* was not isolated from that site by direct plating during this study.

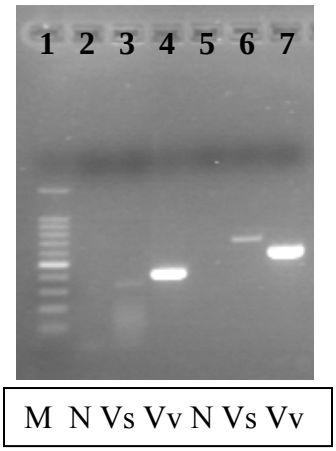

Figure 3.2 - Results of two PCR assays for the *vvhA* gene for *V. vulnificus* and *V. sinaloensis* strains. Lane 1: Molecular weight ladder (100 to 1500 bp) (M). Reactions in lanes 2-4 used Vv 1 & 3 primers while those in lanes 5-7 used Vvh-785F and Vvh-1303R (FDA-recommended primer set). Lanes 2 and 5: negative (no template) controls; lane 3: *V. sinaloensis* isolate Oy 3-3; lanes 4 and 7: *V. vulnificus* 9067-96; lane 6 is *V. sinaloensis* isolate Oy 2-1.

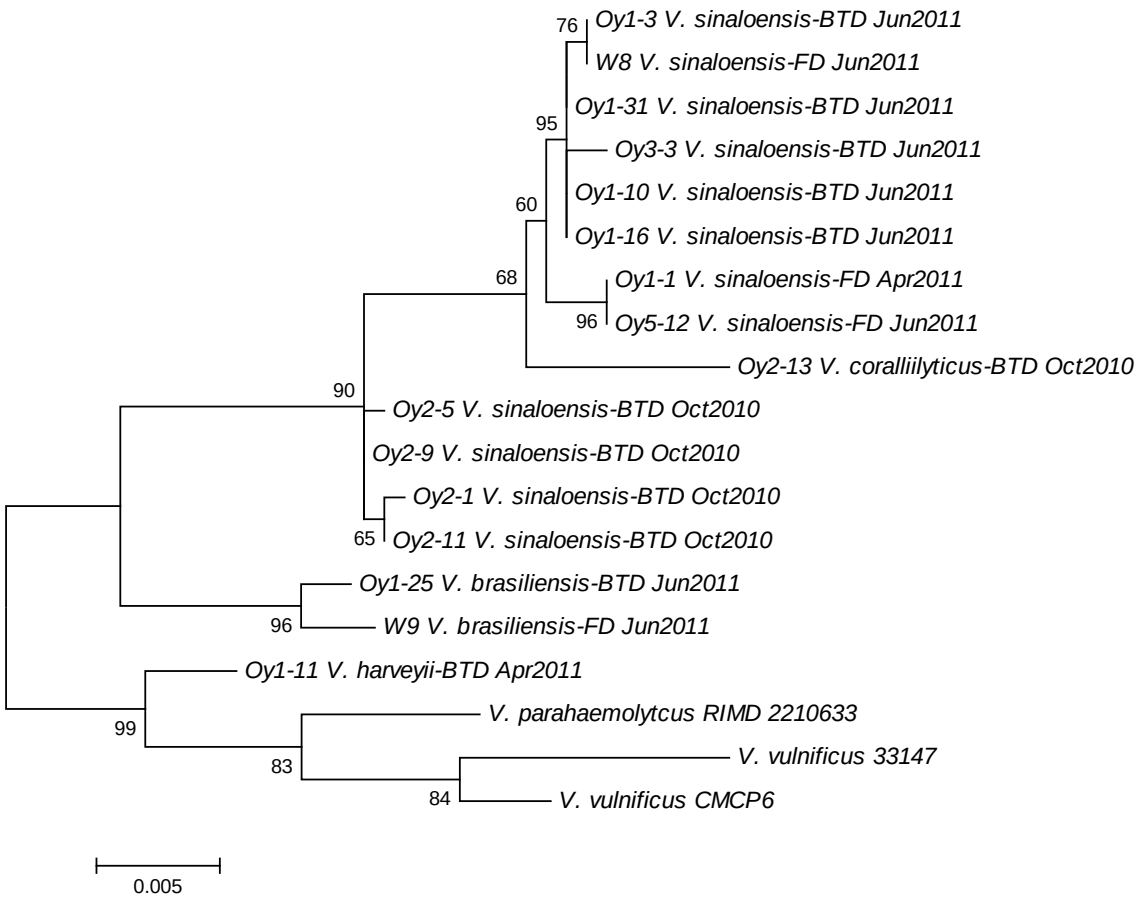

Figure 3.3 - Maximum likelihood tree of partial 16S rRNA sequences (1230 bp) of environmental isolates obtained in this study and reference *Vibrio* spp. (retrieved from GenBank). Isolates designed "Oy" were isolated from oysters while those designated "W" were isolated from water. Bootstrap values were calculated at 500 iterations.

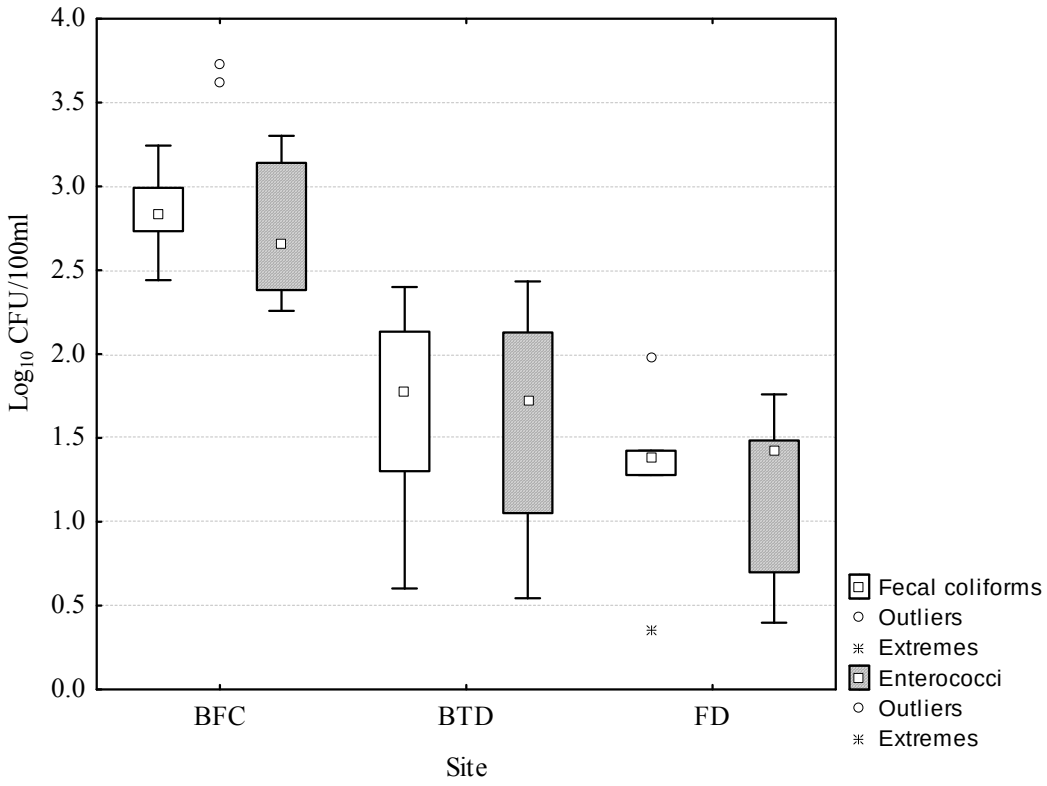

Figure 3.4 - Box and whisker plot of fecal indicator bacteria concentrations (log_{10} CFU × $100ml^{-1}$) in the water column. Inner boxes represent mean concentrations throughout the study period, and box lengths reflect upper and lower quartiles. Whiskers represent one standard deviation from the mean. Outliers were within two standard deviations from the mean while extremes are greater than two standard deviations.

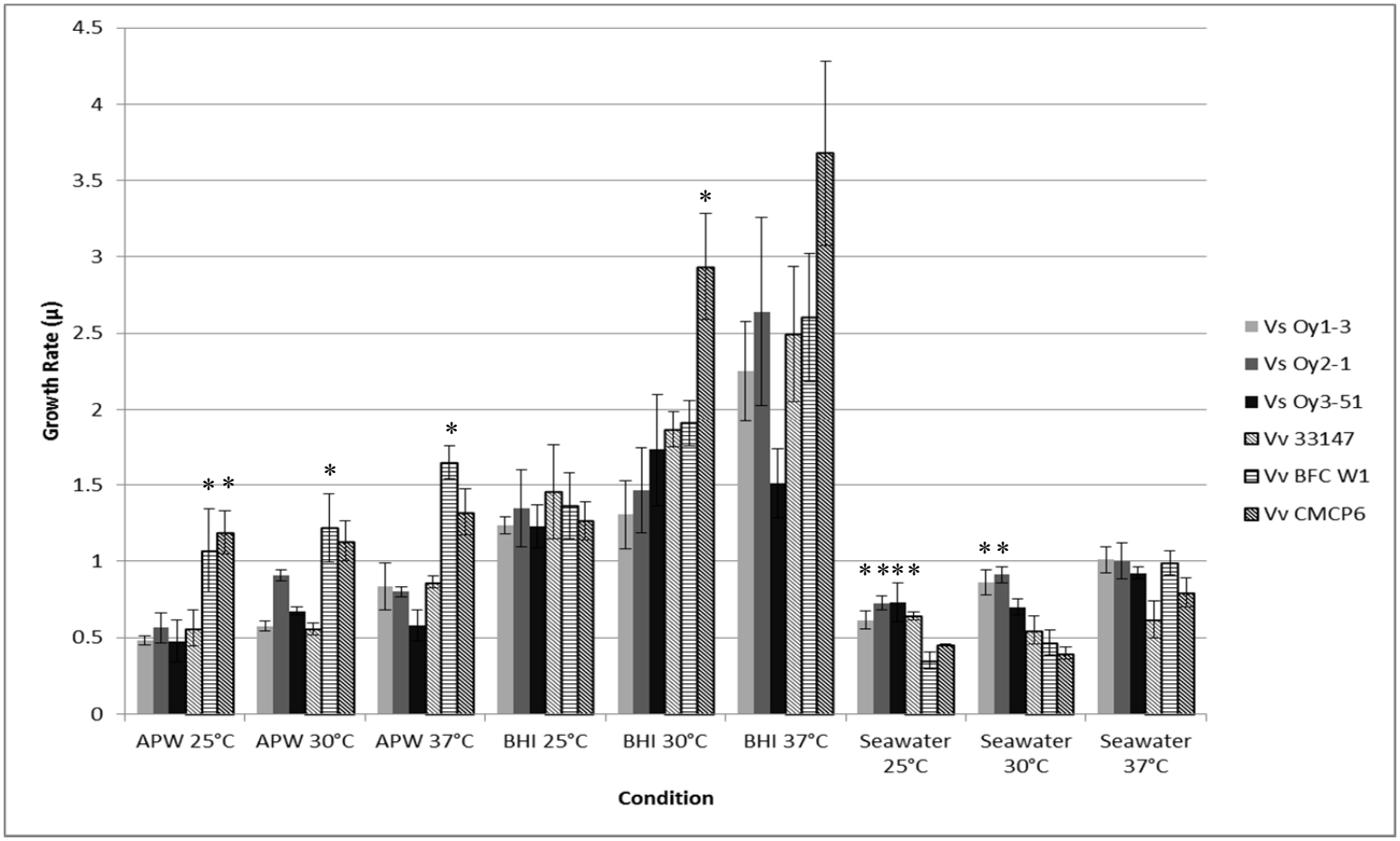

Figure 3.5 - Mean growth rates (μ) of *V. sinaloensis* and *V. vulnificus*. All *V. sinaloensis* strains as well as *V. vulnificus* BFC W1 were isolated from environmental samples. *V. vulnificus* CMCP6 and 33147 were obtained from laboratory stocks and represent biotypes 1 and 2, respectively. μ was assessed in triplicate for each species. Error bars reflect standard deviations. For each condition, asterisks indicate strains which showed significantly higher growth within a particular set of conditions.

CHAPTER 4: DIFFERENTIAL EXPRESSION OF A SODIUM-PHOSPHATE COTRANSPORTER AMONG VIBRIO VULNIFICUS STRAINS[3]

Introduction

Vibrio vulnificus is a gram-negative bacterium with a high degree of genetic diversity (25). The species is autochthonous to estuarine and marine waters and is frequently found in shellfish, particularly oysters (171). *V. vulnificus* strains can be grouped into three biotypes based on differences in genotype and phenotypic characteristics (14, 15, 150). All three biotypes are opportunistic pathogens of humans (1, 14, 75); however, biotype 1 is the predominant human pathogen, and biotype 2 is primarily associated with infection in eels (150). Infection with biotype 1 strains can cause gastroenteritis, septicemia, and wound infections (74), while biotype 3 infections are associated with wound infections in humans following handling of *Tilapia* (14). All strains, regardless of virulence potential, are environmentally-derived, but differences in virulence potential toward humans have resulted in efforts to identify more highly virulent strains (79).

Among biotype 1 strains, specifically, methods exploiting heterogeneity in a virulence correlated gene (*vcg*) have been developed to distinguish between strains common to the environment which are generally not implicated in or isolated from human infections (type E, environmental) vs. those which are believed to be more highly

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virulent and have been isolated from clinical cases (designated type C for clinical) (128). This strain distinction is closely aligned with other typing strategies based on heterogeneity of the 16S rRNA gene or multilocus sequence typing to distinguish potentially less virulent strains (*vcg* type E, 16S type A or AB, lineage II) from those suspected to be more highly virulent (*vcg* type C, 16S type B, lineage II) types (39). While these typing strategies are well correlated with each other as well as strain isolation source, the relationship between strain type and virulence remains imperfect.

