


1-1-2013

# Environmental Pre-Conditioning of Bacterial Populations and Communities to Extreme Acidity

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ENVIRONMENTAL PRE-CONDITIONING OF BACTERIAL  
POPULATIONS AND COMMUNITIES TO EXTREME ACIDITY

by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2013

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## DEDICATION

This dissertation is dedicated to my wife, Summer, who provides me endless love and encouragement.

Also to my children, Sōl, Oliver, Lucy and baby on the way, who have loved and respected me despite my absence for much of the past four years.

## ACKNOWLEDGEMENTS

This research was supported by the Slocum-Lunz Foundation, The Explorers Club Exploration Fund and the F. John Vernberg Bicentennial Fellowship in Marine Science. I would first like to acknowledge, my home institution, Paine College, with special appreciation to Dr. George C. Bradley and Dr. Tina Marshall-Bradley, for providing this opportunity. I am deeply grateful to my advisor, Richard A. Long, Ph.D., for his patience, generosity and insights during my time in his lab. I appreciate the interest and confidence he had in my work, the freedom he provided me to explore and his high standard of excellence in conducting science. Thank you to the members of my dissertation committee, Drs. James L. Pinckney, Ronald Benner, Charles R. Lovell and Raymond Torres. I appreciate the feedback and insights you all provided during this dissertation research. Thanks to the Belle W. Baruch Institute for access to sample sites.

I thank my colleagues and friends at USC, particularly Isaac Hagenbuch and Christopher Brandon, for the critical discussions that strengthened aspects of my research. Thanks to members of the Long Lab, past and present, especially Kennerly Patrick, Thomas Barrineau and Flavio Guidi, who participated in various research projects and helped create an enjoyable and productive work environment. I acknowledge my mother and father and members of my family for supporting me in all of my efforts over my academic career. Finally, heartfelt thanks to my friends Scott and Amber Rankin, for their unwavering hospitality during my time at USC.

I am grateful to you all for the many ways that you have influenced my life by celebrating my accomplishments and helping me to learn from my failures. Thank you.

## ABSTRACT

The ability of opportunistic enteric pathogens to cause disease is effected by their ability to survive the variable and lethal acidity of the mammalian host gastrointestinal milieu. In many of these pathogens, survival is conferred by an acid stress response (ASR). ASR is elicited when bacterial cells are pre-conditioned in sub-lethal acidity (pH 4-6) which induces rapid biochemical and physiological modifications and facilitates survival when extreme acidity (pH 1-3) is encountered. In *Vibrio cholerae*, the causative agent of the diarrheal disease Cholera, ASR was initially investigated in an effort to determine how this ubiquitous aquatic bacterium is capable of causing disease on pandemic scales. It has since been discovered that *V. cholerae* is capable of a robust ASR. However, the conservation of this response among clinical and environmental *V. cholerae* populations has been unexplored. Further, the environmental elicitation of ASR in *V. cholerae* has not been considered. This dissertation examines ASR capabilities among *V. cholerae* populations from clinical and environmental isolation origins. Using the molecular approach, polymerase chain reaction (PCR), nearly fifty *V. cholerae* isolates were screened to establish presence/absence of a subset of genes involved in ASR. A high-throughput technique developed during this dissertation (Chapter 3) was used to evaluate the ability of these isolates to respond to lethal acid stress. ASR positive phenotypes were identified among clinical and environmental *V. cholerae* populations (Chapter 4) and this response was not genotype dependent. *In silico* analysis of *Vibrio* genomes reveals that clinical *V. cholerae* genomes and genomes isolated from Cholera endemic regions, display greater sequence similarity of ASR genes than do environmentally derived genomes. The growth of *V. cholerae* in moderately

acidic freshwater systems has important ecological implications but remains poorly characterized. We examine the population growth dynamics and elicitation of ASR in the clinical *V. cholerae* N16961 strain cultured in environmental swamp water (Chapter 5). Our results suggest this opportunistic pathogen is capable of prolonged growth in swamp water samples at densities above the known minimum infectious dose. Further, cultivation in the swamp water was shown to elicit ASR which facilitated survival of *V. cholerae* N16961 upon exposure to extreme acidity. ASR elicitation was supported by the observation of ASR gene expression profiles similar to previous findings. Finally, the ASR of culturable, heterotrophic bacterial communities from estuarine, freshwater and contaminated sediments is evaluated (Chapter 6). Sediment associated bacterial communities isolated from euphotic and anoxic partitions displayed a robust response to extreme acidity. Sediments contaminated with copper and mixed polyaromatic hydrocarbons (PAHs) were shown to select for bacterial communities capable of ASR. The findings of this dissertation research may be valuable in the development and improvement of tools predicting environmental pathogen distribution and will benefit efforts towards disease mitigation and prevention.

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## CHAPTER 1

### INTRODUCTION

Increased incidence of food and waterborne disease over the past quarter century and the emergence of novel bacterial strains capable of causing infection have renewed interest in the role of aquatic environments in the ecology of infectious disease. *Vibrio cholerae* is a gram-negative, curved rod common in brackish and freshwater systems as a constituent of the autochthonous bacterial communities in free-living forms and in association with algae, insects and crustaceans (West and Lee, 1982; Xu et al., 1985; Lipp et al., 2002; Worden et al., 2006). Pathogenic strains of *V. cholerae* are the causative agent of the diarrheal disease Cholera which, with a mortality rate of ~50% when untreated, causes hundreds of thousands of infections, tens of thousands of deaths and more than \$1 billion human suffering annually, primarily in developing countries (Riedle and Klose, 2002; WHO, 2002).

*V. cholerae* diversity is determined by O-antigen lipopolysaccharide (LPS) composition variation. Strains presenting the O1 antigen have historically been considered clinically relevant and non-O1 strains were regarded as having little infectious significance (Yamai et al., 1997; Jiang et al., 2000). The O1 strains are further classified into the classical and El Tor biotypes. The first recorded Cholera pandemics (1817-1961)

were caused by the extremely virulence, classical *V. cholerae* biotype. The seventh pandemic began in Indonesia with the emergence of the highly adaptive El Tor biotype which quickly eclipsed the classical biotype as the cause of epidemic Cholera (Faruque et al., 1998; Pradhan et al., 2010). In 1992, a new strain of *V. cholerae* emerged and initiated the eighth Cholera epidemic that began in India and spread to neighboring countries. Discovery of *V. cholerae* O139 sparked interest in the effect of genotype on disease causing phenotype as O139 was genetically distinct from the O1 El Tor strain which was the hallmark of disease (Albert et al., 1993; Ramamurthy et al., 1993; Nair et al., 1994; Jiang 2000).

More recently, *V. cholerae* has been isolated from India and non-endemic regions of Australia that are genetically distinct from clinical strains and do not possess the cholera toxin genes but induced Cholera-like symptoms (Sharma et al., 1998; Islam et al., 2013). Studies on whole *V. cholerae* genomes reveal a high degree of conservation (<1% genome variability) among sequenced environmental and clinical genomes (Hang et al., 2000; Heidleberg et al., 2008). However, sequence variability of select genes has been observed in strains isolated from various environments (Dziejman et al., 2005; Nair et al., 2006; Ray et al., 2009; Haley et al., 2010). These findings prompt curiosity towards the role of genetic pre-disposition in observed phenotypes among clinical and environmental *V. cholerae* populations.

Outbreaks of Cholera are seasonal, correlating with regional weather patterns and algal blooms (Huq et al., 1990; Epstein, 1993; Islam et al., 1994; Colwell, 1996; Pascual et al., 2000; Collwell and Huq, 2001; Kirschner et al., 2008). This particle associations in the environment influence *V. cholerae* pathogenicity and survival through lateral

acquisition of virulence genes and colonization of carbon rich strata (Nalin et al., 1979; Blokesch and Schoolnik, 2007; Hsieh et al., 2007; Pruzzo et al., 2008). Recent investigations have detected virulence and antibiotic resistance genes, and genes regulating these pathways, in high frequency among environmental isolates (Goel et al., 2007; Zo et al., 2008; Goel and Jiang, 2010). The uncertainty of how this common aquatic bacterium is capable of causing disease at pandemic scales was partially resolved with the discovery that *V. cholerae* is capable of mounting an acid stress response (Merrell et al., 2001; Merrell and Camilli, 2000; Merrell and Camilli, 2002).

Acid stress response (ASR) is a key adaptive feature involving rapid physiological and biochemical modifications after pre-conditioning in sub-lethal acidity (pH 4-6). This dynamic response ultimately enhances cell survival upon exposure to potentially lethal acidity (pH 1-4) such as that encountered during transit through the mammalian GI tract. Laboratory investigations of *V. cholerae* ASR suggest that ASR bolsters virulence as *V. cholerae* cells expressing ASR demonstrate increased competitive index over acid intolerant isolates in the infant mouse model (Merrell, Hava and Camilli, 1999; Merrell et al., 2001; Merrell and Camilli, 2002).

ASR is conserved among opportunistic enteric pathogens and is often investigated in model bacteria such as *Escherichia coli* and *Salmonella enterica* (reviewed in Swenson et al., 2013). ASR varies by organism, acid classification, bacterial growth phase, nutrient availability and additional environmental cues (Lin et al., 1996; Foster, 1999; Koutsoumanis et al., 2003). ASR may activate additional adaptive responses including virulence, biofilm formation, chemotaxis and antibiotic resistance (Polen et al., 2003; Maurer et al., 2005; Butler et al., 2006; Hayes et al., 2006). This response may also

provide cross protection to other forms of environmental stress (Rallu et al., 2000; Frees et al., 2003; DiAngelis and Gobetti, 2004; Xie et al., 2004).

Environmental survival of opportunistic pathogens in aquatic ecosystems has been shown to effect residence as particle attached cells survive longer than free living cells, thus increasing the duration of risk to the public (Craig et al., 2004; Garcia-Armisen and Servais, 2009). Sediments association also influences environmental fate of opportunistic pathogens as fecal coliform densities are several orders of magnitude greater in some sediment than in the water column (An et al., 2002). The bacterial communities must response to caustic and mercurial conditions such as shifting pH and anoxia resulting from decomposition and exposure to environmental contaminants, anthropogenic compounds and industrial waste. Thus a robust response to stress facilitates environmental bacterial survival.

This dissertation explores the global hypothesis that certain environments pre-condition bacteria to survive extreme acidity through elicitation of ASR which is investigated in the context of bacterial populations and communities. The response of *V. cholerae* to extreme acidity is investigated by examining the genotype profile and phenotypic response within populations from clinical and environmental origins. This work is complimented by *in silico* analysis of *V. cholerae* genomes to assess ASR gene sequence similarities as a function of genome isolation origin. The clinical *V. cholerae* N16961 strain is evaluated for elicitation of ASR during cultivation in environmental swamp water. Finally, the response of bacterial communities from water and sediment partitions from estuarine and freshwater sampling sites is evaluated. This is complimented by investigating the impact of anthropogenic waste on bacterial



community structure and the response of these communities to acid stress. The dissertation is organized into the following chapters:

**Chapter 2.** This chapter consists of a manuscript published in *Frontiers in Biology* (2013) reviewing recent literature on the acid stress response (ASR) in environmental and clinical strains of opportunistic enteric pathogens. Emphasis is placed on transformational molecular assessment of ASR investigated in *Escherichia coli*, *Salmonella enterica* and *Vibrio cholerae*.

**Chapter 3.** This chapter consists of a methods paper published in *Journal of Microbiological Methods* (2012) where we demonstrated the use of fluorometry of microalgal chlorophyll *a* and bacterial cells stained with SYBRGreen™ to assess surface associated microbial abundances. This method was demonstrated as effective in evaluating the response of clinical and environmental bacteria, as well as microalgae, to three common antibiotics.

**Chapter 4.** This chapter evaluates ASR in clinical and environmental strains of *V. cholerae* using culture based and molecular techniques. *In silico* analysis of *V. cholerae* genomes available in PATRIC were also conducted to determine the sequence similarities of previously described ASR genes. ASR was detected in clinical and environmental *V. cholerae* isolates and ASR genotype was not a determinant of ASR positive phenotype. *In silico* assessment reveals a higher degree of sequence similarity among *V. cholerae* genomes isolated from Cholera patients and environments where Cholera is endemic than in genomes isolated from non-endemic regions.

**Chapter 5.** This chapter explores environmental elicitation of ASR in the clinical strain of *V. cholerae* N16961. Growth and ASR elicitation were monitored for this strain using culture based assays and molecular techniques. The findings reported here suggest that *V. cholerae* N16961 is capable of robust growth in environmental swamp water samples (CSW) at growth rates similar to the bacterial community autochthonous to the swamp water. Of greatest interest is the finding that pre-conditioning *V. cholerae* in *in situ* CSW enhanced population survival upon exposure to

extreme acidity. Environmental elicitation of ASR was confirmed by observed variability in the expression of ASR genes consistent with previous findings.