A previous study assessing diversity among biotype 1 strains via BOX-PCR revealed that most type C strains produced a unique DNA fragment compared to type E strains (138). The nucleotide sequence of this DNA fragment corresponded to a portion of a gene encoding a conserved hypothetical protein (*hypB*; VV1_0515 in *V. vulnificus* CMCP6, accession number NP_759506) that was only amplified in type C strains, as assessed by a conventional PCR assay targeting *hypB* (138). The gene is located immediately downstream of the *nptA* gene encoding a sodium-dependent phosphate transporter (VV1_0514 in *V. vulnificus* CMCP6, accession number NP_759505), and both genes were expected to be transcribed as an operon. Amplification of the putative *nptA-hypB* operon was observed from genomic DNA of all strain types; however, transcription of the putative operon via conventional reverse transcriptase PCR (RT-PCR) was only observed in type C strains (139). Based on these studies, the putative operon was hypothesized to play a role in strain virulence. Several previously described methods for gene knockout mutation in vibrios (63, 176) were unsuccessful at knocking out the *nptA* gene in the biotype 1, type C strain CMCP6, suggesting its function may be essential for survival.

The function of NptA has not been widely studied in prokaryotes outside of *V. cholerae* (167). One such study conducted by Lebens, *et al.* (92) found the protein to be homologous to type II sodium-phosphate cotransporters in animals, which facilitate P_i uptake in intestinal and renal cells (73, 111). This group assessed the potential of this protein to act as a high-affinity P_i uptake system in *V. cholerae* (92); however, increased P_i uptake during phosphate starvation could not be attributed to this gene based on expression of a reporter gene under control of the *nptA* promoter. Interestingly, activity of the *V. cholerae* protein did show pH-dependence similar to the animal enzyme (only 50% activity at pH 6.5 vs. pH 9.0), although, the prokaryotic enzyme lacked the REK motif, which affects pH-dependent alterations in activity in animals (44, 92). The authors suggested based on these findings that NptA in *V. cholerae* may act in response to changes in pH, energy, and phosphate availability as the bacterium moves from water to the host environment. Furthermore, they suggested that the protein functions as a low-affinity, high-capacity P_i uptake system in *V. cholerae* which allows rapid growth in nutrient-rich environments (*e.g.* in an animal host); however, the factors affecting regulation of this gene have yet to be determined (92). The *nptA* gene has also been found in a variety of other *Vibrio* spp. whose genomes have been sequenced, including *V. alginolyticus*, *V. cambellii, V. brasiliensis*, *V. parahaemolyticus*, and *V. sinaloensis*, as well as other human pathogens such as *Aeromonas hydrophila*, *Bacillus anthracis*, *Clostridium botulinum*, and *C. difficile* (104).

The *nptA* gene in *V. vulnificus* has a nucleotide sequence that is 75% identical to that of *V. cholerae*, and the protein shares 86% amino acid similarity. We hypothesized that pH, salinity, and/or phosphate concentration may affect transcription levels of this

gene, and that transcript abundance would be elevated under more nutrient-rich conditions (*e.g.* higher phosphate concentration). Furthermore, based on differences in gene expression among strains observed using the RT-PCR assay (139), we hypothesized that relative transcript abundance would vary among strains of differing *vcg* types or biotypes. To test these hypotheses, concentrations of *nptA* transcripts were assessed by quantitative reverse-transcriptase PCR (qRT-PCR) using strains representing each biotype, and biotype 1 genotypes C and E, in chemically-defined media of varying salinity, phosphate, and pH. In addition, genomic analysis of the *nptA* gene from each strain was performed to determine if differences in nucleotide or amino acid sequence reflect potential differences in transcript abundance among strain types.

Materials and Methods

Bacterial Culture Conditions. *V. vulnificus* strains included ATCC 27562 (biotype 1, type E), CMCP6 (biotype 1, type C), 9067-96 (biotype 1, type C) (161), ATCC 33147 (biotype 2) , and 302/99 (biotype 3) (34). For clarity, strains will be referred to throughout as 27562(E), CMCP6(C), 9067-96(C), 33147(2), and 302/99(3), where parenthetic designations specify genotype (C or E for biotype 1) or biotype (2 or 3). Strains were maintained on Difco™ marine agar (Becton Dickson, Sparks, MD). For growth and gene expression studies, cultures were incubated at room temperature (25°C) overnight in 5 ml of BBL™ brain heart infusion (Becton Dickson, Sparks, MD) broth supplemented with 0.5% (wt/vol) NaCl (BHI + 0.5% NaCl). Overnight cultures were streaked onto marine agar to verify pure cultures and for maintenance. Overnight growth in copiotrophic BHI + 0.5% NaCl was performed to yield a substantial inoculum in experimental media the following day such that growth to mid-exponential phase would

be observed in < 4 hrs, allowing for RNA extraction and treatment on the same day (see below). While this methodology likely resulted in a carry-over of some nutrients from the overnight culture, the effect of this carry-over is expected to have been minimal based on the extent of dilution (below). Growth rate and gene expression data observed are presumed to result from experimental conditions since carry-over was kept constant in all trials. Overnight cultures were diluted in duplicate 1:20 (v/v) in 20 ml of defined media modified from (116) [50mM Tris, 10mM NH_4Cl, 0.1mM $CaCl_2$, 1mM $MgSO_4$, 0.1mM $FeCl_2$, 0.2% (wt/vol) Bacto™ yeast extract (Becton Dickson, Sparks, MD), 0.2% (wt/vol) Bacto™ casamino acids (Becton Dickson, Sparks, MD), and 0.2% (wt/vol) glucose (1.1mM)]. The base salinity of the experimental medium was 10‰, which was adjusted to 30‰ with NaCl. Phosphate concentration was adjusted by addition of KH_2PO_4 at concentrations of 5µM or 1mM. pH of the medium was adjusted to 6.0, 7.0, or 8.0, and the medium was filter-sterilized through a 0.45µM pore-sized nitrocellulose filter (Fischer Scientific, Waltham, MA).

In addition to experimental media, growth and *nptA* expression were also assessed in cultures grown in BHI + 0.5% NaCl and 0.2µM filter-sterilized seawater (35‰, pH 8.0) for comparison of copiotrophic vs. oligotrophic conditions. All cultures were grown in duplicate at 37°C with agitation at 155 rpm. Growth was measured using the Nanodrop 2000 spectrophotometer (Fisher Scientific, Waltham, MA) as the absorbance at OD_{600}, and cultures were plated in triplicate at three time points during the exponential growth phase on Difco™ typtic soy agar (TSA) (Becton Dickson, Sparks, MD) to obtain culturable concentrations and calculate specific growth rates (µ). For three culture conditions (15‰, 1mM PO_4, pH 6.0; 10‰, 5µM PO_4, pH 6.0, and 30‰, 1mM PO_4, pH

8.0), pH was monitored during growth to determine the effect of metabolites on pH and assess pH at the time of RNA extraction, and it was observed that pH dropped approximately 0.5 units during growth. This finding is important as a medium with an initial pH of 8.0 corresponds to a pH of 7.5 at the time of RNA extraction, which may affect the relative levels of *nptA* expression among strains.

RNA Extraction. During mid-exponential growth (generally ~10^8 colony forming units (CFU) / ml, OD_{600} = 0.45), 1.8 ml culture was removed from each replicate culture for RNA extraction. RNA was extracted using the RiboPure™-Bacteria Kit (Ambion, Austin, TX) following the manufacturer's instructions with a single 50 µl elution followed by the DNase I treatment as described in the manufacturer's instructions. A second DNase treatment was performed using TURBO DNA-*free* (Ambion, Austin, TX) according to the manufacturer's instructions for routine treatment.

DNA Extraction. For use as a PCR positive control and for construction of the standard curve (described below), *V. vulnificus* CMCP6 was grown overnight at 37°C in 5 ml BHI + 0.5% NaCl. DNA was extracted using the QIAamp DNA Blood MIDI kit (Qiagen, Valencia, CA) following the manufacturer's instructions. For sequence analysis of *V. vulnificus* strains ATCC 27562, 9067-96, 33147, and 302/99, cultures were grown overnight at 37°C in 2 ml BHI + 0.5% NaCl. DNA extraction was carried out using the QIAamp DNA Stool Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The DNA blood MIDI kit yielded higher concentrations of DNA, which were necessary for construction of the standard curve. DNA concentrations were determined using the NanoDrop 2000 spectrophotometer (Fisher Scientific, Waltham, MA).

PCR. RNA extracts were verified to be free of DNA contamination via endpoint PCR targeting a conserved region of *nptA*. Primers are described in Table 4.1. PCR was carried out in 25 µl reactions consisting of 12.5 µl 2X GoTaq Green Master Mix (Promega, Madison, WI), 8.5 µl nuclease-free water (Fisher Scientific, Waltham, MA), 1µl (0.4 µM) of each primer (Integrated DNA Technologies, Coralville, IA), and 2 µl of RNA template. Thermocycling conditions included an initial denaturation step at 94°C for 5 minutes followed by cycling at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for one minute for 30 cycles with a final extension at 72°C for 7 minutes. PCR products were visualized via gel electrophoresis using a 2% agarose gel stained with ethidium bromide. For each set of PCR reactions a no-template-added negative control was included to control for contamination, and genomic DNA from *V. vulnificus* CMCP6 was used as a positive control.

Primer Design. The primer sets used for qRT-PCR are shown in Table 4.1. Primers were designed based on the *nptA* gene sequence of *V. vulnificus* CMCP6 (VV1_0514, accession number NC_004459) using PrimerQuest software (Integrated DNA Technologies, Coralville, IA). Initial qRT-PCR runs failed to amplify the biotype 3 strain. A partial *nptA* sequence was obtained for this strain by direct sequencing using the nptA1/nptA2 primer set (accession number JN420346), and primers (BT3nptA1/BT3nptA2, Table 4.1) were designed that target regions of similarity between the biotype 3 strain and *V. vulnificus* CMCP6. The 16S rRNA was used as an endogenous control for normalization of *nptA* concentration as has been previously reported (38, 95, 148).

Standard Curve. Standard curves for all qRT-PCR targets were constructed using genomic DNA from *V. vulnificus* CMCP6. Target copy numbers were estimated by multiplying the DNA concentration by Avogadro's number and dividing by the product of the genome size and average weight of a base pair (178). Genomic DNA was serially-diluted in AE buffer (Qiagen, Valencia, CA) to final concentrations ranging from 10^1 to 10^6 gene copies/reaction. Each concentration on the standard curve was run in duplicate on each reaction plate. Default settings for the Applied Biosystem 7500 Real-Time PCR System (Carlsbad, CA) were used to establish the baseline fluorescence for each quantitative reverse transcriptase PCR (qRT-PCR) run. Standard curves relating C_T values to target copy concentration were constructed and linear regression was used to evaluate qRT-PCR runs. All runs had correlation coefficients (r^2) > 0.90 with amplification efficiency between 80 and 110%.

qRT-PCR. RNA concentrations were measured using the NanoDrop 2000 spectrophotometer (Fisher Scientific, Waltham, MA) and were diluted to 20 ng/µl in nuclease-free water (Fisher Scientific, Waltham, MA). PCR reactions were carried out in 96-well plates and all reactions were run using the Applied Biosystems 7500 Real-Time PCR System (Carlsbad, CA). The qScript 1-step SYBR green qRT-PCR kit, low ROX (Quanta Biosciences, Gaithersburg, MD) was used for all qRT-PCR reactions. Reactions were 25µl total volume consisting of 12.5 µl 2X qScript master mix, 5.5 µl nuclease-free water, 0.75 µl (0.3µM) of each primer (Integrated DNA Technologies, Coralville, IA), and 5 µl RNA template (100ng). qRT-PCR reaction plates were centrifuged prior to PCR at 1000 × *g* for 1 minute. Thermocycling conditions included an initial reverse-transcriptase step at 50°C for 10 minutes, denaturation at 95°C for five minutes, cycling

at 95°C for 10 seconds and 60°C for 30 seconds, and a final melt curve stage using instrument default settings. The 16S rRNA gene target was set as the endogenous control for normalization of the *nptA* transcript data, and data were reported as normalized mean quantities as calculated by the instrument. Each RNA sample was run in duplicate and two no-template-added controls were included for each target run as negative controls. Melting temperatures for standard curve amplicons were evaluated to assess specificity of amplification, and amplicons which had melting temperatures outside the ranges specified (Table 4.1) were not considered for data analysis.

DNA Sequencing. PCR amplicon was generated for the *nptA* gene of *V. vulnificus* 302/99(3) using the nptA1/nptA2 primers (Table 4.1). The region amplified by the nptA1/nptA2 primer set does not include the region of the gene expected to encode the REK motif related to pH-dependent activity (167). A previously described primer set (nptAF/hypB) (139) includes 991 bp of the *nptA* gene including the region expected to contain the REK motif, so this primer set was used to generate amplicons for *V. vulnificus* 9067-96(C), 27562(E), 33147(2), and 302/99(3) as described above (accession numbers JN646864, JN420347, JN420348, and JN646863, respectively). All PCR amplicons were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Duplicate single-extension sequencing using both primers was performed on amplicons by Macrogen Corp, USA (Rockville, MD).

Sequence Analysis. Contigs for forward and reverse sequences were assembled using DNA Baser software (HeracleBiosoft, Pitesti, Romania). Sequences obtained for *V. vulnificus* strains used in this study were aligned with *nptA* sequences for *V. vulnificus*

strains CMCP6, M06-24/O, YJ016, *V. alginolyticus* 12G01, *V. brasiliensis* CAIM 495, *V. cholerae* O395, and *V. parahaemolyticus* RIMD 2210633, and *V. sinaloensis* CAIM 648 obtained from GenBank (NP_759505, YP_004189734, NP_933474, ZP_01261953, ZP_08097714, YP_001216171, NP_796905, and ZP_08102804). Sequences were aligned by ClustalW using MEGA 5 software (147). Neighbor-joining tree generation, bootstrapping, and distance matrix calculation were also performed using MEGA 5 software (147).

Statistics. Mean quantities of *nptA* mRNA normalized to 16S rRNA were log-transformed to meet the assumption of a normal distribution for all statistical analyses. All statistical analyses were considered significant at the alpha level of 0.05. Differences in *nptA* transcript abundance among experimental media and among strains were compared via two-tailed, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using InStat version 3.00 (GraphPad Software, Inc, La Jolla, CA). Spearman correlations relating salinity, phosphate concentration, pH, growth rate, and *nptA* expression were calculated using IBM SPSS Statistics 19 (SPSS, Chicago, IL). Nested ANOVA analyses were also performed to determined interaction effects between salinity, phosphate concentration, and pH. To compare *nptA* transcript abundance with μ, strains were separated into two groups based on similar patterns of *nptA* expression as well as similarity in *nptA* gene sequence (biotype 1, type C and biotype 3 strains formed the clinically-associated group, while biotype 1, type E and biotype 2 strains formed the environmentally-associated group). Mean μ in each medium were calculated for both groups. Comparisons were not made among individual strains because the power of the analysis was very low; however, grouping the strains increased the statistical power.

Relative growth rates were calculated as the ratio of μ of the clinically-associated group to μ of the environmentally-associated group. Relative concentrations of *nptA* transcripts were also calculated using the same groupings (ratio of clinically and environmentally-associated groups). The relative μ and concentration of *nptA* transcripts in each medium were subjected to Pearson correlation analysis.

Results

nptA Transcript Abundance. Abundance of *nptA* transcripts normalized to that of the 16S rRNA gene is shown for each *V. vulnificus* strain in Figures A1 through A5 (Appendix), and the interaction plot showing the effects of salinity and pH on transcript abundance is shown in Figure 4.1. The effect of phosphate on transcript abundance is not shown as it did not significantly affect transcript abundance among all strains. 16S rRNA transcription was consistently measured on the order of 10^8 copies per reaction for all strains regardless of the culture media. Normalized *nptA* transcript abundance varied under the conditions tested by as much as 3.2 log units, and factors influencing differences in expression levels differed by strain (Figure 4.1 and Figures A1-5). When data from all conditions were pooled, the two biotype 1 (type C) strains (CMCP6(C) and 9067-96(C)) as well as the biotype 3 strain, 302/99(3) showed significantly lower levels of *nptA* transcript abundance (more than 100-fold lower; Figures A1 through A3) than did the biotype 1 (type E) strain (27562(E)) or the biotype 2 strain (33147(2)) (Figures 4.4-5, $P < 0.0001$). Post-hoc tests showed no significant differences in *nptA* transcript abundance among the biotype 1 (type C) strains and biotype 3 strain ($P = 0.3877$). Similarly, transcript abundance between the biotype 1 (type E) strain and biotype 2 strain were not significantly different ($P = 0.3746$). For all strains, *nptA* transcript abundance

was approximately tenfold greater in BHI + 0.5% NaCl compared to sterile seawater, in which the lowest concentrations of transcripts (between -4.3 and -5.3 log units) were measured.

Parameters influencing transcript concentration were similar among biotype 1 (type C) and biotype 3 strains. Mean abundance of *nptA* mRNA for these strains was -4.0 log units (on the order of 10^4 target copies/reaction) in most media; however, in conditions of low salinity (10‰) and high pH (8.0), transcript concentrations for the biotype 1 CMCP6(C) and biotype 3 strains was approximately 1.4 logs greater than in other experimental media (Figures A1 and A3). Mean transcript concentrations were also higher for the biotype 1 9067-96(C) strain in low salinity, alkaline media, but the difference in transcript concentration was not as great compared to other conditions tested in 9067-96C(C) as it was for CMCP6(C) or biotype 3 (Figure A2). Transcript concentration in the biotype 3 strain was significantly negatively correlated with salinity ($r^2 = 0.30$, $P = 0.003$). Salinity alone accounted for 17.7% and 39.4% of variability in transcript abundance for CMCP6(C) and the biotype 3 strain, respectively, and this effect was significant ($P = 0.027$ and < 0.001, respectively). The interaction of salinity with pH was also significant for CMCP6(C) and the biotype 3 strain, accounting for 27.2% and 19.5% of variation in transcript abundance, respectively ($P < 0.0001$). pH and *nptA* transcript concentration were positively correlated for both biotype 1, type C strains ($r^2 =$ 0.18 and 0.40, $P = 0.021$ and < 0.0001 for CMCP6(C) and 9067-96(C), respectively).

Similar concentrations of *nptA* transcripts were observed for biotype 1 (type E) and biotype 2 strains under all conditions tested, and differed from those of the other strains [biotype 1, (type C) and biotype 3 strains] in media at pH 6.0. The biotype 1 (type

E) and biotype 2 strains showed increased transcript abundance (approximately one $\log_{10}$ increase) at low pH regardless of salinity or phosphate concentration and maintained high concentrations in all low salinity media (Figures A4 and A5). Normalized *nptA* transcript abundance ranged from -1.5 log units to -4.6 log units (approximately 10^6 to 10^4 target copies/reaction). pH and *nptA* transcript abundance were negatively correlated for the biotype 1 (type E) strain ($r^2 = 0.14$, $P = 0.042$), but not the biotype 2 strain. Similar to the biotype 3 strain, salinity was significantly negatively correlated with *nptA* abundance for the biotype 1 (type E) and biotype 2 strains ($r^2 = 0.25$ and 0.59, $P = 0.004$ and < 0.001, respectively) with normalized transcript concentrations between -2.7 and -1.1 log units at low salinity. The interaction of salinity with pH also showed a significant effect on transcript abundance, accounting for 15.8% and 14.5% of variability in biotype 1 (type E) and biotype 2 strains ($P \leq 0.001$).

Analysis of Deduced NptA Sequences. Due to the observed influence of pH on *nptA* transcript abundance, partial NptA amino acid sequences were deduced from nucleotide sequences for all *V. vulnificus* strains to determine whether the REK motif previously associated with pH-dependent activity in animals (167) was present. This motif was not observed in the sequences obtained. These sequences were compared with amino acid sequences of *V. vulnificus* strains (CMCP6, YJ016, and MO6-24/O; all biotype 1, type C strains) obtained from GenBank (Figure 4.2). Deduced sequences matched a 302 amino acid segment encoded by 3' terminus of the *nptA* gene in *V. vulnificus* CMCP6(C). Sequences for CMCP6(C), 9067-96(C), and M06-24/O were identical (100% identity) and had 97.8% identity to the partial *nptA* sequence of YJ016. Partial sequences of the biotype 1 (type C) strains used in this study had 96.3% identity to 302/99(3). Sequences

for strains 27562(E) and 33147(2) had 98.2% identity to each other. Sequence identity for both 27562(E) and 33147(2) strains compared to CMCP6(C), 9067-96(C), and 302/99(3) was 85.4 to 89.4%. The gene sequence in *V. parahaemolyticus* had 63.7 to 65.2% identity to *V. vulnificus* strains, and *V. cholerae*, which had the least similar sequence, had 50.5 to 54.5% identity to *V. vulnificus*.

Growth Rates. Mean growth rates (μ) for all strains (pooled data) in each condition are shown in Table 4.2. Mean μ among all strains (pooled) ranged from 0.16 to 1.49 hr^{-1} under the various conditions (Table 4.2). Some significant differences in μ under the differing conditions were observed (Table 4.2). In general, the slowest mean growth rates were observed in media with either low salinity (0.37 hr^{-1}), low pH (0.55 hr^{-1}), or in seawater (0.43 hr^{-1}). When growth rates were compared among strains by pooling data from all media, they did not vary significantly ($P = 0.7314$, Figure 4.3).

We hypothesized that μ of *V. vulnificus* strains would be correlated with *nptA* transcript abundance as the gene was expected to facilitate growth via rapid phosphate uptake. Since biotype 1 (type C) and biotype 3 strains showed similar concentrations of *nptA* transcripts and amino acid sequence identity different from biotype 1 (type E) and biotype 2 strains, these strains were grouped separately. Grouping also served to increase the power of the analyses and accounted for differences in strain numbers between groups. Group I included CMCP6(C), 9067-96 (C), and 302/99(3) while group II included 27562(E) and 33147(2). The relative μ (ratio of mean μ for group I vs. mean μ of group II strains) was calculated for each medium (data from all strains in each group were pooled). A higher relative μ indicates relatively faster growth of group I vs. group II strains (Table 4.2); a relative μ of 1.0 would indicate equal growth rates. The two

conditions in which the group I strains clearly outpaced the growth of the group II strains were low salinity, pH 7.0 (relative μ of 1.77 for high phosphate, and 1.45 for low phosphate). Conversely, the mean μ of the group II strains clearly exceeded that of the group I strains in seawater (ratio of 0.55). Relative μ were significantly positively correlated with relative *nptA* transcript abundance, which was calculated using the same strain groupings as relative μ ($r^2 = 0.30$, $P = 0.0441$).

Discussion

This study revealed differences in *nptA* transcript abundance among *V. vulnificus* strains representing the three known biotypes, and biotype 1, C and E genotypes suggesting a potential for differences in expression of this gene. Biotype 1 (type C) and biotype 3 strains (group I) showed transcript concentrations similar to each other but distinct from and less than biotype 1 (type E) and biotype 2 strains (group II), which were also similar to each other. In addition to differences in *nptA* transcript abundance among the strains, mRNA concentrations were also differentially influenced based on changes in salinity, phosphate concentration, and pH between strain groups. These results suggest that the *nptA* gene may play a divergent role between strain groups, which may affect a given strain's ability to adapt to shifts in changes to environmental conditions. The previous study of NptA in *V. cholerae* suggested that this protein may also aid in host colonization (92). Differences in *nptA* expression between *V. vulnificus* strain groups may reflect differences in host preference or virulence potential, although the relationship between *nptA* expression and either host association or virulence was not assessed in this study.

The *V. cholerae* NptA protein exhibited pH-dependent activity when cloned into *Escherichia coli*, showing half the activity at pH 6.5 compared to pH 9.0; however, expression of *nptA* was not measured (92). In the present study, pH also significantly affected transcript abundance (increased concentrations at low pH) as was observed for biotype 1 (type E) and biotype 2 strains in all pH 6.0 media (regardless of salinity or phosphate concentration), which would help offset a decline in enzyme activity, if it occurs in *V. vulnificus*. For the group II strains, an independent effect of salinity was also observed such that low salinity, regardless of phosphate concentration or pH, also increased mRNA concentrations. In contrast, *nptA* transcript abundance in biotype 1 (type C) and biotype 3 was positively correlated with pH when salinity was low, and a significant increase in *nptA* transcripts was observed under these conditions. Strain-dependent differences in *nptA* transcript concentrations may be related to differences in stress response or host association among the strains.

The pH of experimental media dropped approximately 0.5 units during *V. vulnificus* growth such that an experimental medium with an initial pH of 8.0 would be at pH 7.5 at the time the RNA for the expression assay was extracted. Taking this into consideration, the low salinity (10‰), high phosphate, pH 8.0 medium in which the biotype 1 (type C) and biotype 3 clinically-associated strains showed the greatest *nptA* transcript concentrations would be most similar to human blood chemistry (9‰, pH ~7.4) among the conditions tested (91). Low pH media (representing a pH of ~5.5 at RNA extraction), in which the biotype 1 (type E) and biotype 2 strains showed the highest transcript abundance, may also approximate acidic conditions found in the digestive tissues of oysters (pH 5.5) or fish known to be colonized by vibrios (ranging from pH 1.0

to 5.0, after feeding) (45, 51, 58, 115, 134). These observations suggest that transcript of *nptA* may be up-regulated to facilitate growth in specific host environments. The chemistry of the experimental media tested in this study vs. that of host environments is not directly comparable due to the presence of other ions, serum, etc. in the host, so interpretation of these data regarding a specific role for NptA in host-colonization should be considered cautiously. Interestingly, increased *nptA* abundance in biotype 1 (type C) strains v. biotype 1 (type E) or biotype 2 strains was not observed in seawater (pH 8.0), which may be due to a greater amount of variability associated with the cumulative interactions of pH and salinity for biotype 1 (type C) strains than biotype 1 (type E) or biotype 2 strains. Furthermore, greater concentrations of *nptA* transcripts in the biotype 1 (type E) strain compared to the biotype (type C) strain may be related to generally higher proportions of type E in environmental waters (60, 99).

Analysis of the deduced amino acid sequences showed that the REK motif related to pH-dependent activity of animal NaPi-type II transporters was not present in these *V. vulnificus* strains, which was also the case for *V. cholerae* (92). Alignment of NptA sequences revealed that the amino acid sequences of the biotype 1 (type E) strain were nearly identical to the biotype 2 strain (98.2% identity) but differed from biotype 1 (type C) sequences (86.2% identity). Furthermore, the NptA sequence of the biotype 3 strain, which showed *nptA* transcript levels similar to biotype 1 (type C) strains, showed greater identity (96.3%) to the biotype 1 (type C) strains than the biotype 1 (type E) or biotype 2 strains (89.4% and 88.6% identity, respectively). These data support the existence of at least two separate alleles between the strain groups, and the heterogeneity of the NptA

gene sequence between these groups may account for differences in expression pattern or even gene function, although this was not investigated.

In this study, we observed that *V. vulnificus* strains more commonly isolated from water, oysters, and fish (group II strains) exhibit nearly 100-fold greater transcript abundance of the *nptA* gene compared to group I strains, which are more commonly associated with human infections, and that heterogeneity in the amino acid sequence is related to strain type. Such heterogeneity between biotype 1 *vcg* genotypes has been previously demonstrated in eight virulence-associated and housekeeping genes, and the authors suggested that heterogeneity between type C and type E strains may a divergence of the species into two ecotypes (127). Interestingly, *nptA* transcript abundance appears to be related to relative μ, where higher μ and more *nptA* transcripts characterize the group II strains compared to the group I strains. Salinity, pH, and phosphate concentration all affected the level of *nptA* abundance at certain levels and as a result of interactions between parameters, suggesting that multiple environmental variables contribute to regulation of expression, and that these effects differ by strain. Further studies are necessary to understand the relationship of *nptA* regulation and function to *V. vulnificus* growth in free-living and host-associated states, and may shed some light on the mechanism of host preference and virulence potential of this opportunistic pathogen.

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Table 4.1 – Primer sets used in this study.

Target	Primer	Sequence (5'-3')	Amplicon Size (bp)[a]	Melt Temp[b]	Reference
nptA **(all strains)**	nptA1	TGGTCTTGTGGCGACAGCTCTTAT	691	ND	This study
	nptA2	TACCAATGTTCGCACCCAGAGTGA			
nptA **(biotypes 1 and 2)**	qNptA1	CCTTCCTTTGGAAATGATGTTCGGC	121	81.10 ± 0.16	This study
	qNptA2	TACAGCGTACGTGGCCAAGAGAAA			
nptA **(biotype 3)**	BT3nptA1	CACCCGTGTTCAATAGTGGTGACA	76	77.53 ± 0.21	This study
	BT3nptA2	CCTTCCTTTGGAAATGATGTTCGGC			
16S rRNA	RRSH0701F	ACGACACCACCTTCCTCACAAC	136	84.31 ± 0.23	(95)
	RRSH0701R	ACACGGTCCAGACTCCTACGG			
nptA/hypB	nptAF	GAGTTTGCCTCACACCCTGT	1236	ND	This study
	hypBR	TGCCGCTCTTCCTTGTAGTT			

[a]Amplicon size determined based on the genome sequence of *V. vulnificus* CMCP6

[b]Average melting temperature and standard deviation for qRT-PCR targets. Average melting temperatures were determined from standard curve amplicons measured during the study (n = 169, 79, 199 for *nptA* – biotypes 1 and 2, *nptA* – biotype 3, and 16S rRNA, respectively). Reactions that produced melting temperatures > 0.5°C from the mean melting temperature at the time of analysis were not considered in this calculation. Melting temperatures were not determined (ND) for end-point PCR targets.