**Chapter 6.** Environmental pre-conditioning of aquatic bacterial communities from water and sediment partitions to extreme acidity is the focus of this final chapter. Freshwater, estuarine and experimental mesocosms were sampled for this study. The bacterial communities associated with sediment were capable of robust ASR whereas free-living and interstitial communities were more sensitive. Molecular analysis of bacterial communities from experimental mesocosms reveals that sediments contaminated with copper and polyaromatic hydrocarbons select for a bacterial community capable of robust response to extreme acidity. Sequence analysis reveals that Vibrionales represent a significant fraction of the bacterial community in contaminated sediment as compared to control sediment. Further, contaminated sediment appears to pre-condition ASR in the Alteromonadales with a greater fraction surviving extreme acidity than is observed in control sediments.

## CHAPTER 2

# ACID STRESS RESPONSE IN ENVIRONMENTAL AND CLINICAL STRAINS OF ENTERIC BACTERIA

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*Front Biol*, 2013, 7(6): 495–505.

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## ABSTRACT

The success of many enteric bacteria is hinged on the ability to tolerate environmental stress such as extreme acidity. The acid stress response (ASR) has been investigated in many enteric bacteria and has been shown to involve variable expression of a broad spectrum of genes involved in transcriptional regulation, metabolism, colonization and virulence; representing a linkage between acid tolerance and pathogenicity. Though the majority of ASR studies have been conducted in laboratory conditions and from the perspective of pathogenicity, the role of environmental reservoirs on acid adaptation has recently emerged as an important aspect of pathogenic microbial ecology. This mini-review profiles ASR in three opportunistic enteric pathogens and synthesizes recent work pertaining to the study of this dynamic response.

## INTRODUCTION

In the past 30 years there has been more than a 6-fold increase in the frequency of food outbreaks in the United States resulting from contaminated produce and prompting investigations into the role of environmental reservoirs and food processing techniques on stress conditioning and pathogenicity of infectious bacteria (Doyle and Erickson, 2008; Capozzi et al., 2009).

Opportunistic enteric pathogens occupy unique and austere niches and must maintain the capacity to react to mercurial environmental conditions and the caustic and highly variable acidic conditions of the gastrointestinal milieu. Environmental strains of enteric bacteria may also respond to acid stress in the form of industrial waste, decomposition of organic matter and the chemical constituents of food preservation. As such, robust response to acid stress is central to survivability in the environment and the host. A key adaptive feature of enteric bacteria involves the rapid induction of physiological and biochemical changes upon exposure to extreme acidity (pH 1-4) after sub-lethal (pH 4-6) preconditioning. This mechanism is referred to as acid habituation (Goodson and Rowbury, 1989), acid tolerance (Foster and Hall, 1990) and the acid stress response (ASR); it varies by organism, acid classification (organic vs. inorganic), phase of bacterial growth (logarithmic vs. stationary) and growth media as well as other environmental factors (Foster, 1991; Rowbury, 1995; Lin et al., 1996; Foster, 1999; Koutsoumanis et al., 2003). In addition to protection from lethal acidity, ASR has been shown to provide cross protection to other forms of stress (Rallu et al., 2000; Frees et al., 2003; Xie et al., 2004) and may be elicited upon exposure to various environmental cues (Flahaut et al., 1996; Frees et al., 2001; DeAngelis and Gobetti, 2004). Further, induction of ASR has been demonstrated to activate additional adaptive behavior phenomena including virulence, biofilm formation, chemo taxis and antibiotic

resistance (Leyer and Johnson, 1992; Merrell and Camilli, 2002; Polen et al., 2003; Maurer et al., 2005; Butler et al., 2006; Hayes et al., 2006; Merrell et al., 2001).

The physiologic and biochemical mechanics of acid stress response have frequently been investigated in food borne pathogens such as enterohemorrhagic *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Recently, *Vibrio cholerae*, the waterborne pathogen responsible for Cholera, has emerged as capable of robust ASR. Several studies have investigated ASR in Gram-positive, industrially relevant bacteria, primary pathogens and Archaea (van de Guchte et al., 2002; Merrell et al., 2003; Cotter and Hill, 2003; Beales, 2004; Xie et al., 2004; Ciaramella et al., 2005; Padan et al., 2005; Baker-Austin and Dopson, 2007; Mols et al., 2010); however, reports contextualizing ASR in environmental and clinically derived strains of enteric bacteria are limited. This mini-review will discuss reports on the study of acid stress response and synthesize recent and transform active molecular microbiological investigations of ASR in *E. coli*, *S. typhimurium* and *V. cholerae* in laboratory and environmental conditions and the agricultural phenomena eliciting this response.

### ***Escherichia coli***

*Escherichia coli* is an ubiquitous Gram-negative and rod-shaped bacteria, commonly found in the gastrointestinal track of warm-blooded animals. Though harmless strains exist, enteric *E. coli* is commonly implicated as a food and water borne pathogen. Demonstrating three genetically and physiologically distinct AR strategies, the glucose-repressible oxidative pathway (Lin et al., 1995; Lin et al., 1996), glutamate-dependent acid resistance (GDAR; Hersh et al., 1996) and arginine-dependent ASR (Lin et al., 1996), this opportunistic pathogen is arguably the “gold-standard” in the study of enteric ASR. In the past 20 years, *E. coli* has emerged as an infectious agent when associated with minimally processed foods, thus garnering investigations on stress response and pathogenicity when associated with produce and during food processing (Capozzi et al., 2009).

Arnold et al. (2001) investigated the impact of the food preservative acetate on expression of

ASR genes in enterohemorrhagic *E. coli* O157:H7 reporting > twofold reduction in more than 60 genes; 48 of which are involved in transcription and translational regulation. By contrast, approximately 25 genes were shown have > twofold increased expression; most noteworthy being five *rpoS* sigma factors which have been identified as central for response to acid and peroxide stress as well as heat and osmotic shock (Cheville et al., 1996; Price et al., 2000; Nyström et al., 2004). Utilization of alternative sigma factors (i.e. Rpo family) have also been shown to regulate expression of virulence factors as well as genes that enhance both pathogenicity and environmental viability such as colonization and biofilm formation for *E. coli* and other enterotypes (Joelsson et al., 2007; Dong and Schellhorn, 2010).

Long-term survival in lethal pH is a hallmark of *E. coli* ASR with two separate mechanisms that involve amino acid antiporters. The first employs glutamate antiporters and several key decarboxylase enzymes, which maintain internal pH ( $pH_i$ ; Hersh et al., 1996). It has been reported that all of these metabolic enzymes must be synthesized for extensive ASR at pH 2; however, either decarboxylase coupled with the glutamate antiporter will provide long-term resistance at  $pH > 2.5$  (Foster, 1999, 2004). Prolonged survival to lethal pH is also facilitated by alteration in membrane phospholipid composition (Chang and Cronan, 1999) and the subsequent variance in membrane potential and increased  $pH_i$  (Richard and Foster, 2004). This durational response to extreme pH also involves synthesis of acid shock proteins that vary temporally during pH exposure (Foster, 2004) and sequestration of DNA promoter regions (Choi et al., 2000). Further, it was recently reported that osmolytes such as NaCl, KCl, proline and sucrose facilitate rapid  $pH_i$  homeostasis and therefore hasten the recovery of *E. coli* from acid shock (Kitko et al., 2010). The second long-term survival mechanism is linked to arginine-aggmatine amino acid antiporters, which participate in extreme acid response (XAR) and are highly conserved phylogenetically (Iyer et al., 2003). Sun et al. (2011) recently investigated the role of ATP dependent DNA repair machinery in *E. coli* XAR citing marked reduction in cell viability at

extreme pH when genes coding for the ATP biosynthesis enzymes (i.e. *purA*, *purB* and *adk*) were knocked out. These results suggest that ATP dependent metabolic processes such as select DNA repair systems, contribute to long-term survival at extreme pH. The phenomenon of DNA repair during stress induced mutagenesis was reviewed by Foster (2007) who reports that in response to environmental cues such as low pH, many alternative sigma factors (i.e. RpoS) moderate increased expression of error prone polymerases (i.e. Pol IV) and downregulate error-correcting enzymes (i.e. RecO/A) to increase mutations that vary progeny genotypes and facilitate survival.

Recent molecular advancements provide for wholesale screening of microbial transcriptomes enabling nuanced understanding of the diverse factors expressed during enteric ASR and the environmental conditions eliciting this response. Maurer et al. (2005) observed expression variation of approximately 760 genes in response to pH, identifying genes involved in flagellar motility and chemotaxis, oxidative stress and catabolism being variably moderated in response to pH. Further, this study demonstrated the induction of regulons involved in heat shock and oxidative stress response upon exposure to low pH. In an expansive survey of the *E. coli* genome, Kang et al. (2005) suggest a role for the DNA binding protein, FNR, in regulation of nearly 300 genes, including 189 operons, 5% of which are expressed during acid stress under anaerobic conditions. By contrast, Hayes et al. (2006) report on the impact of oxygen which moderates expression of > 260 genes upon exposure to extreme acid with aeration; though core constituents of these enzymes were expressed independent of oxygen. These ASR factors include hydrogenases and enzymes involved in sugar fermentation, antibiotic resistance and cell membrane composition.

In a microarray survey, Tucker et al. (2002) identified 28 novel genes expressed during GDAR representing functional groups involved in metabolism, cell envelope composition and modification, chaperoning and transcriptional regulation. These findings suggest clustering of nine acid-inducible genes in the *gadA* region involved in GDAR. Though the exact role of each of



these genes during ASR is unclear, mutations to these factors reduced acid tolerance. Using molecular techniques and bioinformatics, Hommais et al. (2004) report clustering of *gadE* into chromosomal fitness islands which are highly conserved across multiple *E. coli* strains. The *in silico* analysis suggested preferential denaturing of promoters required for pH homeostasis during acid adaptation. These findings suggest a highly conserved fitness island for ASR in the *E. coli* chromosome. This study is complimented by identifying the role of *gadW* in activation of *gadE* promoters under acidic conditions which resulted in improved colonization capabilities in the infant mouse model (Tucker et al., 2003). Ma et al. (2004) characterized the GDAR pathway and identified the novel regulatory factors, EvgA and YdeO, during *gadA* activation in exponential and stationary ASR. These factors were identified across several ASR pathways in *E. coli* and involve multiple EvgA interactions along inverted repeat promoters in response to acid stress (Masuda and Church, 2003). Additionally, Ma et al. (2003) reported on the regulatory role of acid induced GadE/X and W during ASR as they govern expression of glutamine decarboxylases encoded by *gadA* and *gadB* and the glutamate:  $\gamma$ -aminobutyric acid (GABA) antiporter, GadC. These results suggest that GadE is central to expression of these factors regardless of media or growth conditions, illustrating the complexity of this stress response cascade which involves five regulatory proteins, two sigma factors and multiple feedback loops.

Molecular investigations have resulted in the discovery of additional key factors that directly govern regulatory cascades during *E. coli* ASR. Krin et al. (2010a) identified H-NS as being near the top of the governance hierarchy during glutamate-, arginine- and lysine-dependent acid resistance. Krin et al. (2010b) also reported that the director of these three ASR strategies is moderated by RcsB. This is facilitated by RcsB-P/GadE complexes that interact with at least one other alternative regulator (i.e. H-NS, HdfR, CadC or AdiY) and subsequently regulate overlapping ASR pathways. It was later shown that RcsB is conserved and required for ASR in *E. coli* K12 and O157:H7 and interactions with GadE occur when cells are in logarithmic phase. In

stationary phase however, ASR is controlled by RcsB interactions with promoters that are not GadE regulated (Johnson et al., 2011). The elaborate interactions governing enteric ASR were further elucidated by Zwir et al. (2005) in a study characterizing the complex regulatory cascade of promoter interactions for the alternative regulatory proteins, PhoP/PhoQ. Using the Gene Promoter Scan technique, this study identified members of the PhoP regulon and characterized previously unknown regulatory interactions, thus mapping the complex network of the transcriptional regulatory cascade involved in moderating ASR. Very recent work has employed systems biologic approaches combining molecular and phenotypic analysis combined with computational modeling to identify the transcriptional networks underlying *E. coli* ASR; this resulted in identification of the outer membrane family of proteins (Omp) as central to robust response to mild and extreme acid in aerobic and anaerobic conditions (Stincone et al., 2011).

Studies to assess the role of fermentation and food production on ASR elicitation have been conducted. In one such study, Polen et al. (2003) employed molecular techniques to investigate the impact of exposing *E. coli* to the short chain fatty acids propionate and acetate. This study reports increased expression of families of genes involved in flagellar function and biosynthesis (*fli*, *fth* and *flg*), antibiotic resistance (*mar*), chemotaxis (*che*) and fimbriae morphology (*fim*) as well as the reduced expression of carbon uptake and utilization genes (*cst*, *gal*, *srl*, *ebg*, *mal* and *mgl*) based on short-term exposure to propionate and/or acetate. A related study investigates the role of fermentation byproducts (formate and acetate) on *E. coli* ASR (Kirkpatrick et al., 2001) citing increased expression of RpoS and periplasmic transporters in the presence of acetate but reduced expression of this regulatory factor when exposed to formate. A recent investigation of *E. coli* K12 and O157:H7 exposure to complex acid cocktails revealed a more robust response on the part of the enterohemorrhagic strain involving molecular mechanisms not identified in the laboratory strain suggesting improved ASR capability in conditions mimicking those that occur during digestion which subsequently aid in survival in the host GI

tract (King et al., 2010). Bergholz et al. (2009) identified more than 330 genes, 104 of which are specific to O157:H7, that are upregulated during cultivation of *E. coli* in apple juice (pH 3.5), including genes involved in osmotic, acid and oxidative stress. Price et al. (2000) identified the diet of cattle and the fermentative conditions therein, which subsequently induce RpoS, as central to shedding of viable, acid-adapted *E. coli* into the environment thus implicating ASR in carriage and cell survival in the bovine GI tract. Later, Price et al. (2000) were the first to report on the utilization of select acid response systems based on the chemical profile of the various acidic environments encountered by the pathogen. They demonstrate the crucial role of RpoS for survival in apple cider (pH 3.5) and in cattle intestines, thus prompting efforts to target this regulatory protein in calves colonized by *E. coli* O157:H7. Additionally, RpoS has been implicated in the release and survival of other acid adapted enteric pathogens into aquatic environments (Merrell and Camilli, 2000) and has been shown to regulate multiple stress response pathways of environmentally derived pathogens (Small et al., 1994; Cheville et al., 1996; Nyström, 2004; Bhagwat et al., 2008). Further, results of a genome-wide expression study suggest that nearly 10% of all *E. coli* genes are under the direct or indirect regulatory control of RpoS which governs all cell physiology under non-optimal growth conditions (Weber et al., 2005).

In an effort to assess the acid adaptive capability of environmentally derived enterohemorrhagic *E. coli* (EHEC), Bhagwat et al. (2005) surveyed 82 isolates from 35 countries to examine their GDAR response through traditional laboratory techniques and molecular characterization. Their findings reveal that nearly 40% of the isolates were defective in inducing GDAR under aerobic conditions while approximately 5% of the isolates were defective under both aerobic and fermentative conditions. However, introduction of *rpoS* on a low-copy-number plasmid resulted in the restoration of GDAR under aerobic growth in nearly 80% of acid sensitive isolates, emphasizing the significance of this regulatory agent in *E. coli* ASR. Further,

environments where GDAR positive EHEC isolates originated may represent conditioning reservoirs for acid resistance and pathogenicity.

### ***Salmonella enterica* serovar Typhimurium**

*Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is a Gram-negative, neutralophile commonly found in the intestines of animals such as birds, reptiles and humans making it another model organism in the study of enteric ASR. It is also found in contaminated and polluted pond water and within macrophage phagolysosomes; and, it has been shown to colonize a broad variety of fruits, vegetables, nuts and poultry products resulting in its distinction as the most common foodborne pathogen (Foster and Hall, 1990; Faucher et al., 2006; Hanning et al., 2009). The acid adaptive capabilities of *S. typhimurium* vary according to log or stationary growth and involve synthesis of early and late stage acid shock proteins (ASPs) which moderate cell surface features (i.e. hydrophobicity and outer membrane porins), cytoplasmic pH and macromolecular repair (Foster, 1991; Foster, 1993; Bearson et al., 1998). The various stages of *S. typhimurium* acid resistance are governed by the regulatory proteins RpoS, Fur and PhoP/PhoQ, with each responding to a wide array of environmental cues and regulating separate subsets of ASR (Foster, 1991; Foster, 1993; Bader et al., 2003). Collectively these regulatory factors provide cross protection to numerous forms of environmental stress such as temperature, oxidative damage, salinity and antimicrobial agents (Leyer and Johnson, 1993; Foster and Spector, 1995; Bader et al., 2003; Greenacre et al., 2006) and enhance the expression of multiple virulence and colonization factors (Foster and Hall, 1990; Wilmes-Riesenberg et al., 1997; Dong and Schellhorn, 2010).

*S. typhimurium* has been investigated with regards to ASR variability based on population growth phase and acid classification. During logarithmic growth, *S. typhimurium* ASR involves induction of the alternative sigma factor (*rpoS*) which moderate multiple ASPs in

response to short chain fatty acids but play a reduced role during inorganic acid exposure (Bearson et al., 1998). During stationary phase, ASR is determined in an RpoS-independent manner, which involves induction of outer membrane porins (Omp) in response environmental cues such as pH and salinity; *V. cholerae* has been shown to respond in a similar manner (Foster, 1999). As is reported in other enteric pathogens, this pathway involves the regulatory factors PhoP/PhoQ which differentiate between acids (inorganic vs. organic), Mg concentrations and enhance expression of virulence factors when exposed to moderate levels of organic acids and when cultivated in mammalian phagosomes (Bearson et al., 1998; Prost et al., 2007); phenomena conserved in *S. typhimurium* Typhi in response to host immune response (Faucher et al., 2006).

Regarding the impact of food processing on elicitation of enteric ASR, adaptation in inorganic acid has been demonstrated as sufficient for *S. typhimurium* and several other *Salmonella* species to remain viable and virulent for up to two months in organically acidic cheeses (Leyer and Johnson, 1992). Further, *S. typhimurium* cultivated in acidic conditions typical of food processing displayed robust acid tolerance at a lower pH (pH 4.5) than was observed for *Listeria monocytogenes* (pH 5.5) or *E. coli* O157:H7 (pH 5.0), suggesting enhanced ASR induction capability, and reduced sensitivity to environmental pH, compared to the common food pathogens *L. monocytogenes* and *E. coli* (Koutsoumanis and Sofos, 2004). Acid habituation of *S. typhimurium* during food production has been shown to provide cross protection to environmental stress such as heat, salt and lactoperoxidases, and confers tolerance to cell wall destroying agents and antibiotics (Leyer and Johnson, 1992). However, short-term exposure of *S. typhimurium* to the food preservative lactic acid was recently shown to reduce resistance to hydrogen peroxide via downregulation of the OxyR regulon which imparts protection to oxidative stress (Greenacre et al., 2006).

The interactions between enteric pathogens and the organisms they colonize may play a significant role in elicitation of ASR and was recently investigated by Bhagwat (2006) in a report

on the linkage between plant stress response and acid adaptation in *S. typhimurium*. In this study, *S. typhimurium* cultivated on cut or damaged plant displayed elevated percent population survival upon subsequent exposure to lethal pH in laboratory conditions (pH 2, 37°C, 2 h). In addition to the mildly acidic conditions of these wounded fruits and vegetables, apoplastic fluid from decomposing plants colonized by soft rot pathogens may induce acid resistance of *S. typhimurium* (Nachin and Barras, 2000). The antimicrobial peptides that are part of a wounded plants immune response result in upregulation of ASR factors in *S. typhimurium*'s *sap* operon which enhance survival upon subsequent exposure to the mammalian gut (Parra-Lopez et al., 1993; López-Solanilla et al., 1998, 2001). This pathogen has been shown to respond to cues from bacterial community constituents on spoiled plant biomass via acyl-homoserine lactones (AHLs) produced by the bacterial community (Ahmer, 2004), prompting the hypothesis that this inter-cellular communication is upregulating determinants of human pathogen growth on plants during decomposition (Brandl, 2006). Additionally, these interactions have been shown to enhance population densities of *S. typhimurium* in wounded fruits and vegetables when co-occurring with other bacterial species (Wade and Beuchat, 2003) or proteolytic yeasts (Wade et al., 2003); with the latter being implicated as a transmission vector of enterics into plant tissue (Richards and Beuchat, 2005). It was recently demonstrated that *S. typhimurium* associated with the food vacuoles of the protist *Tetrahymena*, alter the expression of approximately 1000 and 1200 genes compared to water and culture broth respectively, including enhanced expression of alternative electron acceptors and factors involved in anaerobic respiration (Rehfuss et al., 2011). Further, expression of arginine-dependent ASR factors (i.e. AdiA, AdiY) were increased in *S. typhimurium* cells within the food vacuole, suggesting that elicitation of ASR confers resistance to digestion from the protist, provides survival advantage upon egestion and may represent an ASR pre-conditioning reservoir in the contamination cycle of *S. typhimurium*.

The role of plants as disease vectors during outbreaks of salmonellosis has been realized since the 1990s when the shift in food contamination became clearly linked to irrigation and plant health (Hanning et al., 2009). Since then, there has been renewed interest in the role of aquatic reservoirs and the bacterial load thereof, in conditioning enteric pathogens and displacing them to fruit and vegetable products in various agricultural settings. Freshwater systems have commonly been a source of irrigation (Assadian et al., 1999; Garcia et al., 2001) and assessing density, virulence and elicitation of stress response of *Salmonella* in these aquatic reservoirs has become essential to risk assessment and infection reduction (Baudart et al., 2000). In one such study employing general media and media selecting for, and differentiating between, enteric pathogens, *Salmonella* was shown to be proportionally dominant in samples taken from beaches and freshwater reservoirs in North-eastern Spain (Polo et al., 1998). It has also been demonstrated, via traditional culturing techniques, that *Salmonella* is a dominant component of the cultivable bacterial community in the water column and in sediment systems impacted by point and non-point sources of pollution in a coastal Mediterranean system; with residence in marine sediments being central to its long-term viability in these aquatic reservoirs (Baudart et al., 2000). Cultivation in these environmental conditions has been shown to increase expression of key virulence factors, with variability in pH being cited as the environmental parameter regulating of virulence in samples taken from regions along the Rio Grande in southern Texas (Nutt et al., 2003).

### ***Vibrio cholerae***

*Vibrio cholerae* is the highly motile, gram-negative, curve-shaped bacteria responsible for the gastro-intestinal disease Cholera. This microorganism follows the oral route of infection, survives the low pH conditions of the stomach (pH 1-3) and colonizes the small intestine (pH 6-8). Here, it produces the cholerae toxin (CT) that results in the loss of copious volumes of fluid from the host, and returns virulent strains to the aquatic environment. The mystery of how this environmentally

ubiquitous bacteria, which is categorized as acid-sensitive but is capable of causing gastrointestinal disease in epidemic proportions, has been deciphered by the discovery that *V. cholerae* mounts a robust ASR when pre-conditioned at sub-lethal pH (Merrell and Camilli, 2002). In general, *V. cholerae* ASR involves overlapping responses to inorganic and organic acids, increased expression of > 60 and decrease in > 50 protein species with functions including transcriptional regulation, cell wall maintenance, catabolism, colonization, DNA repair, Na<sup>+</sup> homeostasis and membrane transport; collectively resulting in reduced sensitivity to acidic conditions and a greater capacity to cause harm in the mammalian host (Merrell and Camilli, 1999, 2000, 2002; Peterson, 2002; Reidl and Klose, 2002). Acid adapted *V. cholerae* display increased colonization capabilities and competitive advantage; thus requiring a reduced infective dose compared to non-adapted cells (Merrell and Camilli, 1999). This enhanced pathogenicity results from greater reproductive potential within the host, as opposed to increased expression of virulence factors such as cholera toxin (*ctx*) or toxin co-regulated pili (*tcp*) (Angelichio et al., 2004).

The first groundbreaking studies on *V. cholerae* ASR employed signature tagged mutagenesis (STM) to identify novel factors involved in acid adaptation (Chiang and Mekalanos, 1998; Merrell and Camilli, 1999, 2002). Merrell and Camilli (1999) were the first to identify the ASR factor CadA in *V. cholerae*, which moderates pH<sub>i</sub> via lysine consumption in the bacterial cytoplasm. Since this study, the Cad family of proteins has been implicated in moderating pH<sub>i</sub> during *S. typhimurium* ASR (Greenacre et al., 2006) and in the aquatic pathogen *V. vulnificus* (Rhee et al., 2004). Screening large pools of virulence attenuated *V. cholerae* resulted in identification of 9 novel ASR factors involved in sodium and potassium homeostasis, cell wall maintenance, DNA repair and protein synthesis (Merrell et al., 2002a). Acid-adapted *V. cholerae* with enhanced expression of these ASR factors were shown to be more competitive mutants and non-adapted cells when challenged to the suckling mouse model.