Table 4.2 – Mean μ in each medium (data for all strains pooled). Relative μ and relative *nptA* refer to the ratios of the average μ and *nptA* expression levels, respectively, of pooled clinically-associated strains [CMCP6(C), 9067-96(C), 302/99(3)] to environmentally-associated strains [27562(E) and 33147(2)].

Media	Mean[1]	Relative μ	Relative *nptA*
BHI + 0.5% NaCl	0.97 ± 0.23^{A}	0.93	1.27
Seawater	0.43 ± 0.15^{B}	0.55	1.13
30ppt, 1mM PO4, pH 6	$0.68 \pm 0.20^{A,C}$	0.64	1.74
30ppt, 1mM PO4, pH 7	0.93 ± 0.18^{A}	0.86	1.38
30ppt, 1mM PO4, pH 8	0.79 ± 0.15^{A}	1.05	1.58
30ppt, 5μM PO4, pH 6	$0.68 \pm 0.10^{A,C}$	0.88	1.98
30ppt, 5μM PO4, pH 7	0.91 ± 0.33^{A}	0.75	1.50
30ppt, 5μM PO4, pH 8	$0.78 \pm 0.25^{A,C}$	0.91	1.36
15ppt, 1mM PO4, pH 6	0.46 ± 0.09^{C}	0.72	2.22
10ppt, 1mM PO4, pH 7	0.40 ± 0.11^{B}	1.77	2.07
10ppt, 1mM PO4, pH 8	0.30 ± 0.15^{B}	0.64	1.56
10ppt, 5μM PO4, pH 6	0.41 ± 0.11^{B}	0.87	2.06
10ppt, 5μM PO4, pH 7	0.34 ± 0.17^{B}	1.45	2.72
10ppt, 5μM PO4, pH 8	0.29 ± 0.06^{B}	0.79	1.18

[1]Mean μ of all strains, pooled, in each medium.

[A,B,C] Statistical comparison of mean μ (all strains pooled) in each medium. Growth rates in media sharing the same superscript are not significantly different ($P < 0.05$).

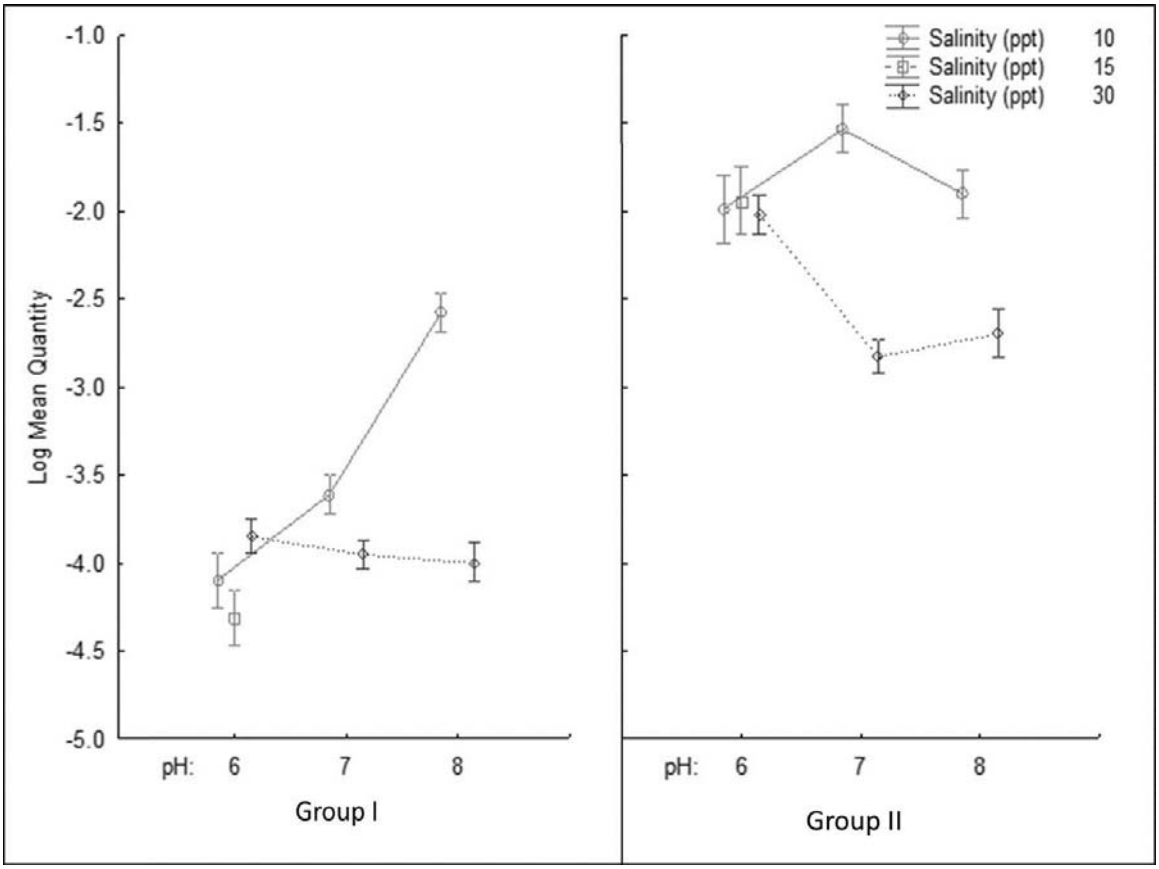

Figure 4.1 – Interaction plots of transcript abundance data at varying salinity and pH. Strains were grouped according to similar transcript abundance values under each condition where Group I includes biotype 1 (type C) and biotype 3 strains and Group II includes biotype 1 (type E) and biotype 2 strains. Log mean quantities are the $\log_{10}$ values of *nptA* transcript abundance normalized to that of 16S rRNA.

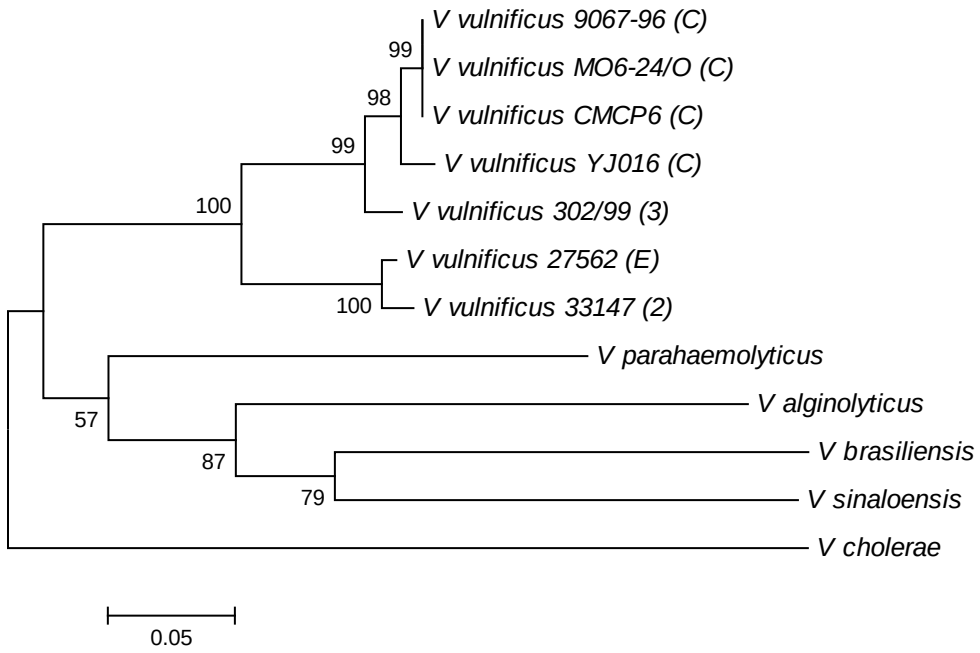

Figure 4.2 – Neighbor-joining tree of deduced NptA amino acid sequences. Sequences were obtained for stains 9067-96(C), 302/99(3), 27562(E), and 33147(2) and aligned with a 302 amino acid segment encoded by the 3'-terminus of the *nptA* gene of *V. vulnificus* CMCP6(C).. Numbers represent bootstrap values at 500 iterations.

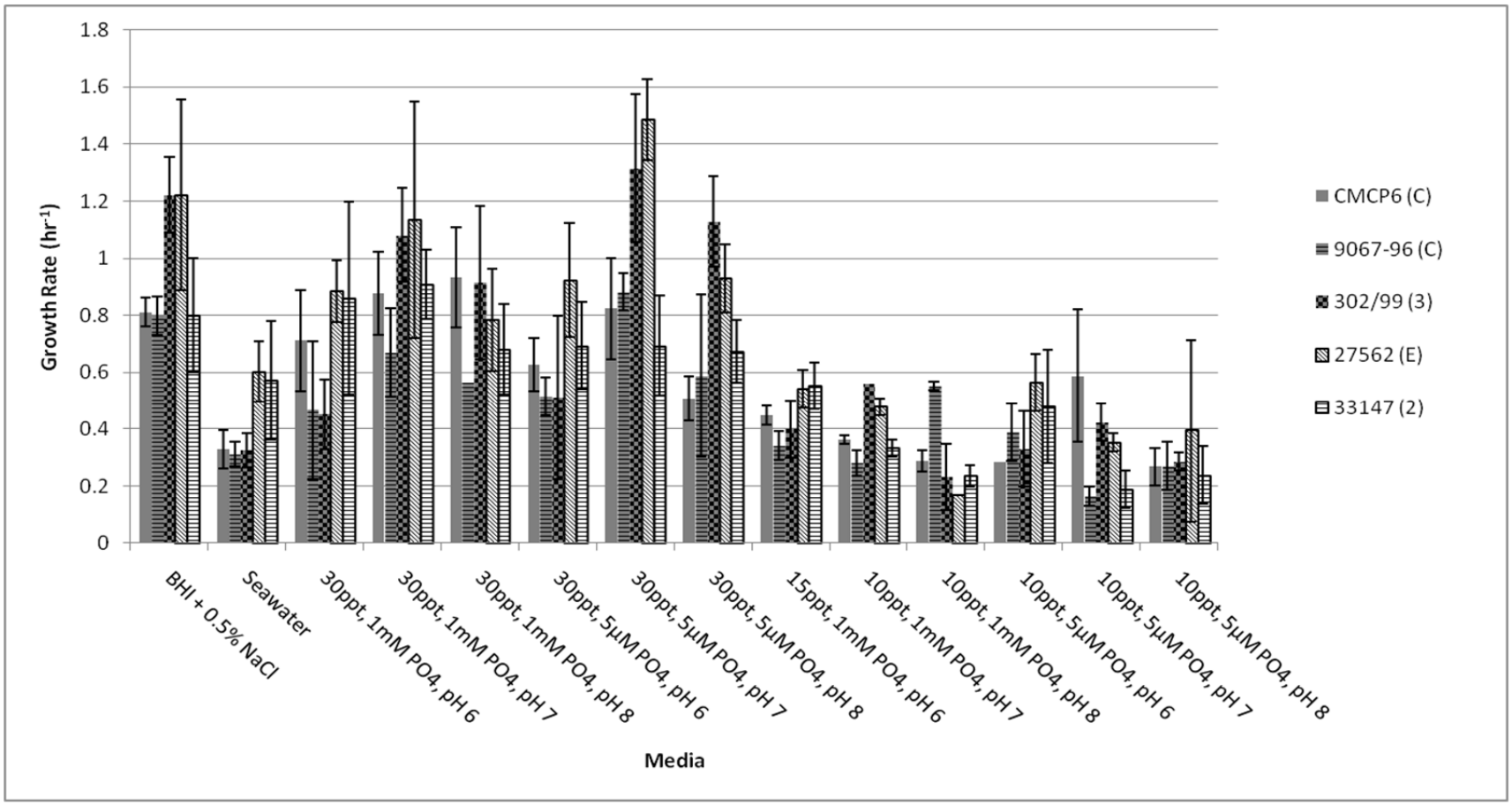

Figure 4.3 – Mean growth rates for *V. vulnificus* strains grown under differing conditions. Error bars represent standard deviations. Darker patterns represent biotype 1 (type C) strains CMCP6(C) and 9067-96(C) and the biotype 3 strain 302/99(3) while lighter patterns represent the biotype 1 (type E) strain 27562(E) and the biotype 2 strain 33147(2).

CHAPTER 5: REFERENCES

1. **Amaro, C., and E. G. Biosca.** 1996. *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. Applied and Environmental Microbiology **62:**1454-1457.

2. **American Public Health Association.** 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed, Washington, D.C.

3. **Anderson, K. L., J. E. Whitlock, V. J. Harwood, and K.** 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Applied and Environmental Microbiology **71:**3041-3048.

4. **Anonymous** 2006, posting date. 2006 Aquatic Plant Monitoring Guidelines. [Online.]

5. **Aznar, R., W. Ludwig, R. I. Amann, and K. H. Schleifer.** 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. Int J Syst Bacteriol **44:**330-7.

6. **Badgley, B. D., F. I. M. Thomas, and V. J. Harwood.** 2010. The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. Environmental Microbiology **12:**1271-1281.

7. **Badgley, B. D., F. I. M. Thomas, and V. J. Harwood.** 2011. Quantifying environmental reservoirs of fecal indicator bacteria associated with sediment and submerged aquatic vegetation. Environmental Microbiology **13:**932-942.

8. **Bales, R. C., S. M. Li, K. M. Maguire, M. T. Yahya, C. P. Gerba, and R. W. Harvey.** 1995. Virus and bacteria transport in a sandy aquifer, Cape Cod, MA. Ground Water **33:**653-661.