As is observed among enteric species, *V. cholerae* ASR involves activation of the Rpo family of transcriptional regulators which are central to quorum sensing (Joelsson et al., 2007), expression of virulence factors (Dong and Schellhorn, 2010) and secretion of outer membrane vesicles that improve colonization of the intestinal milieu (Song et al., 2008). Additionally, the Rpo family moderates biofilm formation and expression of the virulence pathogenicity island (VPI) during low and high cell density via the transcriptional regulators, AphA and HapR (Zhu and Mekalanos, 2003; Kovacikova et al., 2010; Rutherford et al., 2011). These factors have been shown to govern ASR based on environmental conditions, with AphB moderating virulence in response to low pH and an aerobiosis (Kovacikova et al., 2010) and HapR governing protease production, motility, biofilm formation and expression of virulence factors in vivo (Zhu and Mekalanos, 2003).

Investigations into the role of the outer membrane porins (Omp) in *V. cholerae* ASR have revealed important interactions between this family of proteins and the intercellular regulatory agent's central to gene expression. Kovacikova and Skorupski (2002) report on the role of the Omp family in moderating the regulatory protein RpoE and enhancing infectivity and colonization capabilities of *V. cholerae* and *E. coli*. Mathur et al. (2007) demonstrate that OmpU acts as an environmental sensor in the signal transduction pathway which regulates the alternative sigma factor and confers resistance to antibiotics such as polymixin B and human gut derived bacteriocidal proteins. Mathur and Waldor (2004) report on OmpU moderated ToxR expression as essential for antimicrobial resistance and the expression of virulence factors. Though not acid shock proteins per se, as Omp expression is not moderated exclusively by acid, this family of porins has been demonstrated as responsive to physiochemical signals such as bile and organic acids and responsible for regulating alternative sigma factors central to *V. cholerae* ASR (Li et al., 2000; Provenzano and Klose, 2000; Merrell and Camilli, 2000; Matson et al., 2007). Recent molecular investigations detecting genes involved in acid stress response (i.e. *ompU* and *toxR*), as well as other virulence and regulatory factors in *V. cholerae*, report a high degree of conservation

of these factors among genetically diverse strains of *V. cholerae* isolated from aquatic environments within countries where it is endemic (Zo et al., 2009; Goel and Jiang, 2010). The conservation of such stress factors is imperative for environmental survival which is emphasized by the finding that *V. cholerae* from pH stressed environments is capable of growth at rates comparable to the natural assemblages in alkaline lake water (pH 7.8-9.1; Kirschner et al., 2008).

The Tox family (ToxT, ToxR and ToxS) has been shown central to transcriptional regulation for myriad factors involved in *V. cholerae* ASR. These regulators moderated by the Omp family of proteins which stimulate transcription in response to environmental cues by binding *toxbox* regions upstream of promoter elements (Withey and DiRita, 2005, 2006). Subsequently, tox proteins govern a cascade of ASR factors involved in virulence (*ctx*, *tcp*), colonization (*msh*), antimicrobial resistance (*mex*), cellular metabolism (*gal*, *lac*, *mrs*) and DNA repair (*rec*); they are activated by an array of metabolic intermediates, regulatory messengers and environmental conditions such as temperature, salinity and pH (Merrell and Camilli, 2002; Peterson, 2002; Reidl and Klose, 2002; Tischler and Camilli, 2004; Abuaita and Withey, 2009).

Several studies have aimed to characterize the expression profile of ASR genes as *V. cholerae* transitions between the host GI tract and aquatic reservoirs. In vivo results suggest that *V. cholerae* that has passed through the host GI tract demonstrates enhanced virulence upon re-introduction to the host (Merrell and Camilli, 2002; Reidl and Klose, 2002). This increased infectivity is facilitated by reduced expression of chemotaxis factors such as CheW-1; a phenotype maintained in the aquatic environment and only elicited post-infection (Butler et al., 2006; Merrell et al., 2001). Schild et al. (2007) investigated *V. cholerae* in transit through the host and report increased expression of six unique genes in the latter stage of infection which play limited roles during infection, are critical for survival upon introduction to the aquatic reservoir and are expressed prior to exiting the host. In this study, viability of *V. cholerae* expressing late stage genes after transfer to various environments (i.e. rice water stools, pond water and nutrient

broth) was monitored and resulted in identification of multiple factors functioning in transcriptional regulation, substrate level phosphorylation and nutrient transport; all of which improve the fitness of *V. cholerae* upon introduction to environmental reservoirs.

Investigations of *V. cholerae* transitioning between the aquatic environment and the mammalian host have revealed the role of quorum sensing induced biofilm formation as implicit in surviving the host defenses and inducing infection upon entering the GI tract (Kamruzzaman et al., 2010). Biofilm formation has been shown to involve activation of the regulatory protein, HapR upon exposure to bile acid and is central for *V. cholerae* survival in the aquatic environment (Zhu and Mekalanos, 2003). Further, Faruque et al. (2006) report that biofilm aggregated *V. cholerae* display enhanced viability in the environment after passage through the mammalian host, suggesting a role of ASR for survival in aquatic reservoirs. This study is complimented by the finding that biofilm formation in the environment results in a hyper-infective phenotype upon introduction to the host GI tract (Tamayo et al., 2010). Beyhan et al. (2006) report increased expression of genes involved in biofilm formation (*vps*), extracellular protein secretion (*eps*) and mannose-sensitive hemagglutinin (*msh*) as well as reduction of flagellar gene expression in response to variability in environmental concentrations of the second messenger 3', 5'-cyclic diguanylic acid (c-di-GMP) which aids in the integration of environmental stimuli and effects cell physiology for many microorganisms.

The genetic predisposition of *V. cholerae* to transition between environmental and pathogenic lifestyles was recognized upon sequencing its two chromosomes (Heidelberg et al., 2000). Results indicate that the small chromosome has a greater proportion of hypothetical genes (59%) than the large chromosome (42%) and has more genes originating in plasmids and other  $\gamma$ -Proteobacteria. In addition to providing evidence that the small chromosome originated as a mega-plasmid which was acquired by an ancestral *V. cholerae*, this study provides a foundation for deciphering how these environmental bacteria emerged as enteric pathogens.

Additional factors contributing to successful transition at the environment-host interface were identified by Vezzulli et al. (2008) who identified dual role colonization factors (DRCF) such as the cholera toxin (*ctx*), toxin co-regulated pili (*tcp*), alternative sigma factor (*rpo*) and quorum sensing genes (*lux*) that are necessary for successful colonization of both the gastrointestinal epithelia and the chitinous exoskeleton of copepods in the aquatic environment. Further, Kirn et al. (2005) recently recognized a gene (*gpbA*) involved in chitin colonization in the aquatic environment that is also necessary for successful colonization of the epithelia in the mammalian host. Exoskeleton associations have been shown to be central for environmental survival, biofilm formation and intra-species gene exchange, as well as pathogenicity and pre-conditioning of ASR (Nalin, 1976; Nalin et al., 1979; Blokesch and Schoolnik, 2007; Pruzzo et al., 2008). The significance of these associations is further illustrated by the observation that Cholera epidemics are seasonal and strongly linked to algal blooms due to *V. cholerae* associations with phytoplankton and the chitinous exoskeleton of copepods within aquatic systems (Colwell, 1996; Hsieh et al., 2007).

## CONCLUSION

Since the discovery of acid stress response and its characterization in *E. coli* and *S. typhimurium*, there has been a broad array of highly conserved acid stress response factors observed in Gram-negative and Gram-positive pathogens with new model organisms being reported. The use of large-scale, signature-tagged mutagenesis (STM), microarrays and metatranscriptomic analysis have led to rapid discovery of novel factors for acid resistance and provide insight into this bacterial survival strategy. However, the role of various environmental reservoirs on acid adaptation and elicitation of the ASR in the context of pathogen ecology remains largely unexplored. Studies have focused upon bacterial pathogen response either within the mammalian host, in laboratory conditions mimicking the host or in industrially relevant food products during food preservation and processing. While understanding of ASR is immensely valuable and central

to the control of microbial pathogens, gaining a deeper understanding of environmental reservoirs that condition for acid adaptation and the elicitation of stress response pathways will enable disruption of the fecal-environmental-oral cycling of these enteric pathogens. Such insights would aid in developing reliable models of pathogen distribution that effectively incorporate the functional niche of microorganisms and the environmental 'hot spots' of pathogenicity. Finally, characterizing these stress response pathways in environmental and clinical strains of enteric bacteria will provide a broader and more complete context of pathogenicity which could facilitate improved methods for bacterial control during food and water preparation and potentially aid in disease mitigation.

## CHAPTER 3

# FLUOROMETRIC ESTIMATION OF SURFACE ASSOCIATED MICROBIAL ABUNDANCES

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Swenson G.J., Hagenbuch I.M., Pinckney J.L., and Long R.A. 2012. *Journal of Microbiological Methods*. 88: 297–303

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## ABSTRACT

Surface associated microbes have historically been difficult to accurately and effectively enumerate. In the current study, we propose a rapid and simple method for estimating abundance of surface associated microbial cells by fluorescence of SYBRGreen stained bacteria and *in vivo* chlorophyll *a* fluorescence of benthic diatoms in 24 and 48-well microtiter plates. The effectiveness of this high-throughput technique is demonstrated by assessing sensitivity of a clinical strain of *Vibrio cholerae*, a benthic bacterial isolate and the benthic microalgae *Cylindrotheca closterium* to three antibiotics - tylosin, lincomycin and ciproflaxacin. We report on the significant linear relationships between spectral chl *a* fluorescence and cell abundance and between microalgal growth rates derived from cell counts and fluorescence. Additionally, we provide a simplified and improved method for preparation of a silica gel matrix (SGM), which is an ideal plating media for fluorescence applications. These findings indicate that spectrofluorometry is an inexpensive tool for rapidly estimating abundance of surface associated microbiota and can be employed for assessing antibiotic sensitivity.

## INTRODUCTION

A central tenet of microbial investigation, including pathogenicity, environmental contamination, ecology and ecotoxicology involves the quantification of microbial abundance. The capacity to rapidly and accurately enumerate abundance of surface associated microbial cells is imperative for multiple disciplines within the field of microbial ecology. Many observational methods have been employed to assess abundances of these microorganisms. Direct observations include various forms of microscopy (*i.e.*, light, epifluorescence, confocal microscopy, electron microscopy). Though epifluorescence microscopy has been shown in the past to result in density estimation errors (Blackman and Frank, 1996), microscopy remains central in quantifying surface associated microbes (Lad and Costerton, 1990; Lindsay and von Holy, 1997).

Fuches et al. (2002) report on a technique using thin light sheet microscopy (TLSM) for observation of microbes associated with individual particles at the sub-millimeter scale in suspension; however, this is time intensive. Flow cytometry has been shown to be an effective alternative to microscopy (Diaper and Edwards, 1994) but is not applicable for enumerating particle associated cells, bacterial aggregates or biofilm communities while they are surface associated. Wegley et al. (2006) recently identified bulk fluorescence as a novel tool for quantifying mixed bacterial populations suspended in the water column citing enhanced precision in estimating microbial numbers using bulk fluorescence as compared to direct counts via epifluorescence microscopy. However this technique was not employed for quantification of surface associated cells. Msken et al. (2010) report on a protocol to measure bacterial density and antibiotic sensitivity of *Pseudomonas aeruginosa* using fluorescent live-dead stains and automated confocal microscopy equipment to qualitatively and quantitatively evaluate biofilm



formation but requires specialized equipment, 16 procedural steps, ~60 hours of preparation time and ~5 hours per plate of data analysis.

Several indirect observational methods such as plate counts coupled with sonication, vortexing and scraping have been examined to enumerate surface associated microbes by first detaching and then quantifying the cells. However, many of these techniques are considered inaccurate, unreliable and inconsistent (Lind, 1985; Poulsen, 1999; Patil and Anil, 2005; Goeres et al., 2009). The use of microtiter plates is an indirect means of microbial observation that is easily modified for biofilm and colonization assays (An and Friedman, 2000). Applications have successfully employed multi-well microtiter plates to assess biofilm formation by measuring absorbance of crystal violet (CV) stained biofilms (Christensen et al., 1985; Djordjevic et al., 2002; Silva et al., 2009). This is a rapid approach to qualifying biofilm formation but is limited in analytical power and sensitivity.

Quantification of benthic microalgal species is complex given the heterogeneous nature of sediments and the propensity of cells to form biofilms incorporating multiple species of microalgae and bacteria (Miller et al., 1996). The use of fluorescent staining of environmentally derived samples has been limited due to the non-specific binding of the dyes and subsequent high background fluorescence (Diaper and Edwards, 1994). Additionally, this approach requires time intensive microscopy and automated image analysis software for observation based enumeration.

In the current study, we present a simple, rapid, reliable and inexpensive protocol for quantifying surface associated microbiota that will be of interest to a wide range of investigators from a variety of microbiological disciplines. We have eliminated the need to concentrate microbial cells via membrane filtration, mitigated the influence of non-specific dye binding and media derived background noise and improved the reproducibility of abundance estimates using multi-well microtiter plates. We demonstrate the applicability of this protocol in antibiotic

sensitivity assays and provide a simplified protocol for the preparation of silica gel for bacterial cultivation.

## MATERIALS AND METHODS

### *1.1. Silica gel matrix preparation*

The silica gel matrix (SGM) was prepared according to Dietz and Yayanos (1978) with several modifications to reduce preparation time and volume. This was accomplished by making a 1.0M solution of sodium metasilicate pentahydrate ( $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ ; VWR, West Chester, PA) rather than the suggested 0.5M sodium silicate meta ( $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ ) solution to produce a stable, solid and transparent SGM with desiccation resistance. Finally, the tryptone-glucose-yeast extract was excluded from the media to discourage growth of plated bacteria and to reduce background noise during the fluorescence assays. During fluorescence assays, nutrient amended SGM was prepared by enriching with 10% and 20% final (V/V) ZoBell broth.

A strong cation exchange resin, Dowex® C-211,  $\text{H}^+$  (Fisher Scientific; Pittsburg, PA) was packed 1cm in diameter and 30cm high. A plug of autoclaved cheese cloth 1.5cm thick was inserted into the base of the graduated burette to prevent outflow of cation exchange resin particles. The exchange column was regenerated with 50mL 10% HCl and rinsed with 250mL distilled water. Once the water drained to the top of the resin column, flow was interrupted and 50mL of 1.0M  $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ , meta solution was gradually added to the column. A flow rate up to 100mL per min was sufficient for adequate cation exchange as previously reported (Dietz and Yayanos, 1978).

During silica sol collection, the first 50mL of effluent was discarded, as this is the void volume, *i.e.*, primarily water from the secondary column rinse. Up to 50mL of effluent was then collected and the pH was adjusted to 1.5 with 4N HCl. The silica sol was then autoclaved at 121°C for 20 min and stored at room temperature until processing. At neutral pH, gelation of

silica sol will occur rapidly when at  $\geq 25^{\circ}\text{C}$ . To slow the rate of gelation, the temperature of the sol was reduced to approximately  $4^{\circ}\text{C}$  before adjusting the pH to 7.2-7.5 with NaOH; gelation occurs within 10-12 min.

Previous protocols were described for generating large volumes (1L), thus requiring a large resin exchange column (Taylor, 1950; Thatcher and Weaver, 1974; Dietz and Yayanos, 1978). We found that smaller volumes of gel sol could be prepared in either a 50mL graduated burette, a 250mL glass separatory funnel or a 30cm glass chromatography column for cation exchange.

## 2.2. Strains, culture conditions and media preparation

### 2.2.1. Bacterial cultivation

*Vibrio cholerae* N16961 was incubated in 10mL of ZoBell broth at  $37^{\circ}\text{C}$  overnight. The cultures were vortexed for  $\sim 5$  min to homogenize culture. Sterile Roll & Grow™ Plating Beads (Qbiogene, Inc.; Solon, OH) were added to the culture prior to vortexing to aid in disrupting bacterial aggregates during resuspension. Samples were then serially diluted two-fold (*i.e.*, 1:1, 1:2...1:64) in ZoBell broth.

The benthic bacterial unknown (GSBB01) was isolated from the sediment near Winyah Bay (Georgetown, SC), cultured on ZoBell agar and incubated overnight at  $22^{\circ}\text{C}$ . One colony of this growth was transferred to 10mL of ZoBell broth, incubated overnight at  $22^{\circ}\text{C}$  and vortexed for  $\sim 30$  sec before inoculating micro-titer plates.

### 2.2.2. Eukaryotic cultivation

The benthic diatom *Cylindrotheca closterium* (CCMP 1855) was grown in filtered, autoclaved, f/2 + Si enriched seawater at  $23^{\circ}\text{C}$  on a 12 hr light/dark cycle at an irradiance of  $\sim 85 \mu\text{mol m}^{-2}\text{s}^{-1}$ .

During assay setup, subcultures were gently vortexed for 30 sec to disrupt flocs before being distributed into 24-well microtiter plates.

### 2.2.3. *Bacterial culture plate preparation*

SGM was prepared as described above. ZoBell agar (ZBA) and ZoBell broth (ZBB) were prepared according to ZoBell (1941) and 1.5% agar plates were prepared with BactoAgar (BA) in 90% filtered seawater and 10% Nanopure water. After preparation, 500 $\mu$ L of medium was transferred to each well of a standard clear, 48-well plate (Greiner Bio-One; Monroe, NC). Plates were solidified in a sterile biosafety hood at 25°C for 25 min.

100 $\mu$ L of culture broth from each dilution factor was transferred to each well of the previously prepared 48-well plate. After sample transfer, the plates were incubated at 4°C for 30 min to allow diffusion of sample broth and adsorption of bacterial cells on surface matrices. All sample and control wells were stained with 50 $\mu$ L SYBRGreen (1X final concentration; Molecular Probes, Inc., Carlsbad, CA) and incubated in the dark at 25°C for 15 min.

### 2.2.4. *Microalgal culture plate preparation*

All preparatory work was done inside a laminar flow hood. Each 6-well row of a 24-well microtiter plate was used as a complete dilution series of the given antibiotic including a control. The following was added to each well - 4 $\mu$ L concentrated antibiotic solution, 2mL f/2 +Si, and 25 $\mu$ L *Cylindrotheca closterium* culture and homogenized via resuspension. Plates were incubated at 22°C for 2 hrs to allow the diatom cells to settle before initial and following microscopic enumeration and measurements.

### 2.3. *Fluorometry*

Plates were read on a SpectraMax GeminiEM spectrofluorometer (Molecular Devices; Sunnyvale, CA, USA). For SYBRGreen bacterial estimates, samples were read from above with

the following parameters; excitation at 485nm and emission at 538nm with a cutoff long band pass 530nm filter. Three replicate wells per dilution factor were measured. Control wells, which consisted of 100µL sterile ZBB on 500µL of respective surface matrix, were also measured in triplicate. For microalgal assays, the optical parameters were excitation wavelength for chl *a* at 460nm and emission wavelength at 685nm with a cutoff long band pass 665nm filter. In microalgal assays, fluorescence was detected from below the clear well plates, allowing *in vivo* chlorophyll *a* fluorescence to be measured closer to the cells, improving the signal/noise ratio. Fluorescence data were collected and analyzed using SoftMaxPro™. Each well was read at empirically determined parameters for well scan reading (fill), density (3), spacing (1.13mm), sensitivity (normal) and total points (9) with each setting being variably adjusted but uniformly applied for entire plates, depending on the density of the samples. All fluorescence results are reported as relative fluorescence units (RFU).

#### *2.4. Cell density estimates*

##### *2.4.1. Bacterial enumeration*

Direct counts by microcapillary cytometer were used to enumerate bacterial abundance. Duplicate 1:10 diluted cultures were fixed with borate buffered formalin (2% final concentration) and stained with 1X SYBRGreen. Abundances were measured in duplicate for each dilution factor and were determined using the Guava Easy Cyte Plus Microcapillary Cytometer and Guava Check Express Pro version 5.3 (Guava Technologies, Hayward, CA).

##### *2.4.2. Microalgal enumeration and growth rate*

Direct algal cell counts were conducted daily using microscopic quadrant (grid) counts. Counts were made prior to fluorometry measurements using an eyepiece micrometer grid attached to an inverted epifluorescent microscope. Counts of five grids were made at one magnification in each well and only cells exhibiting chl *a* fluorescence were counted. Plates were allowed to sit in a

dark room for 15 min before their fluorescence was quantified in the plate reader to allow dark acclimation. Growth rates were calculated by fitting an exponential function to the population estimates and RFU values.

## 2.5. Antibiotic assays

### 2.5.1. Bacterial sensitivity

The clinical strain *Vibrio cholerae* N16961 and the environmental bacteria GSBB01 were grown overnight in ZoBell broth at 37°C and 22°C respectively. In a 48-well microtiter plate, 50µL of each antibiotic solution was added to 500µL of molten ZoBell agar per well and mixed.

Antibiotics used were ciprofloxacin (concentration gradient,  $3 \times 10^{-2}$  -  $3 \times 10^{-5}$  g mL<sup>-1</sup>), lincomycin ( $5 \times 10^{-2}$  -  $5 \times 10^{-5}$  g mL<sup>-1</sup>) and tylosin ( $9 \times 10^{-2}$  -  $9 \times 10^{-5}$  g mL<sup>-1</sup>). After solidification of the media, the surface was inoculated with 100µL of bacterial culture and incubated at 37°C and 22°C overnight for *V. cholerae* and GSBB01, respectively. After incubation, each well was stained with SYBRGreen (1X FC), incubated in the dark for 15 min and the fluorescence was measured as described previously.

### 2.5.2. Microalgal sensitivity

24-well microtiter plates were prepared and incubated as previously described with the same antibiotic compounds as in the bacterial assays, however surface matrices were not employed. Final concentrations (g mL<sup>-1</sup>) of antibiotics were ciprofloxacin ( $1 \times 10^{-2}$  -  $1 \times 10^{-6}$ ), lincomycin ( $4 \times 10^{-2}$  -  $4 \times 10^{-6}$ ) and tylosin ( $2 \times 10^{-2}$  -  $2 \times 10^{-6}$ ). Because each antibiotic was differently soluble, the maximum concentrations of each antibiotic also differed. Concentrations for each compound in bacterial and microalgal assays were normalized to the maximum concentration of that compound to provide a common unit (concentration factor) for all three antibiotics. Longer generation times for this diatom necessitated longer assay duration consisting of measurements once per day for up to 5 days. Assays were terminated when cultures became too dense to enumerate via epifluorescent microscopy.

## 2.6. Statistical analysis

Univariate analysis of variance, linear regression and post hoc analyses were conducted with SPSS 19.0 software (IBM; Armonk, NY, USA). The Ryan-Einot-Gabriel-Welsch F or Q tests were used to determine antibiotic subset identity.

## RESULTS AND DISCUSSION

### 3.1. Auto-fluorescence of surfaces matrices and growth media

#### 3.1.1. Surface matrices

In devising a protocol for rapid assessment of bacterial cells on surface matrices, we first examined the background signal of SYBRGreen and the auto-fluorescence of the respective matrices by measuring the fluorescence of SYBRGreen stained amended and unamended surface (Fig.1). We found that the auto-fluorescence of BA was nearly half of that observed for SGM. When SGM was amended with 10% growth media (bacto-peptone, yeast extract; SGM+ZBB10%), we observed background fluorescence elevated by <1% whereas SGM+ZBB20% displayed ~30% increase in auto-fluorescence. When BA was amended with full strength growth media, the background noise increased 255%. The elevated control signal of enriched matrices is likely due to the autofluorescence of compounds in the bacto-peptone and yeast extract and associated opalescence. However, when employed in bacterial surface association fluorometric assays, the background signal did not significantly interfere with fluorometric detection of SYBRGreen stained bacterial cells, even at low densities (section 3.2.1).

#### 3.1.2. Microalgal growth medium

The f/2 + Si medium used in microalgal assays exhibited no significant auto-fluorescence and did not affect fluorescent detection even at very low population densities. The mean RFU of 288 wells before algal addition was  $0.260 \pm 0.036$  RFU, which is an order of magnitude below the

lowest single RFU observed after algal cells were added. This suggests that f/2 + Si enriched seawater is an ideal medium for use in benthic microalgal fluorescent bioassays of this kind because it contributes no auto-fluorescent noise or variability to assay.

### 3.2. Validation of fluorometric estimation of microbial abundance

#### 3.2.1. Bacterial abundance assessment

Fluorescent emission of SYBRGreen stained *V. cholerae* N16961 was strongly linear and statistically significant ( $R^2 > 0.900$ ,  $p < 0.001$  for all surfaces) in surface associated assay and across a broad bacterial concentration gradient (Fig. 2A). Regarding specific effect of surface matrices, linear model fits for SGM and BA ( $R^2=0.999$ ,  $p<0.001$  for both) were slightly better than nutrient amended surface matrices. Linear models of SGM surfaces amended with 10% and 20% ZBB ( $R^2=0.997$ ,  $R^2=0.980$ , respectively;  $p<0.001$  for both) accounted for slightly less variability than the SGM and BA. ZBA demonstrated the poorest linear fit but maintained a high coefficient of determination and statistical significance ( $R^2=0.967$ ,  $p<0.001$ ). Each of the matrices appears to be effective in surface associated fluorometric assays, emitting a fluorescence signal that is consistent, detectable and linear across a broad concentration gradient.