9. **Bell, S. S., A. Tewfik, M. O. Hall, and M. S. Fonseca.** 2008. Evaluation of seagrass planting and monitoring techniques: Implications for assessing restoration success and habitat equivalency. Restoration Ecology **16:**407-416.

10. **Bernhard, A. E., and K. G. Field.** 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Applied and Environmental Microbiology **66:**1587-1594.

11. **Bernhard, A. E., and K. G. Field.** 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. Appl Environ Microbiol **66:**4571-4.

12. **Biosca, E. G., J. D. Oliver, and C. Amaro.** 1996. Phenotypic characterization of *Vibrio vulnificus* biotype 2, a lipopolysaccharide-based homogeneous O serogroup within *Vibrio vulnificus*. Appl Environ Microbiol **62:**918-27.

13. **Birkenhauer, J. M., and J. D. Oliver.** 2003. Use of diacetyl to reduce the load of *Vibrio vulnificus* in the Eastern oyster, *Crassostrea virginica*. J Food Prot **66:**38-43.

14. **Bisharat, N., V. Agmon, R. Finkelstein, R. Raz, G. Ben-Dror, L. Lerner, S. Soboh, R. Colodner, D. N. Cameron, D. L. Wykstra, D. L. Swerdlow, and J. J. Farmer, 3rd.** 1999. Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Israel *Vibrio* Study Group. Lancet **354:**1421-4.

15. **Bisharat, N., D. I. Cohen, R. M. Harding, D. Falush, D. W. Crook, T. Peto, and M. C. Maiden.** 2005. Hybrid *Vibrio vulnificus*. Emerging Infectious Diseases **11:**30-35.

16. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. N Engl J Med **300:**1-5.

17. **Bofill-Mas, S., S. Pina, and R. Girones.** 2000. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. Appl Environ Microbiol **66:**238-45.

18. **Bogard, R. W., and J. D. Oliver.** 2007. Role of iron in human serum resistance of the clinical and environmental *Vibrio vulnificus* genotypes. Appl Environ Microbiol **73:**7501-5.

19. **Bonadonna, L., R. Briancesco, M. Ottaviani, and E. Veschetti.** 2002. Occurrence of *Cryptosporidium* oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. Environmental Monitoring and Assessment **75:**241-252.

20. **Bourdage, K., A. M. Starks, and P. Gulig.** 2003. The RtxA toxin is a major cytotoxic factor but plays a minor role in virulence of *Vibrio vulnificus*, Annu. Meet. Am. Soc. Microbiol.

21. **Brasher, C. W., A. DePaola, D. D. Jones, and A. K. Bej.** 1998. Detection of microbial pathogens in shellfish with multiplex PCR. Current Microbiology **37:**101-107.

22. **Brauns, L. A., M. C. Hudson, and J. D. Oliver.** 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Appl Environ Microbiol **57:**2651-5.

23. **Brownell, M. J., V. J. Harwood, R. C. Kurz, S. M. McQuaig, J. Lukasik, and T. M. Scott.** 2007. Confirmation of putative stormwater impact on water quality at a Florida beach by microbial source tracking methods and structure of indicator organism populations. Water Res **41:**3747-57.

24. **Bryant, R. G., J. Jarvis, and J. M. Janda.** 1987. Use of sodium dodecyl sulfate-polymyxin B-sucrose medium for isolation of *Vibrio vulnificus* from shellfish. Applied and Environmental Microbiology **53:**1556-1559.

25. **Buchrieser, C., V. V. Gangar, R. L. Murphree, M. L. Tamplin, and C. W. Kaspar.** 1995. Multiple *Vibrio vulnificus* strains in oysters as demonstrated by clamped homogeneous electric field gel electrophoresis. Appl Environ Microbiol **61:**1163-8.

26. **Byappanahalli, M. N., and R. S. Fujioka.** 1998. Evidence that tropical soil environment can support the growth of *Escherichia coli*. Water Science and Technology **38:**171-174.

27. **Byappanahalli, M. N., D. A. Shively, M. B. Nevers, M. J. Sadowsky, and R. L. Whitman.** 2003. Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga Cladophora (*Chlorophyta*). FEMS Microbiology Ecology **46:**203-211.

28. **Byappanahalli, M. N., R. L. Whitman, D. A. Shively, M. J. Sadowsky, and S. Ishii.** 2006. Population structure, persistence, and seasonality of autochthonous *Escherichia coli* in temperate, coastal forest soil from a Great Lakes watershed. Environmental Microbiology **8:**504-513.

29. **Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin.** 1982. Swimming-associated gastroenteritis and water quality. American Journal of Epidemiology **115:**606-616.

30. **Calif, E., N. Pick, U. Dreyfuss, and S. Stahl.** 2002. Upper extremity infections following common carp fish (*Cyprinus carpio*) handling. Journal of Hand Surgery-British and European Volume **27B:**78-82.

31. **Campbell, M. S., and A. C. Wright.** 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl Environ Microbiol **69:**7137-44.

32. **Centers for Disease Control and Prevention.** 2008. Surveillance for Waterborne Disease and Outbreaks Associated with Recreational Water Use and Other Aquatic Facility-Associated Health Events --- United States 2005-2006, Atlanta, GA.

33. **Chan, W. L., C. H. S. Chan, and T. Y. K. Chan.** 1999. *Vibrio vulnificus* septicaemia and necrotizing fasciitis after a prick from the dorsal fin of a tilapia. Transactions of the Royal Society of Tropical Medicine and Hygiene **93:**174-174.

34. **Chase, E., and V. J. Harwood.** 2011. Comparison of the effects of environmental parameters on growth rates of *Vibrio vulnificus* biotypes I, II, and III by

culture and quantitative PCR analysis. Applied and Environmental Microbiology **77:**4200-4207.

35. **Chatzidaki-Livanis, M., M. A. Hubbard, K. Gordon, V. J. Harwood, and A. C. Wright.** 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Appl Environ Microbiol **72:**6136-41.

36. **Chen, Y. C., M. C. Chang, Y. C. Chuang, and C. L. Jeang.** 2004. Characterization and virulence of hemolysin III from *Vibrio vulnificus*. Current Microbiology **49:**175-179.

37. **Cheung, W. H., K. C. Chang, R. P. Hung, and J. W. Kleevens.** 1990. Health effects of beach water pollution in Hong Kong. Epidemiology and Infection **105:**139-162.

38. **Choi, J., D. Shin, and S. Ryu.** 2007. Implication of quorum sensing in *Salmonella enterica* serovar typhimurium virulence: the *luxS* gene is necessary for expression of genes in pathogenicity island 1. Infection and Immunity **75:**4885-4890.

39. **Cohen, A. L. V., J. D. Oliver, A. DePaola, E. J. Feil, and E. F. Boyd.** 2007. Emergence of a virulent clade of *Vibrio vulnificus* and correlation with the presence of a 33-kilobase genomic island. Applied and Environmental Microbiology **73:**5553-5565.

40. **Colford, J. M., T. J. Wade, K. C. Schiff, C. C. Wright, J. F. Griffith, S. K. Sandhu, S. Burns, M. Sobsey, G. Lovelace, and S. B. Weisberg.** 2007. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology **18:**27-35.

41. **Cornelison, C. D., P. A. Gillespie, M. Kirs, R. G. Young, R. W. Forrest, P. J. Barter, B. R. Knight, and V. J. Harwood.** 2011. Motueka River plume facilitates transport of ruminant faecal contaminants into shellfish growing waters, Tasman Bay, New Zealand. New Zealand Journal of Marine and Freshwater Research **45:**477-495.

42. **Cottingham, K. L., D. A. Chiavelli, and R. K. Taylor.** 2003. Environmental microbe and human pathogen: the ecology and microbiology of *Vibrio cholerae*. Frontiers in Ecology and the Environment **1:**80-86.

43. **Davies, C. M., J. A. H. Long, M. Donald, and N. J. Ashbolt.** 1995. Survival of fecal microorganisms in marine and fresh water sediments. Applied and Environmental Microbiology **61:**1888-1896.

44. **de la Horra, C., N. Hernando, G. Lambert, I. Forster, J. Biber, and H. Murer.** 2000. Molecular determinants of pH sensitivity of the type IIa Na/P_i cotransporter. Journal of Biological Chemistry **275:**6284-6287.

45. **Depaola, A., G. M. Capers, and D. Alexander.** 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. Applied and Environmental Microbiology **60:**984-988.

46. **DePaola, A., M. L. Motes, D. W. Cook, J. Veazey, W. E. Garthright, and R. Blodgett.** 1997. Evaluation of an alkaline phosphatase-labeled DNA probe for enumeration of *Vibrio vulnificus* in Gulf Coast oysters. Journal of Microbiological Methods **29:**115-120.

47. **DePaola, A., J. L. Nordstrom, A. Dalsgaard, A. Forslund, J. Oliver, T. Bates, K. L. Bourdage, and P. A. Gulig.** 2003. Analysis of *Vibrio vulnificus* from market oysters and septicemia cases for virulence markers. Appl Environ Microbiol **69:**4006-11.

48. **Dorevitch, S., N. J. Ashbolt, C. M. Ferguson, R. Fujioka, C. D. McGee, J. A. Soller, and R. L. Whitman.** 2010. Meeting Report: Knowledge and Gaps in Developing Microbial Criteria for Inland Recreational Waters. Environmental Health Perspectives **118:**871-876.

49. **Englebert, E. T., C. McDermott, and G. T. Kleinheinz.** 2008. Effects of the nuisance alga, *Cladophora*, on *Escherichia coli* at recreational beaches in Wisconsin. Sci Total Environ **404:**10-17.

50. **Farjalla, V. F., F. A. Esteves, R. L. Bozelli, and F. Roland.** 2002. Nutrient limitation of bacterial production in clear water Amazonian ecosystems. Hydrobiologia **489:**197-205.

51. **Farrell, A. P., C. J. Brauner, and M. Grosell.** 2010. Feeding, Digestion and Absorption of Nutrients, Fish Physiology: The Multifunctional Gut of Fish. Elsevier Science, Burlington, NC.

52. **Fayer, R.** 2004. *Cryptosporidium*: a water-borne zoonotic parasite. Veterinary Parasitology **126:**37-56.

53. **Ferguson, C. M., B. G. Coote, N. J. Ashbolt, and I. M. Stevenson.** 1996. Relationships between indicators, pathogens and water quality in an estuarine system. Water Research **30:**2045-2054.

54. **Field, K. G., and M. Samadpour.** 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research **41:**3517-3538.

55. **Florida Department of Agriculture and Consumer Services Division of Aquaculture** 2011, posting date. Shellfish Harvesting. [Online.]

56. **Florida Department of Environmental Protection.** 2004. Criteria for Surface Water Quality Classifications. F.A.C. 62-302-530.

57. **Fuhrman, J. A., X. L. Liang, and R. T. Noble.** 2005. Rapid detection of enteroviruses in small volumes of natural waters by real-time quantitative reverse transcriptase PCR. Applied and Environmental Microbiology **71:**4523-4530.

58. **Galtsoff, P. S.** 1964. The American oyster, *Crassostrea virginica* Gmelin. Fish B-NOAA **64:**1-480.

59. **Gomez-Gil, B., E. Fajer-Avila, J. Pascual, M. C. Macian, M. J. Pujalte, E. Garay, and A. Roque.** 2008. *Vibrio sinaloensis* sp nov., isolated from the spotted rose snapper, *Lutjanus guttatus* Steindachner, 1869. International Journal of Systematic and Evolutionary Microbiology **58:**1621-1624.

60. **Gordon, K. V., M. C. Vickery, A. DePaola, C. Staley, and V. J. Harwood.** 2008. Real-time PCR assays for quantification and differentiation of *Vibrio vulnificus* strains in oysters and water. Appl Environ Microbiol **74:**1704-9.

61. **Graczyk, T. K., R. Fayer, and M. R. Cranfield.** 1997. Zoonotic transmission of *Cryptosporidium parvum*: implications for water-borne cryptosporidiosis. Parasitology Today **13:**348-351.

62. **Gulig, P. A., K. L. Bourdage, and A. M. Starks.** 2005. Molecular pathogenesis of *Vibrio vulnificus*. J Microbiol **43:**118-31.

63. **Gulig, P. A., M. S. Tucker, P. C. Thiaville, J. L. Joseph, and R. N. Brown.** 2009. USER friendly cloning coupled with chitin-based natural transformation enables rapid mutagenesis of *Vibrio vulnificus*. Applied and Environmental Microbiology **75:**4936-4949.

64. **Harwood, V. J., M. Brownell, S. Wang, J. Lepo, R. D. Ellender, A. Ajidahun, K. N. Hellein, E. Kennedy, X. Y. Ye, and C. Flood.** 2009. Validation and field testing of library-independent microbial source tracking methods in the Gulf of Mexico. Water Research **43:**4812-4819.

65. **Harwood, V. J., K. V. Gordon, and C. Staley.** 2011. Validation of Rapid Methods for Enumeration of Markers for Human Sewage Contamination in Recreational Water. Water Environment Research Foundation. PATH3C09.

66. **Harwood, V. J., A. D. Levine, T. M. Scott, V. Chivukula, J. Lukasik, S. R. Farrah, and J. B. Rose.** 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Applied and Environmental Microbiology **71:**3163-3170.

67. **Harwood, V. J., and C. Staley.** 2007. Preliminary Assessment of Bacteriological Water Quality in Lake Carroll (Tampa, FL). University of South Florida.

68. **Harwood, V. J., J. Whitlock, and V. Withington.** 2000. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. Appl Environ Microbiol **66:**3698-704.

69. **Harwood, V. J., B. Wiggins, C. Hagedorn, R. D. Ellender, J. Gooch, J. Kern, M. Samadpour, A. C. Chapman, B. J. Robinson, and B. C. Thompson.** 2003. Phenotypic library-based microbial source tracking methods: efficacy in the California collaborative study. J Water Health **1:**153-66.

70. **Harwood, W. J., J. Butler, D. Parrish, and V. Wagner.** 1999. Isolation of fecal coliform bacteria from the diamondback terrapin (Malaclemys terrapin centrata). Applied and Environmental Microbiology **65:**865-867.

71. **Helsel, D. R.** 1990. Less than obvious - statistical treatment of data below the detection limit. Environmental Science & Technology **24:**1766-1774.