#### 3.2.2. Microalgal abundance assessment

Chl *a* fluorescence of *C. closterium* control-wells exhibited a linear relationship to the number of cells present (Fig. 2B,  $R^2 = 0.667$ ,  $p < 0.001$ ). With the exception of three outliers, two from the lincomycin assays and one from the ciprofloxacin assays, the relationship between cell number and fluorescence was consistent across a wide range of cell concentrations and population ages.

### 3.3. Antibiotic sensitivity analysis

#### 3.3.1 Bacterial response



Each of the three antibiotic compounds at various concentrations was shown to inhibit the densities of *V. cholerae* N16961 and GSBB01 populations as measured by significantly lower fluorescent emissions detected after 18 hrs (Fig. 3A, 3B;  $p < 0.001$ ). Based on RFU values, ciprofloxacin was shown to have the greatest inhibitory effect on growth and resulting densities of both *V. cholerae* and GSBB01 (Fig. 3A, 3B;  $p < 0.001$ ). By comparison, tylosin inhibited GSBB01 growth in the two most concentrated treatments, while *V. cholerae* was sensitive to only the most concentrated treatment (Fig. 3A, 3B). Similarly, lincomycin inhibited the growth of the benthic bacteria and *V. cholerae* in the two most concentrated antibiotic solutions and in the most concentrated treatments, respectively (Fig. 3A, 3B). That all concentrations affected GSBB01 indicates that it was more sensitive than *V. cholerae* to low concentrations of antibiotic, regardless of compound.

### 3.3.2. Microalgal response

Each antibiotic substantially reduced endpoint chl *a* fluorescence emission of *C. closterium* (Fig. 3C). Endpoint (115-120 hrs) fluorescent emissions were significantly different between compounds and concentrations ( $p < 0.001$  in both cases). The compound specific response was unique, with each antibiotic compound having a more pronounced effect on the diatoms than was observed in bacteria. The highest antibiotic concentrations had a significant effect on chl *a* fluorescence, whereas intermediate doses were more difficult to separate and the effect of the two lowest concentrations were indistinguishable from the control.

Cell-specific growth rates of *C. closterium* were consistent with the rates derived using RFU (Fig. 4). Cell-specific rates span  $-0.04 - 1.34 \text{ r}^{-\text{d}}$  and RFU-specific rates ranged  $-0.56 - 0.82 \text{ r}^{-\text{d}}$ , with distinct antibiotic and concentration-specific patterns. Tylosin had the most pronounced effect (Fig. 3C, 4A), followed closely by lincomycin. These differences were observed in both cell and RFU growth rates (Fig. 4B). Two of the ciprofloxacin RFU growth rates exhibited

relatively high coefficients of variation of 1.03 and 3.81 (Fig. 4C), which may be due to crystallization, and subsequent fluorescence, of ciprofloxacin in treatment wells. Nevertheless, the responses induced by ciprofloxacin were consistent between cell counts and chl *a* fluorescence.

### 3.4. Evaluation of fluorometric method

#### 3.4.1. Bacterial

Estimating abundance of surface associated microbial cells has historically been intensive, time-consuming, deleterious to the integrity of cells, and prone to very high estimation errors (Lin et al., 1985; Blackman and Frank, 1996; Patil and Anil, 2005; Goeres et al., 2009). We have modified a previous fluorometric protocol (Wegley et al., 2006) to use low concentrations of SYBRGreen stained, surface associated bacterial cells in 48-well microtiter plates. Where previous fluorometric techniques have been reported as inconsistent in assessing bacterial densities and requiring expensive fluorescent stains at higher concentrations (Peeters et al., 2007), the method proposed in the current study is inexpensive and sensitive, showing strongly linear, reproducible results across a broad concentration gradient of cells associated with a variety of surface matrices. Application of this technique is demonstrated in the effective assessment of antibiotic sensitivity of three model microbial organisms, which has in the past been time-intensive, cost-prohibitive and reliant on highly specialized instrumentation (Müsken et al., 2010). As such, this assay provides an inexpensive and rapid alternative for estimating surface associated microbial abundances and may be modified for additional applications.

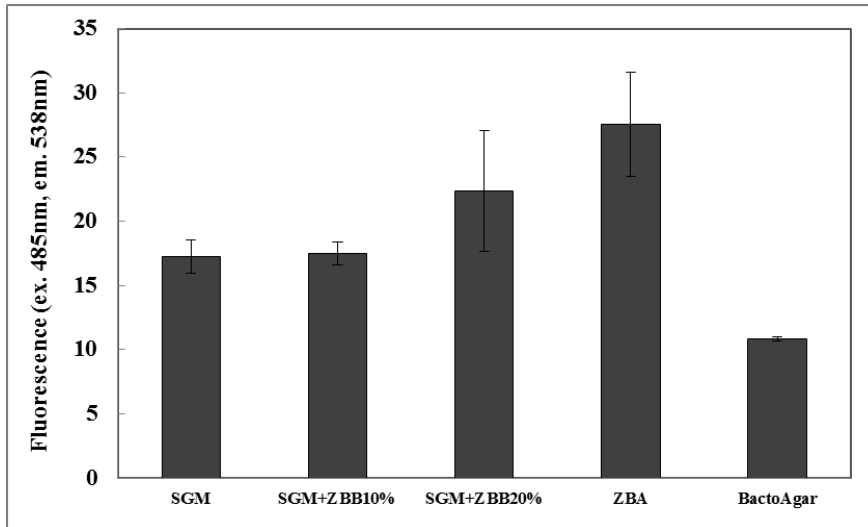
#### 3.4.2. Microalgal

Previous studies have employed benthic diatom microtiter assays to investigate various questions, including heterotrophy (Tuchman *et al.* 2006). However, the use of *in vivo* chl *a* fluorescence to estimate surface-associated diatom populations in microtiter antibiotic assays is novel. The

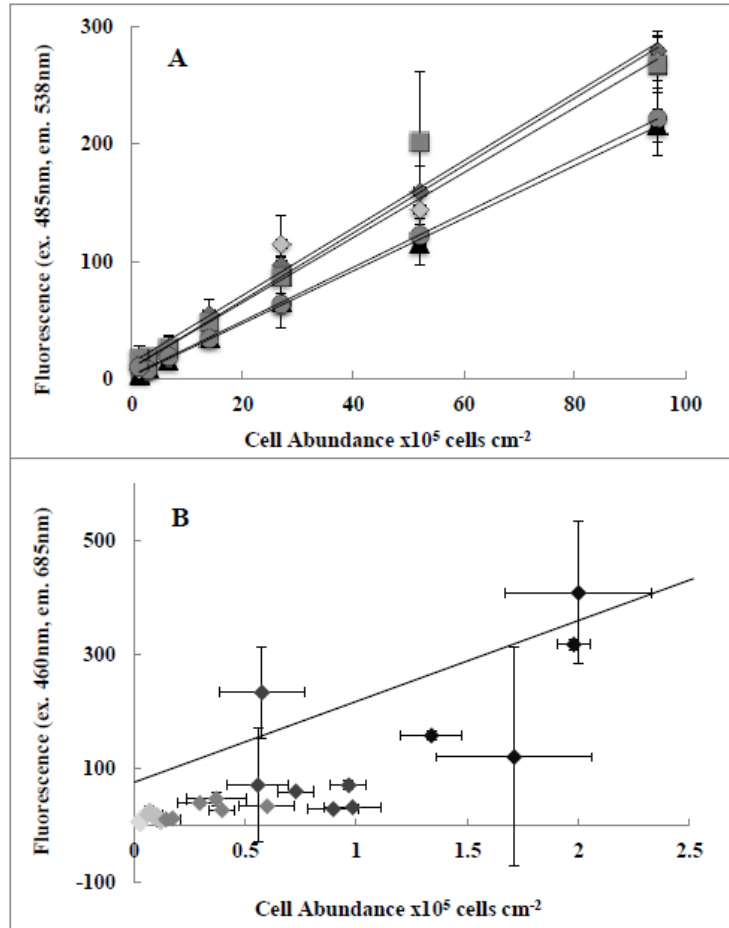
present study demonstrates that microtiter fluorometric assays are useful for quickly and accurately estimating surface associated microalgal population with the relationship between chl *a* fluorescence and population size being linear and statistically significant. Because chl *a* concentrations vary depending on environmental and nutrient conditions, previous studies have avoided linking chl *a* concentration to the number of algal cells, focusing instead upon chl *a* concentration (Lorenzen, 1966; Vyhnalek *et al.* 1993). The consistent growth rate estimations of surface-associated microalgae via fluorescence is therefore significant. And, given the time differential of microscopy counts versus fluorometric quantification of chl *a*, it is worth noting the similarity and consistency between growth rates derived using the two methods. This is extremely useful for investigators using dose-response assays where absolute growth rates are unnecessary and quantification is done using indices based on relative changes between controls and treatments (Halling-Sørensen, 2000; Eguchi *et al.*, 2004).

## CONCLUSION

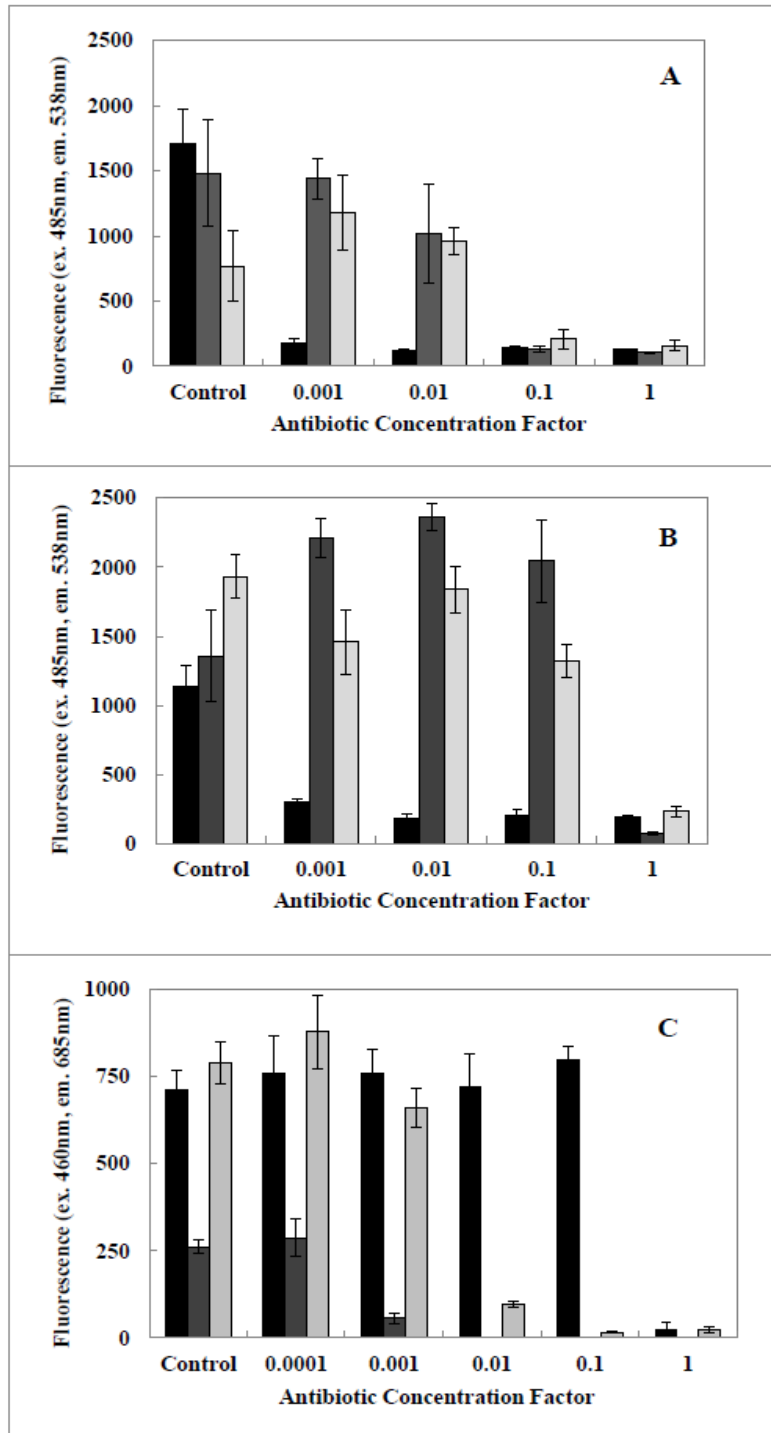
In the present study, surface associated abundance of three microbial species from cultured samples is fluorometrically assessed across broad concentration gradients. Fluorescent staining of clinical and environmental bacterial isolates and the autofluorescence of chl *a* in a benthic microalgae, is employed for quantification of microbial abundance, as well as microalgal growth rates, of surface associated cells in 24 and 48-well microtiter plates. We have shown that *in vivo* chl *a* fluorescence and SYBRGreen, reliably facilitate detection with results showing strong linearity and robust statistical significance. This technique is validated as an inexpensive and high-throughput means of assessing microbial sensitivity to several antibiotic compounds at various concentrations.