72. **Heuvel, A. V., C. McDermott, R. Pillsbury, T. Sandrin, J. Kinzelman, J. Ferguson, M. Sadowsky, M. Byappanahalli, R. Whitman, and G. T. Kleinheinz.** 2010. The green alga, *Cladophora*, promotes *Escherichia coli* growth and contamination of recreational waters in Lake Michigan. Journal of Environmental Quality **39:**333-344.

73. **Hilfiker, H., O. Hattenhauer, M. Traebert, I. Forster, H. Murer, and J. Biber.** 1998. Characterization of a murine type II sodium-phosphate cotransporter expressed in mammalian small intestine. Proceedings of the National Academy of Sciences of the United States of America **95:**14564-14569.

74. **Hlady, W. G., and K. C. Klontz.** 1996. The epidemiology of *Vibrio* infections in Florida, 1981-1993. J Infect Dis **173:**1176-83.

75. **Hlady, W. G., R. C. Mullen, and R. S. Hopkin.** 1993. *Vibrio vulnificus* from raw oysters. Leading cause of reported deaths from food-borne illness in Florida. J. Fla. Med. Assoc. **80:**536-538.

76. **Høi, L. J. L. L., I. Dalsgaard, and A. Dalsgaard.** 1998. Occurrence of *Vibrio vulnificus* in Danish marine environments. Appl Environ Microbiol **64:**7-13.

77. **Hooper, L. V., and J. I. Gordon.** 2001. Commensal host-bacterial relationships in the gut. Science **292:**1115-1118.

78. **Interstate Shellfish Sanitation Conference.** 2003. Issue relating to a *Vibrio vulnificus* risk management plan for oysters, ISSC, Columbia, SC.

79. **Jackson, J. K., R. L. Murphree, and M. L. Tamplin.** 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. J Clin Microbiol **35:**2098-101.

80. **Jeng, H. W. C., A. J. England, and H. B. Bradford.** 2005. Indicator organisms associated with stormwater suspended particles and estuarine sediment. Journal of Environmental Science and Health Part A-Toxic/Hazardous **40:**779-791.

81. **Jin, Y., and M. Flury.** 2002. Fate and transport of viruses in porous media. Advances in Agronomy, Vol 77 **77:**39-+.

82. **Johnson, C. N., A. R. Flowers, N. F. Noriea, A. M. Zimmerman, J. C. Bowers, A. DePaola, and D. J. Grimes.** 2010. Relationships between environmental factors and pathogenic vibrios in the Northern Gulf of Mexico. Applied and Environmental Microbiology **76:**7076-7084.

83. **Jones, M. K., and J. D. Oliver.** 2009. *Vibrio vulnificus*: disease and pathogenesis. Infection and Immunity **77:**1723-1733.

84. **Jones, S. H., and B. Summer-Brason.** 1998. Detection of pathogenic *Vibrio* spp. in a northern New England estuary, USA. J. Shellfish Res. **17:**1665-1669.

85. **Kaysner, C. A., DePaola A.** 2004, posting date. *Vibrio*. [Online.]

86. **Kim, M. S., and H. D. Jeong.** 2001. Development of 16S rRNA targeted PCR methods for the detection and differentiation of *Vibrio vulnificus* in marine environments. Aquaculture **193:**199-211.

87. **Kim, Y. R., and J. H. Rhee.** 2003. Flagellar basal body *flg* operon as a virulence determinant of *Vibrio vulnificus*. Biochemical and Biophysical Research Communications **304:**405-410.

88. **Korajkic, A., B. D. Badgley, M. J. Brownell, and V. J. Harwood.** 2009. Application of microbial source tracking methods in a Gulf of Mexico field setting. Journal of Applied Microbiology **107:**1518-1527.

89. **Korajkic, A., M. J. Brownell, and V. J. Harwood.** 2011. Investigation of human sewage pollution and pathogen analysis at Florida Gulf coast beaches. J Appl Microbiol **110:**174-183.

90. **Lane, D.** 1991. 16S/23S rRNA sequencing, p. 115-148. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. Wiley, Chichester.

91. **Le, T., V. Bhushan, and D. A. Rao.** 2008
First Aid for the USMLE Step 1. McGraw Hill, New York, NY.

92. **Lebens, M., P. Lundquist, L. Soderlund, M. Todorovic, and N. I. A. Carlin.** 2002. The *nptA* gene of *Vibrio cholerae* encodes a functional sodium-dependent phosphate cotransporter homologous to the type II cotransporters of eukaryotes. Journal of Bacteriology **184:**4466-4474.

93. **Leclerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk.** 2001. Advances in the bacteriology of the Coliform Group: Their suitability as markers of microbial water safety. Annual review of Microbiology **55:**201-234.

94. **Lee, B. C., M. S. Kim, D. Cho, S. H. Choi, and T. S. Kim.** 2010. Co-culture supernatants from *Vibrio vulnificus*-infected INT-407 cells induce IL-8 production in intestinal epithelial cells: Crucial role of *V. vulnificus rtxE*. International Journal of Molecular Medicine **26:**651-659.

95. **Lee, B. C., J. H. Lee, M. W. Kim, B. S. Kim, M. H. Oh, K. S. Kim, T. S. Kim, and S. H. Choi.** 2008. *Vibrio vulnificus* rtxE is important for virulence, and its expression is induced by exposure to host cells. Infect Immun **76:**1509-17.

96. **Lee, J. H., M. W. Kim, B. S. Kim, S. M. Kim, B. C. Lee, T. S. Kim, and S. H. Choi.** 2007. Identification and characterization of the *Vibrio vulnificus rtxA* essential for cytotoxicity in vitro and virulence in mice. J Microbiol **45:**146-52.

97. **Lemarchand, K., and P. Lebaron.** 2003. Occurrence of *Salmonella* spp. and *Cryptosporidium* spp. in a French coastal watershed: relationship with fecal indicators. Fems Microbiology Letters **218:**203-209.

98. **Leskinen, S. D., V. J. Harwood, and D. V. Lim.** 2009. Rapid dead-end ultrafiltration concentration and biosensor detection of enterococci from beach waters of Southern California. J Water Health **7:**674-84.

99. **Lin, M. L., and J. R. Schwarz.** 2003. Seasonal shifts in population structure of *Vibrio vulnificus* in an estuarine environment as revealed by partial 16S ribosomal DNA sequencing. Fems Microbiology Ecology **45:**23-27.

100. **Linkous, D. A., and J. D. Oliver.** 1999. Pathogenesis of *Vibrio vulnificus*. Fems Microbiology Letters **174:**207-214.

101. **Litwin, C. M., T. W. Rayback, and J. Skinner.** 1996. Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. Infection and Immunity **64:**2834-2838.

102. **Liu, L., M. S. Phanikumar, S. L. Molloy, R. L. Whitman, D. A. Shively, M. B. Nevers, D. J. Schwab, and J. B. Rose.** 2006. Modeling the transport and inactivation of *E. coli* and enterococci in the near-shore region of Lake Michigan. Environmental Science & Technology **40:**5022-5028.

103. **Lund, V.** 1996. Evaluation of *Escherichia coli* as an indicator for the presence of *Campylobacter jejuni* and *Yersinia enterocolitica* in chlorinated and untreated oligotrophic lake water. Water Research **30:**1528-1534.

104. **Markowitz, V. M., I. M. A. Chen, K. Palaniappan, K. Chu, E. Szeto, Y. Grechkin, A. Ratner, I. Anderson, A. Lykidis, K. Mavromatis, N. N. Ivanova, and N. C. Kyrpides.** 2010. The integrated microbial genomes system: an expanding comparative analysis resource. Nucleic Acids Research **38:**D382-D390.

105. **Massad, G., and J. D. Oliver.** 1987. New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. Appl Environ Microbiol **53:**2262-4.

106. **McQuaig, S. M., T. M. Scott, V. J. Harwood, S. R. Farrah, and J. O. Lukasik.** 2006. Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. Appl Environ Microbiol **72:**7567-74.

107. **McQuaig, S. M., T. M. Scott, J. O. Lukasik, J. H. Paul, and V. J. Harwood.** 2009. Quantification of human polyomaviruses JC Virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. Appl Environ Microbiol **75:**3379-88.

108. **Moganedi, K. L. M., E. M. A. Goyvaerts, S. N. Venter, and M. M. Sibara.** 2007. Optimisation of the PCR-*invA* primers for the detection of *Salmonella* in drinking and surface waters following a pre-cultivation step. Water Sa **33:**195-202.

109. **Moreno, M. L., and M. Landgraf.** 1998. Virulence factors and pathogenicity of *Vibrio vulnificus* strains isolated from seafood. J Appl Microbiol **84:**747-51.

110. **Motes, M. L., A. DePaola, D. W. Cook, J. E. Veazey, J. C. Hunsucker, W. E. Garthright, R. J. Blodgett, and S. J. Chirtel.** 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Applied and Environmental Microbiology **64:**1459-1465.

111. **Murer, H., M. Lotscher, B. Kaissling, M. Levi, S. A. Kempson, and J. Biber.** 1996. Renal brush border membrane Na/P_i cotransport: molecular aspects in PTH-dependent and dietary regulation. Kidney International **49:**1769-1773.

112. **Nilsson, W. B., R. N. Paranjype, A. DePaola, and M. S. Strom.** 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J Clin Microbiol **41:**442-6.

113. **Noble, R. T., and J. A. Fuhrman.** 2001. Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of Santa Monica Bay, California: low correlation to bacterial indicator levels. Hydrobiologia **460:**175-184.

114. **Noble, R. T., S. B. Weisberg, M. K. Leecaster, C. D. McGee, J. H. Dorsey, P. Vainik, and V. Orozco-Borbon.** 2003. Storm effects on regional beach water quality along the southern California shoreline. J Water Health **1:**23-31.

115. **Noël-Lambot.** 1981. Presence in the intestinal lumen of marine fish of corpuscles of high cadmium-, zinc-, and copper-binding capacity: a possible mechanism of heavy metal tolerance. Mar Ecol Prog Ser **4:**175-181.

116. **Oh, W. S., Y. S. Im, K. Y. Yeon, Y. J. Yoon, and J. W. Kim.** 2007. Phosphate and carbon source regulation of alkaline phosphatase and phospholipase in *Vibrio vulnificus*. Journal of Microbiology **45:**311-317.

117. **Oliver, J. D.** 2005. *Vibrio vulnificus*. *In* B. a. Colwell (ed.), Oceans and Health: Pathogens in the Marine Environment. Springer, New York, NY.

118. **Oliver, J. D.** 2006. *Vibrio vulnificus*. *In* B. A. F.L. Thompson, and J. Swings (ed.), The Biology of Vibrios. ASM Press, Washington, D.C.

119. **Oliver, J. D., K. Guthrie, J. Preyer, A. Wright, L. M. Simpson, R. Siebeling, and J. G. Morris.** 1992. Use of colistin-polymyxin B-cellobiose agar for isolation of *Vibrio vulnificus* from the Environment. Applied and Environmental Microbiology **58:**737-739.

120. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl Environ Microbiol **45:**985-98.

121. **Parvathi, A., H. S. Kumar, I. Karunasagar, and I. Karunasagar.** 2005. Study of the occurrence of *Vibrio vulnificus* in oysters in India by polymerase chain reaction (PCR) and heterogeneity among *V. vulnificus* by randomly amplified polymorphic DNA PCR and *gyrB* sequence analysis. Environmental Microbiology **7:**995-1002.

122. **Parveen, S., K. M. Portier, K. Robinson, L. Edmiston, and M. L. Tamplin.** 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. Appl Environ Microbiol **65:**3142-7.

123. **Pfeffer, C. S., M. F. Hite, and J. D. Oliver.** 2003. Ecology of *Vibrio vulnificus* in estuarine waters of eastern North Carolina. Appl Environ Microbiol **69:**3526-31.

124. **Randa, M. A., M. F. Polz, and E. Lim.** 2004. Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. Appl Environ Microbiol **70:**5469-76.

125. **Reckhow, K. H.** 1999. Water quality prediction and probability network models. Canadian Journal of Fisheries and Aquatic Sciences **56:**1150-1158.

126. **Reifel, K. M., S. C. Johnson, P. M. DiGiacomo, M. J. Mengel, N. P. Nezlin, J. A. Warrick, and B. H. Jones.** 2009. Impacts of stormwater runoff in the Southern California Bight: Relationships among plume constituents. Continental Shelf Research **29:**1821-1835.

127. **Rosche, T. M., E. A. Binder, and J. D. Oliver.** 2010. *Vibrio vulnificus* genome suggests two distinct ecotypes. Environmental Microbiology Reports **2:**128-132.

128. **Rosche, T. M., Y. Yano, and J. D. Oliver.** 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiol Immunol **49:**381-9.

129. **Roslev, P., L. Iversen, H. L. Sonderbo, N. Iversen, and S. Bastholm.** 2009. Uptake and persistence of human associated *Enterococcus* in the mussel *Mytilus edulis*: relevance for faecal pollution source tracking. Journal of Applied Microbiology **107:**944-953.

130. **Sanjuan, E., F. Gonzalez-Candelas, and C. Amaro.** 2011. Polyphyletic origin of *Vibrio vulnificus* biotype 2 as revealed by sequence-based analysis. Applied and Environmental Microbiology **77:**688-695.

131. **Schlundt, J., H. Toyofuku, J. Jansen, and S. A. Herbst.** 2004. Emerging food-borne zoonoses. Revue Scientifique Et Technique De L Office International Des Epizooties **23:**513-533.

132. **Schoonover, J. E., and B. G. Lockaby.** 2006. Land cover impacts on stream nutrients and fecal coliform in the lower Piedmont of West Georgia. Journal of Hydrology **331:**371-382.

133. **Scott, T. M., T. M. Jenkins, J. Lukasik, and J. B. Rose.** 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. Environ Sci Technol **39:**283-7.

134. **Sera, H., Y. Ishida, and H. Kadota.** 1972. Bacterial flora in digestive tracts of marine fish - IV. Effect of H^+ concentration and gastric juices on indigenous bacteria. Bulletin of the Japanese Society of Scientific Fisheries **38:**859-&.

135. **Simpson, L. M., V. K. White, S. F. Zane, and J. D. Oliver.** 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infection and Immunity **55:**269-272.

136. **Smith, V. H., and D. W. Schindler.** 2009. Eutrophication science: where do we go from here? Trends in Ecology & Evolution **24:**201-207.