**Figure 3.1.** Comparison of auto-fluorescence signal from sterile, un-inoculated surface matrices; (n=7 for all). Error bars are SD.

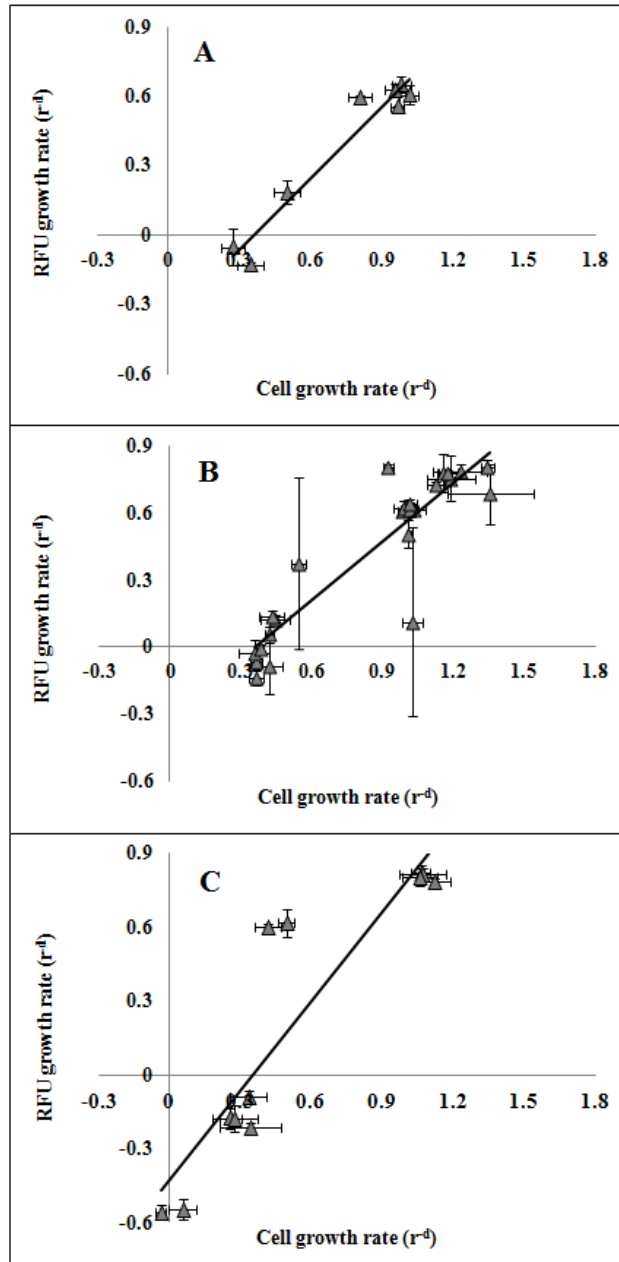


**Figure 3.2.** Linear regression of cell abundances and corresponding fluorescence signals. (A) Surface associated SYBRGreen stained *V. cholerae* N16961; SGM (black triangles),  $y=2.2456x+1.7893$ ; SGM+10% ZBB (grey diamonds),  $y=2.8783x+9.0091$ ; SGM+20% ZBB (light grey diamonds),  $y=2.7726x+9.2517$ ; ZBB (grey boxes),  $y=2.8759x+13.312$ ; BA (grey circles),  $y=2.3051x+2.5036$ . Multiple correlation coefficients are 0.999, 0.997, 0.980, 0.967 and 0.999, respectively;  $p<0.001$  for all surfaces, ( $n=3$ ). SD of the slopes are 73.3, 94.4, 91.8, 97.6 and 76.4, respectively. (B) Chl *a* fluorescence from day 0 (white diamonds) through day 5 (black diamonds) for the benthic diatom *C. closterium* populations in f/2 enriched seawater in 24-well microtiter plates,  $y = 0.0014x - 11.065$ ,  $R^2 = 0.667$ ,  $p < 0.001$ , ( $n=4$ ).



**Figure 3.3.** Antibiotic sensitivity as measured by fluorescence for three microbial isolates in serially diluted antibiotic treatments. For bacterial cultures, antibiotic concentrations start at  $1=3 \times 10^{-2} \text{ g mL}^{-1}$  (ciprofloxacin; black bars),  $1=9 \times 10^{-2} \text{ g mL}^{-1}$  (tylosin; dark grey bars) and  $1=5 \times 10^{-2} \text{ g mL}^{-1}$  (lincomycin; light grey bars); control wells are microbial growth on surfaces free of antibiotic. (A) GSBB01 and (B) *V. cholerae* N16961 in 48-well microtiter plates and treated with antibiotic compounds; (n=3 for each treatment at each dilution factor). Error bars are SD. (C) *C.*

*closterium* at assay termination (hour 71-120 of exposure), antibiotic concentrations start at  $1 = 1 \times 10^{-2} \text{g mL}^{-1}$  (ciprofloxacin; black bars),  $1 = 4 \times 10^{-2} \text{g mL}^{-1}$  (lincomycin; dark grey bars) and  $1 = 2 \times 10^{-2} \text{g mL}^{-1}$  (tylosin; light grey bars); (n=4). Error bars are SD.



**Figure 3.4.** Comparison of *C. closterium* growth rates ( $r^d$ ) in antibiotic exposures and controls as determined by chl *a* fluorescence and microscopy cell counts. With the exception of some few outliers, growth rate estimates under all treatments are consistent and exhibit high coefficients of determination and degrees of significance ( $p < 0.001$  in all cases). Y-axis, RFU ( $r^d$ ) excitation 460nm and emission 685nm. Each panel includes the matching controls: A) growth rates under ciprofloxacin,  $y = 0.8775x - 0.3164$ ,  $R^2 = 0.8201$ ; B) lincomycin,  $y = 1.026x - 0.3703$ ,  $R^2 = 0.932$ ; C) tylosin,  $y = 1.203x - 0.4239$ ,  $R^2 = 0.810$ . Antibiotic treatments ranged from 412-0.0104 g mL<sup>-1</sup>. Error bars are SD.



## CHAPTER 4

### ACID STRESS RESPONSE OF ENVIRONMENTAL AND CLINICAL *VIBRIO CHOLERAE* ISOLATES

## ABSTRACT

*Vibrio cholerae*, the causative agent of the diarrheal disease Cholera, is an opportunistic enteric pathogen commonly isolated from aquatic ecosystems. This bacterium must survive the extreme acidity encountered in the host GI tract in order to colonize the small intestine and cause disease; an acid stress response (ASR) confers this survival. ASR has primarily been investigated in foodborne and clinical enteric pathogens and the ASR capabilities of environmentally derived bacteria remains largely unexplored. Using molecular and culture based techniques, we screened 42 environmental and four clinical *V. cholerae* (*El Tor* N16961, O1, O139 and O395) strains for ASR genotype and phenotype. *In silico* analysis was used to investigate sequence similarity of five ASR genes in *V. cholerae* genomes from various clinical and environmental origins. Our results indicate that the entire subset of ASR genes was detected in 71% of isolates and the ASR regulatory factor (*toxT*) was detected in <7% of isolates; yet, 48% of isolates display an ASR positive phenotype. *In silico* analysis suggests a higher degree of ASR gene sequence similarity among clinical genomes and those environmental genomes derived from Cholera endemic regions. These findings suggest that ASR is not observed only in clinical strains of *V. cholerae* but that ASR gene sequences are highly conserved among clinical *V. cholerae* genomes. This suggests that ASR may contribute to success of *V. cholerae* in the environment and that additional regulatory factors may influence

ASR phenotype. Finally, ASR gene sequence conservation among clinical genomes may influence infectious dose of *V. cholerae*.

## INTRODUCTION

*Vibrio cholerae* is a gram-negative, curved rod bacterium ubiquitously distributed in brackish and freshwater environments (From Jiang et al., 2000; Xu et al., 1985).

Pathogenic *V. cholerae* are responsible for the waterborne diarrheal disease Cholera which can result in daily loss of up to 20L of fluids and has a mortality of ~50% when untreated (Riedle and Klose, 2002). Current estimates report hundreds of thousands of Cholera infections, tens of thousands of deaths and more than one billion dollars (US) in human suffering annually, primarily in developing countries (WHO, 2002).

Cholera outbreaks are seasonal, correlating strongly with regional weather patterns and algal blooms (Epstein, 1993; Islam et al., 1994; Colwell, 1996; Pascual et al., 2000; Collwell and Huq, 2001; Kirschner et al., 2008). Physicochemical parameters such as temperature and salinity have historically been strong determinants of *V. cholerae* growth and distribution in aquatic ecosystems (Kaper et al., 1979; Singleton et al., 1982; Huq et al., 2005). *V. cholerae* is a constituent of autochthonous bacterial communities (West and Lee, 1982) within these systems in free-living forms and associated with copepods, zooplankton and algae (Worden et al., 2006; Lipp et al., 2002) the latter serving as an important pre-conditioning site influencing *V. cholerae* pathogenicity and environmental survival (Nalin et al., 1979; Blokesch and Schoolnik, 2007; Pruzzo et al., 2008).

*V. cholerae* diversity is determined by serotyping cells based on epitope variation in O-antigen lipopolysaccharide (LPS) composition. Strains carrying the O1 antigen have historically been considered the most clinically relevant; non-O1 strains were accepted as having little infectious significance (Yamai et al., 1997; Jiang et al., 2000). Emergence of the O139 strain responsible for the 1992 Cholera epidemic that began in India and spread to neighboring countries spurred heightened interest in the effect of *V. cholerae* genotype on disease causing phenotype as the O139 strain was genetically similar, but distinct, from the O1 strain which was the hallmark of disease (Albert et al., 1993; Nair et al., 1994; Ramamurthy et al., 1993; Jiang 2000).

The increased incidence of cholera-like infections in Calcutta, India prompted Sharma et al. (1998) to evaluate the genotypes of *V. cholerae* from nearby freshwater lakes. They identified *V. cholerae* that did not possess the cholera toxin genes (ctxAB) yet were capable of causing disease symptoms. A separate study isolated non-O1/O139 *V. cholerae* strains from Australian water where Cholera is not endemic. They report no correlation with detection of virulence factors and the ability of the isolates to induce Cholera symptoms in infant mice (Islam et al., 2013).

*V. cholerae* populations isolated from the Chesapeake Bay have been observed as experiencing seasonal shifts in community structure linked to environmental conditions (Jiang et al., 2000). Additional studies have focused on temporal succession of genotype diversity in environmental *V. cholerae* populations from California (Jiang et al., 2003). Jiang et al. (2003) argue that the cholera toxin (ctxA) has an environmental origin due to unexpectedly high frequency in the detection of this gene in the isolates derived from this non-epidemic region. Further studies have detected presence of virulence and antibiotic

resistance genes, and genes involved in the regulation thereof, in high frequency among environmental isolates (Goel et al., 2007; Zo et al., 2008; Goel and Jiang, 2010). While reported cases of Cholera are uncommon in developed countries, several studies have isolated El Tor strains of *V. cholerae* from freshwater, brackish and marine ecosystems in California, the Chesapeake Bay, Asia and Africa (Jiang et al., 2000; Nair et al., 2006; Goel et al., 2008). Amplified fragment length polymorphism (AFLP) of *V. cholerae* from endemic regions reveals toxigenic strains that appear to be derived from environmental strains (Jiang et al., 2000). This is supported by molecular characterization of environmental *V. cholerae* isolates observed to have a high degree of genome similarity compared to some clinical strains (Hang et al., 2000). Yet several studies reveal high sequence variability in select genes of isolated strains (Dziejman et al., 2005; Nair et al., 2006; Ray et al., 2009; Haley et al., 2010).

The uncertainty of how *V. cholerae*, which is commonly isolated from aquatic ecosystems, is capable of causing disease at pandemic scales was addressed with the discovery that *V. cholerae* is capable of mounting an acid stress response (ASR; Merrell et al., 2001; Merrell and Camilli, 2000; Merrell and Camilli, 2002). ASR is an adaptive feature that facilitates survival of the bacterium during transit through the variable acidity of the mammalian host GI tract. This phenomenon involves rapid induction of biochemical and physiological changes upon exposure to extreme acidity (pH 1-4) after pre-conditioning in sub-lethal acidity (pH 4-5). ASR provides cross-protection to other forms of stress (Rallu et al., 2000; Frees et al., 2003; Xie et al., 2004) and may activate additional adaptive responses such as virulence, biofilm formation, chemotaxis and antibiotic resistance (Leyer and Johnson, 1992; Merrell et al., 2001; Merrell and Camilli,

2002; Poloen et al., 2003; Maurer et al., 2005; Butler et al., 2006; Hayes et al., 2006). ASR has been investigated using model opportunistic enteric pathogens in laboratory settings and in the context of food production (i.e., fermentation) where it has been shown to vary by organism, acid (organic vs. inorganic), stage of growth (logarithmic vs. stationary) and may be elicited by various types of growth media and environmental cues (Lin et al., 1996; Foster, 1999; Koutsoumanis et al., 2003). The biochemical and physiological mechanics of ASR have been investigated in pathogens such as *Escherichia coli* and *Salmonella enterica* (reviewed in Swenson et al., 2013) yet reports on *V. cholerae* ASR, especially in an environmental context, are limited.