137. **Solo-Gabriele, H. M., M. A. Wolfert, T. R. Desmarais, and C. J. Palmer.** 2000. Sources of *Escherichia coli* in a coastal subtropical environment. Applied and Environmental Microbiology **66:**230-237.

138. **Staley, C., and V. J. Harwood.** 2007. Characterization of a putative virulence marker in *Vibrio vulnificus* unique to clinically-associated strains, 107th General Meeting of the American Society for Microbiology, Toronto, Canada.

139. **Staley, C., and V. J. Harwood.** 2008. A conserved hypothetical protein may aid in typing *Vibrio vulnificus*, 108th General Meeting of the American Society for Microbiology, Boston, MA.

140. **Staley, C., M. K. Jones, A. C. Wright, and V. J. Harwood.** 2011. Genetic and quantitative assessment of *Vibrio vulnificus* populations in oyster (*Crassostrea virginica*) tissues. Environ. Microbiol. Rep. **3:**543-549.

141. **Stoeckel, D. M., and V. J. Harwood.** 2007. Performance, design, and analysis in microbial source tracking studies. Appl Environ Microbiol **73:**2405-15.

142. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect **2:**177-88.

143. **Sun, Y., and J. D. Oliver.** 1995. Value of cellobiose-polymyxin B-colistin agar for isolation of *Vibrio vulnificus* from oysters. Journal of Food Protection **58:**439-440.

144. **Taguchi, F., J. Kajioka, and T. Miyamura.** 1982. Prevalence rate and age of acquisition of antibodies against JC virus and BK virus in human sera. Microbiology and Immunology **26:**1057-1064.

145. **Tamplin, M., G. E. Rodrick, N. J. Blake, and T. Cuba.** 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl Environ Microbiol **44:**1466-70.

146. **Tamplin, M. L., A. L. Martin, A. D. Ruple, D. W. Cook, and C. W. Kaspar.** 1991. Enzyme immuno assay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. Applied and Environmental Microbiology **57:**1235-1240.

147. **Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution **accepted**.

148. **Tasara, T., and R. Stephan.** 2007. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. Fems Microbiology Letters **269:**265-272.

149. **Thompson, R. C. A.** 2004. The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. Veterinary Parasitology **126:**15-35.

150. **Tison, D. L., M. Nishibuchi, J. D. Greenwood, and R. J. Seidler.** 1982. *Vibrio vulnificus* biogroup 2 - new biogroup pathogenic for eels. Applied and Environmental Microbiology **44:**640-646.

151. **Traub, R. J., P. T. Monis, I. Robertson, P. Irwin, N. Mencke, and R. C. A. Thompson.** 2004. Epidemiological and molecular evidence supports the zoonotic transmission of *Giardia* among humans and dogs living in the same community. Parasitology **128:**253-262.

152. **U.S. Food and Drug Administration.** 2001. *Vibrio cholerae, V. parahaemolyticus, V. vulnificus*, and other *Vibrio* spp. Bacteriol. Anal. Manual, Ch 9. URL http://www.cfsan.fda.gov/~ebam/bam-9.html.

153. **United States Environmental Protection Agency.** 1986. Ambient Water Quality Criteria for Bacteria –1986. Available: http://www.epa.gov/waterscience/beaches/files/1986crit.pdf.

154. **United States Environmental Protection Agency.** 2002. EPA-823-B-02-003. Implementation Guidance for Ambient Water Quality Criteria for Bacteria. .

155. **United States Environmental Protection Agency.** 1996. EPA 600/R95/178. ICR Microbial Laboratory Manual.

156. **United States Environmental Protection Agency.** 2002. Method 1600: enterococci in water by membrane filtration using membrane-enterococcus indoxyl-B-D-glucoside agar (mEI).

157. **United States Environmental Protection Agency.** 2002. Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC).

158. **United States Environmental Protection Agency.** 2005. Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA.

159. **United States Environmental Protection Agency.** 2000. The quality of our nation's waters. **EPA841-S-00-001**.

160. **Viau, E. J., K. D. Goodwin, K. M. Yamahara, B. A. Layton, L. M. Sassoubre, S. L. Burns, H. I. Tong, S. H. C. Wong, Y. A. Lu, and A. B. Boehm.** 2011. Bacterial pathogens in Hawaiian coastal streams - associations with fecal indicators, land cover, and water quality. Water Research **45:**3279-3290.

161. **Vickery, M. C., W. B. Nilsson, M. S. Strom, J. L. Nordstrom, and A. Depaola.** 2007. A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. J Microbiol Methods **68:**376-84.

162. **Wade, T. J., R. L. Calderon, E. Sams, M. Beach, K. P. Brenner, A. H. Williams, and A. P. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environmental Health Perspectives **114:**24-28.

163. **Wade, T. J., N. Pai, J. N. S. Eisenberg, and J. M. Colford.** 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environmental Health Perspectives **111:**1102-1109.

164. **Warner, E., and J. D. Oliver.** 2007. Refined medium for direct isolation of *Vibrio vulnificus* from oyster tissue and seawater. Appl Environ Microbiol **73:**3098-100.

165. **Watkins, W. D., and V. J. Cabelli.** 1985. Effect of fecal pollution on *Vibrio parahaemolyticus* densities in an estuarine environment. Applied and Environmental Microbiology **49:**1307-1313.

166. **Weis, K. E., R. M. Hammond, R. Hutchinson, and C. G. Blackmore.** 2010. *Vibrio* illness in Florida, 1998-2007. Epidemiol Infect**:**1-8.

167. **Werner, A., and R. K. H. Kinne.** 2001. Evolution of the Na-P-i cotransport systems. American Journal of Physiology-Regulatory Integrative and Comparative Physiology **280:**R301-R312.

168. **Wilson, W. H., N. G. Carr, and N. H. Mann.** 1996. The effect of phosphate status on the kinetics of cyanophage infection in the oceanic cyanobacterium *Synechococcus* sp. WH7803. Journal of Phycology **32:**506-516.

169. **Wolff, A. H., N. D. Henderson, and G. L. Mccallum.** 1948. *Salmonella* from dogs and the possible relationship to Salmonellosis in man. American Journal of Public Health **38:**403-408.

170. **Wright, A. C., V. Garrido, G. Debuex, M. Farrell-Evans, A. A. Mudbidri, and W. S. Otwell.** 2007. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. Applied and Environmental Microbiology **73:**7477-7481.

171. **Wright, A. C., R. T. Hill, J. A. Johnson, M. C. Roghman, R. R. Colwell, and J. G. Morris.** 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. Applied and Environmental Microbiology **62:**717-724.

172. **Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris, Jr.** 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl Environ Microbiol **59:**541-6.

173. **Wright, A. C., and J. G. Morris.** 1991. The extracellular cytolysin of *Vibrio vulnificus* - inactivation and relationship to virulence in mice. Infection and Immunity **59:**192-197.

174. **Wright, A. C., L. M. Simpson, and J. D. Oliver.** 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infection and Immunity **34:**503-507.

175. **Wright, A. C., L. M. Simpson, J. D. Oliver, and J. G. Morris.** 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infection and Immunity **58:**1769-1773.

176. **Yamamoto, S., H. Izumiya, M. Morita, E. Arakawa, and H. Watanabe.** 2009. Application of lambda red recombination system to *Vibrio cholerae* genetics: simple methods for inactivation and modification of chromosomal genes. Gene **438:**57-64.

177. **Yoshida, S., M. Ogawa, and Y. Mizuguchi.** 1985. Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. Infect Immun **47:**446-51.

178. **Yun, J. J., L. E. Heisler, Hwang, II, O. Wilkins, S. K. Lau, M. Hyrcza, B. Jayabalasingham, J. Jin, J. McLaurin, M. S. Tsao, and S. D. Der.** 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. Nucleic Acids Res **34:**e85.

179. **Zmirou, D., L. Pena, M. Ledrans, and A. Letertre.** 2003. Risks associated with the microbiological quality of bodies of fresh and marine water used for recreational purposes: summary estimates based on published epidemiological studies. Archives of Environmental Health **58:**703-711.

APPENDIX A:

nptA TRANSCRIPT ABUNDANCE GRAPHS BY STRAIN

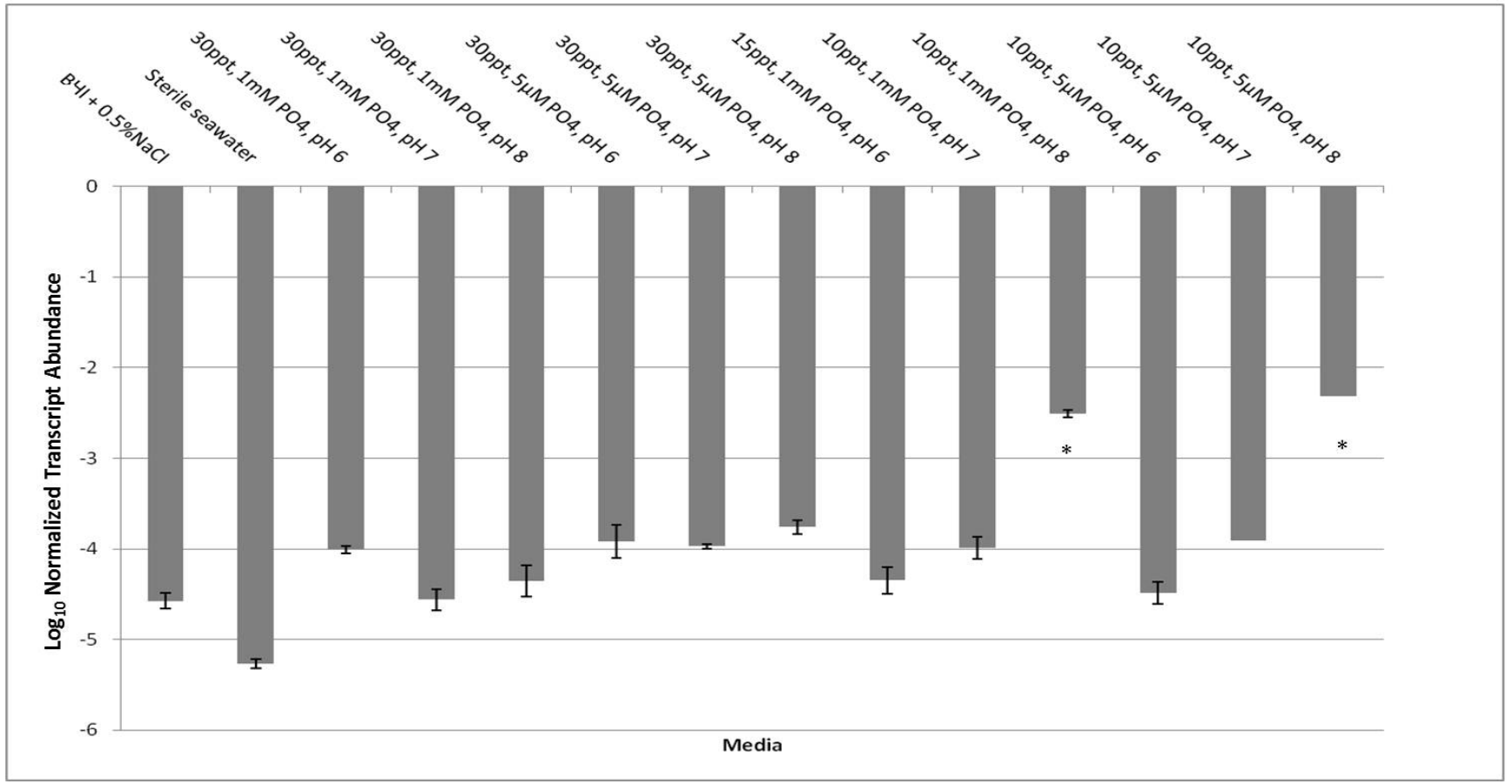

Figure A1 – *nptA* transcript abundance normalized to that of the 16S rRNA gene in *V. vulnificus* CMCP6 (biotype 1, type C) under differing conditions. Asterisks (*) indicate conditions under which the highest levels of *nptA* transcript were measured ($P < 0.05$). No significant difference in abundance was measured between conditions marked with an asterisk.

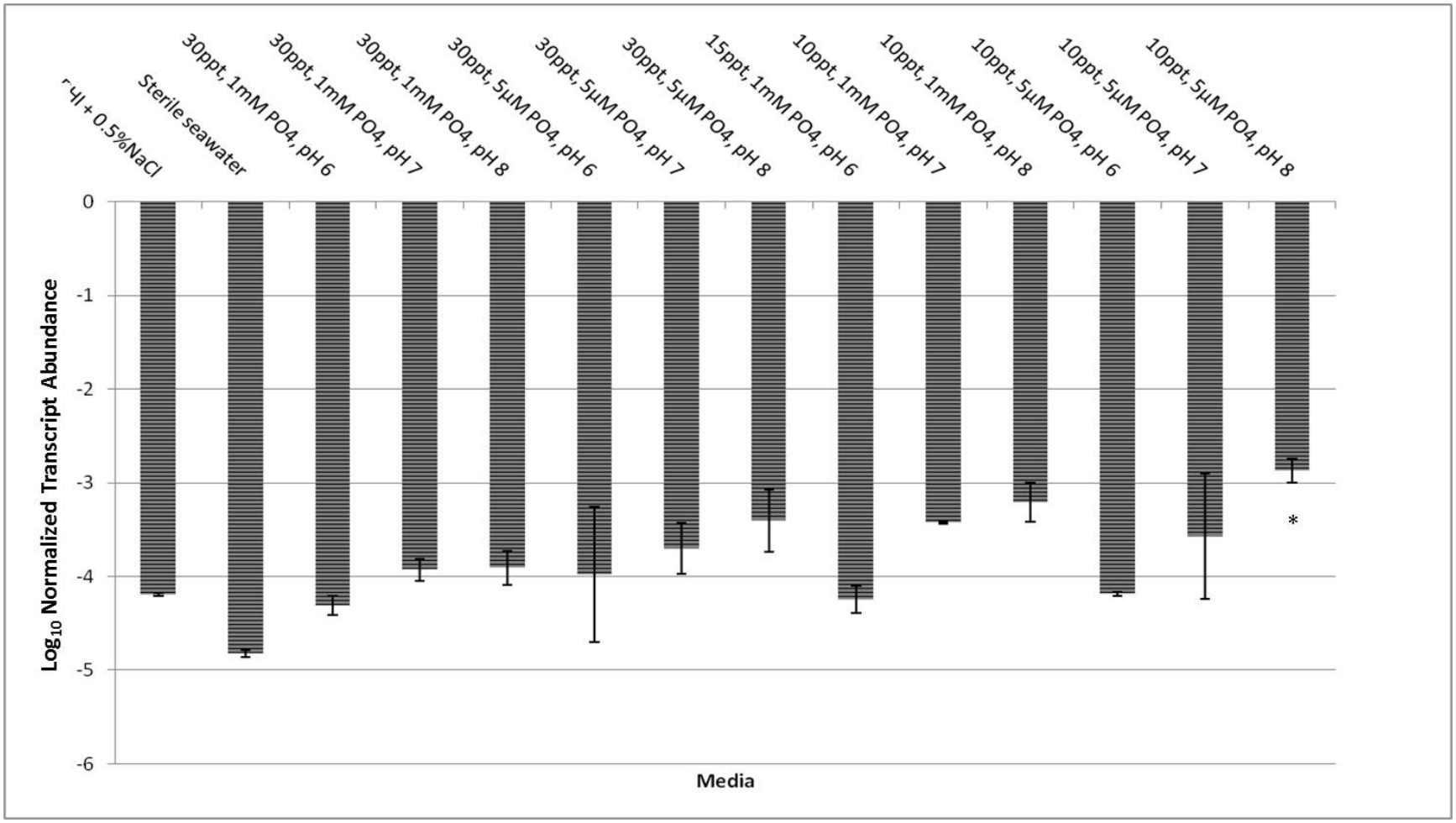

Figure A2 – *nptA* transcript abundance normalized to that of the 16S rRNA gene in *V. vulnificus* 9067-96 (biotype 1, type C) under differing conditions. Asterisks (*) indicate conditions under which the highest levels of *nptA* transcript was measured, although abundance is not significantly higher ($P > 0.05$) than all other media.

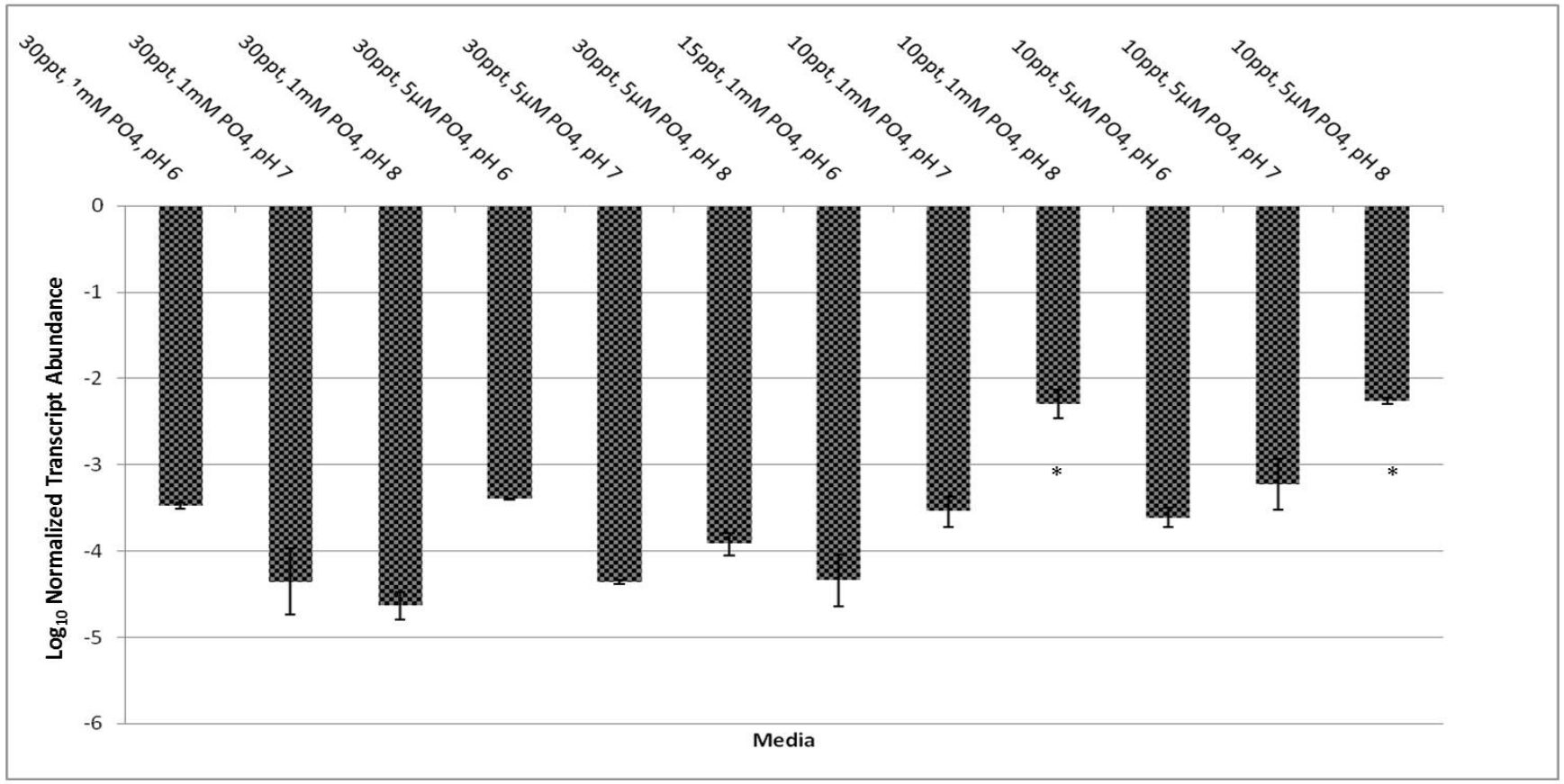

Figure A3 – *nptA* transcript abundance normalized to that of the 16S rRNA gene in *V. vulnificus* 302/99 (biotype 3) under differing conditions. Asterisks (*) indicate conditions under which the highest levels of *nptA* transcript were measured ($P < 0.05$). No significant difference in abundance was measured between conditions marked with an asterisk. The primer set for the biotype 3 assay was not developed until after BHI and seawater data were collected, and, due to financial limitations, these experiments could not be repeated.

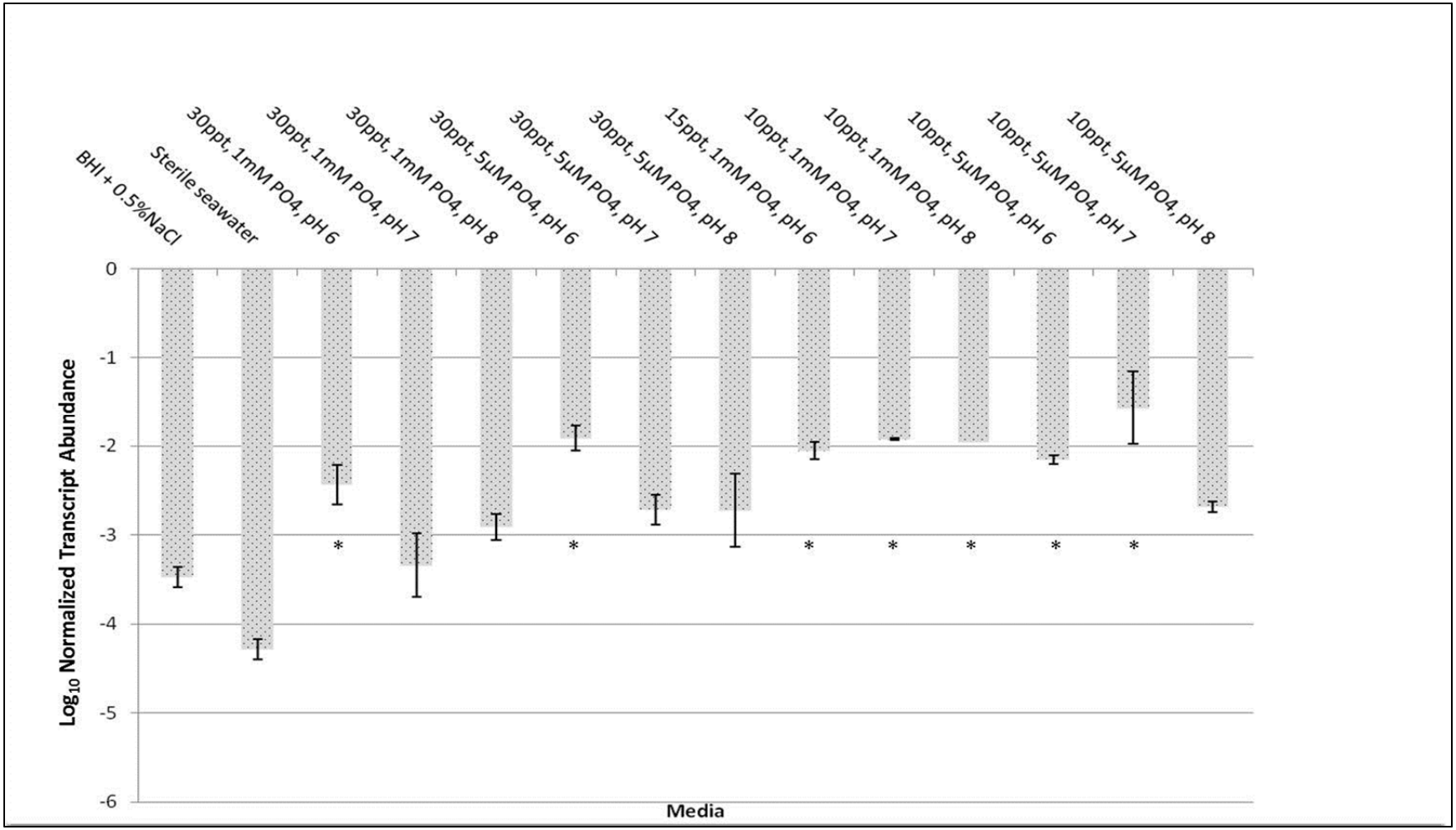

Figure A4 – *nptA* transcript abundance normalized to that of the 16S rRNA gene in *V. vulnificus* 27562 (biotype 1, type E) under differing conditions. Asterisks (*) indicate conditions under which the highest levels of *nptA* transcript were measured ($P < 0.05$). No significant difference in abundance was measured between conditions marked with an asterisk.

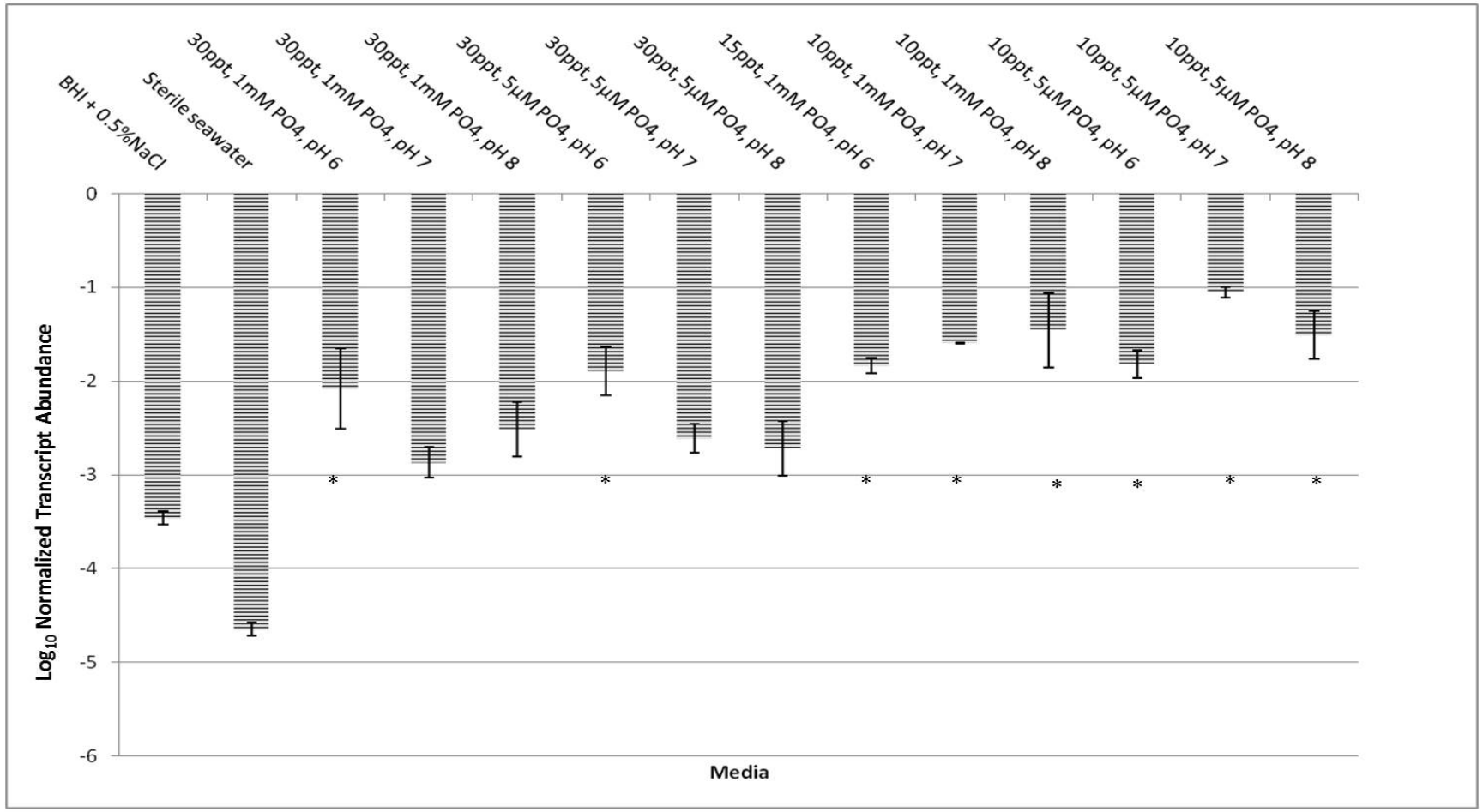

Figure A5 - *nptA* transcript abundance normalized to that of the 16S rRNA gene in *V. vulnificus* 33147 (biotype 2) under differing conditions. Asterisks (*) indicate conditions under which the highest levels of *nptA* transcript were measured ($P < 0.05$). No significant difference in abundance was measured between conditions marked with an asterisk.