*V. cholerae* ASR has primarily been investigated in nutrient rich laboratory cultures (Merrell et al., 2001; Merrell and Camilli, 2002). These studies demonstrate that *V. cholerae* ASR involves rapid protein translation variability which bolsters virulence. Acid adapted *V. cholerae* N16961 demonstrated increased competition in the infant mouse model as compared to acid intolerant isolates. Signature tagged mutagenesis (STM) studies reveal that inducing mutation in known ASR factors reduces the ability of *V. cholerae* to elicit ASR (Merrell and Camilli, 2002).

The distribution and conservation of genes involved in ASR among clinical and environmental *V. cholerae* isolates remains unexplored. This study aims to assess detection frequency of five genes (*recO*, *cpdA*, *mrsA*, *hepA*, *nqrA*) central to *V. cholerae* ASR (Merrell and Camilli, 2002), four ASR regulatory factors (*toxT*, *toxR*, *ompU*, *ompW*) and genes encoding the cholera toxin (*ctxA*, *ctxB*) among four clinical and 42 environmental *V. cholerae* isolates. This is complimented by *in silico* assessment of

these genes among 43 *V. cholerae* and 8 additional *Vibrio* genomes from clinical and environmental origins.

With few exceptions (Waterman and Small, 1996; Bhagwhat, 2005), the response of environmentally derived bacterial isolates to lethal acidity remains unexplored. To our knowledge no studies have surveyed such a broad collection of clinical and environmentally derived *V. cholerae* isolates for known ASR factors nor has the sequence similarities of these ASR genes been evaluated in sequenced *V. cholerae* genomes. As such, it is the aim of this project to test the hypotheses that a) this subset of ASR genes will be detected in greater frequency among the clinical strains of *V. cholerae*, b) clinical *V. cholerae* will be more capable of mounting an ASR and c) clinically derived *V. cholerae* genomes will have greater sequence similarities in the ASR genes than will environmental genomes. The findings from this study will be important in understanding the role of ASR in pathogenicity of clinical *V. cholerae* strains and will be suggestive of the role of ASR in survival of *V. cholerae* in the environment.

## MATERIALS AND METHODS

**Bacterial Culturing.** All environmental *Vibrio cholerae* isolates were the generous gift of Sunny Jiang (UCI) and clinical strains of *V. cholerae* were obtained through Farooq Azam (SIO, UCSD). Fresh source plates were maintained for all clinical and environmental isolates by scratch inoculating from frozen stock (-75°C) onto Luria Broth Agar (LBA; 1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Agar, 1L distilled H<sub>2</sub>O) and incubating overnight at 30°C.



**Molecular Detection of ASR Genes.** DNA template of each isolate was prepared by transferring biomass from a singular bacterial colony into 200µl tubes containing 40µl of the commercially available lysing detergent, Lyse-and-Go™ (Thermo Scientific; USA). Samples were briefly vortexed, loaded onto Mastercycler ep gradient thermalcyclers (Eppendorf; Germany) and run through cycling parameters specified by the Lyse-and-Go™ manufacturer instructions. DNA template was stored at -20°C until polymerase chain reaction (PCR) analysis.

PCR was employed to screen all *V. cholerae* isolates for the presence of genes involved in acid stress response, environmental sensing, transcriptional regulation of ASR and the Cholera toxins (**Table 1**). 10µl PCR reactions were carried out using a Mastercycler ep gradient. Reaction mixtures contained Quiagen Hot Start Blue Master Mix 1.5mM MgCl<sub>2</sub> (1.0x final concentration), 2µM of the respective forward and reverse primers (**Table 2**), 2µl of Lyse-N-Go™ DNA template suspension and PCR grade water to volume. Cycling parameters were as follows: 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, followed by a final extension step of 72°C for 10 min. Detection of ompU and ctxA were determined using the multiplex technique, using the same reaction concentrations and cycling parameters as previously described. PCR products were loaded into 1.5% Omnipure PCR Plus Agarose (Millipore; Germany) gels and run at 100V for 45 minutes to ensure ample band migration. Gel images were visualized using Lab Works (Cambridge, UK) and inspected visually for detection of target amplicons. The presence of the gene of interest was scored as positive if the amplicon was detected twice. Negative results were scored during initial gel run and validated upon at least two subsequent investigations. Any

ambivalent results were re-evaluated until positive or negative confirmation was achieved. Adequate DNA template was confirmed at initiation and termination of PCR by amplifying the highly conserved rDNA gene, 16S, with bacteria specific primers validated by Frank et al. (2008).

**Organic Acid Challenge.** Fresh cultures of *V. cholerae* isolates were grown individually in 10mL of Luria Broth (LBB; 1% tryptone, 0.5% yeast extract, 1% NaCl, 1L distilled H<sub>2</sub>O; pH 7) at 30°C with aeration. Overnight cultures of each isolate were partitioned into 2 x 2mL samples for use during organic acid challenge. Prior to the acid challenge assays, which follow the protocol of Merrell and Camilli (2002), a 10x organic acid cocktail (87 mM acetic acid, 25 mM butyric acid, 37 mM propionic acid) was prepared in LBB. Cells were pelleted by centrifugation (10,000g; 10 min) and supernatant was aspirated. Cells were organic acid adapted by re-suspending the pellets in the pre-conditioning broth comprised of LBB adjusted to a pH of 5.7 with 16N HCl and amended with 0.75x organic acid cocktail (6.5mM acetic acid, 1.9mM butyric acid; 2.8mM propionic acid; final pH 4.5). During acid adaptation, the cultures incubated at 30°C with aeration for 1.5 hours. Unadapted cells incubated in LBB pH 7 in parallel. Post-adaptation, cells were pelleted (10,000g; 10min) and resuspended in acid shock broth. The organic acid shock broth was comprised of LBB adjusted to pH 4.5 with 16N HCl and amended with 1x organic acid cocktail (8.7mM acetic acid, 2.5mM butyric acid; 3.7mM propionic acid; final pH 3.5). Cells were exposed to lethal acidity for 1 hour at 30°C with aeration. Unadapted cells were pelleted via centrifugation and transferred directly from LBB pH 7 into acid shock broth for 1 hour at 30°C with aeration.

Prior to the organic acid challenge, 48 well microtiter plates by transferring 500µl of LBA into each well and allowing overnight solidification. During organic acid challenge, subsamples were transferred along a 1 hour time course at 0, 30 and 60 min. At each time point, 100µl subsamples of broth cultures were diluted 1:10 in isotonic sterile saline to reduce the culture acidity prior to transfer to microtiter plates. 15µl subsamples of these dilute cell suspensions were transferred in triplicate into 48 well microtiter plates. Inoculated microtiter plates incubated overnight at 30°C. The density of the overnight biomass was assessed the following day fluorometrically.

**Detection of *V. cholerae* ASR.** To assess the response of adapted and unadapted *V. cholerae* isolates to extreme acidity, a fluorometric assay was employed as per Swenson et al. (2012), with minor modifications, to determine the density of the overnight cultures. Modifications to the method for optimization involved reducing the transferred culture volume to enhance sensitivity of biomass estimation. The density of post-acid shocked *V. cholerae* isolate biomass was determined by staining cultures in microtiter plates with the nucleic acid fluorescent stain, SYBR®Green (FC 1x; Invitrogen; USA). These samples incubated for 15 minutes at 20°C in the dark. Densities of unadapted and acid adapted isolates was quantified on a Spectramax Gemini EM plate reader (Molecular Devices; USA), using Softmax Pro software (Molecular Devies; USA). Each isolate (adapted and unadapted) was screened in triplicate at each time point and the experiment was conducted in duplicate. Additionally, isolates that had not been shocked (i.e., growth maintained in LBB pH7) were plated on LBA in triplicate and in parallel with acid shocked isolates to ensure the viability of cells prior to acid exposure during assay. ASR was determined by setting thresholds of control-corrected samples which were

representative of the ASR query (*V. cholerae* N16961) such that post shock growth at  $T_{30}$  was  $\geq 20\%$  of the  $T_0$  value and  $T_{60}$  was  $\geq 15\%$  of the densities observed at  $T_0$  for the isolate. After each reading, samples were visualized to ensure the presence of bacterial biomass.

**Genomic Fingerprinting of *V. cholerae* Isolates.** Enterobacterial repetitive intergenic consensus sequencing PCR (ERIC-PCR) of clinical and environmental *V. cholerae* isolates was performed as described in Rivera et al. (1995) and with primer sets (**Table 1**), cycling parameters and condition modifications described in Jiang et al. (2003). One modification employed in the present study involves use of the cell lysis detergent, Lyse-N-Go™ to harvest the DNA from the *V. cholerae* isolates which subsequently served as the DNA template for ERIC-PCR. A 1-kb DNA stepladder (Phenix) was used in multiple places within gels for normalization between gels. *V. cholerae* isolates were grouped into neighboring gel lanes according to empirically determined similarity of banding patterns during preliminary ERIC-PCR analysis. All isolates were run in 2% high resolution agarose gel. Digitized fingerprints of amplicon banding patterns were analyzed using LabWorks; the target lanes and bands were automatically detected and manually refined. Banding pattern comparison for hierarchical clustering was used to create a similarity matrix according to the similarity coefficient defined by Jaccard. The dendrogram was created in IBM SPSS Statistics 20 using Wards clustering method according to squared Euclidean distances.

***In Silico* Genotyping.** *In silico* analysis was conducted using completed *V. cholerae* genomes available at the time of analysis (March 2012) in the National Center for Biotechnology Information (NCBI) genome database. The query nucleotide sequence of

each ASR gene was derived from the clinical isolate *V. cholerae* N16961. This query sequence was then compared to a library of completed *V. cholerae* genomes using a custom search on NCBI's nucleotide blast. Genomes that reported greater than 90% query coverage were included in the results. These isolates' Max ID to the query sequence was then included in a heat map displaying the percent identity of genes matching the query sequence (**Table 3**).

Phylogenetic similarities of *V. cholerae* genomes used in this study were demonstrated using Multi Locus Sequence Typing (MLST) by concatenating nucleotide sequences for the housekeeping genes *recA*, *rpoA* and 16S rDNA which were extracted from the *Vibrio cholerae* genomes used in the *in silico* assessment using PATRIC and NCBI's genome library. After concatenation, the nucleotide sequences for each of these housekeeping genes from each genome were stored as FASTA files for sequence alignment. The nucleotide sequences were entered into Molecular Evolutionary Genetics Analysis (MEGA 5) software and aligned by Clustal W. After sequence alignment, a neighbor-joining tree was constructed with the MEGA software, using complete deletion to remove error introduced by gaps and using 600 iterations.

## RESULTS AND DISCUSSION

### **ASR Genotyping of *V. cholerae* Isolates**

Our findings suggest a high frequency of detection among the clinical and environmental *V. cholerae* isolates screened for the subset of genes involved in ASR. However, there was no distinct genotype/phenotype relationship when considering the ASR genes as had previously been determined central to the ASR capability of *V. cholerae* N16961 (Merrell and Camilli, 2002). *V. cholerae* isolates in which some ASR genes were not detected

demonstrated an ASR positive phenotype (i.e., SJ 72). The entire subset of ASR genes was detected in 33/46 (71%) isolates with nearly half (48%) of all isolates exhibiting an ASR positive phenotype (**Table 3**). Of the 4 clinical and 42 environmental *V. cholerae* isolates screened in the present study, only two isolates (4%), the clinical strains N16961 and O1, were positive for every ASR gene of interest. Of the remaining clinical strains, *V. cholerae* O395 tested positive for 55% of the genes of interest but was unable to mount a detectable ASR. *V. cholerae* O139 tested positive for only 1/11 (9%) genes of interest and exhibited an ASR negative phenotype. All 46 isolates tested positive for at least one ASR gene. The gene expressing phosphoglucomutase (*mrsA*) hypothesized to function in cell wall maintenance was detected in 93% of isolates. The DNA repair mechanism gene (*recO*) and the transcriptional regulator gene (*cpdA*) were detected in 91% of the isolates. An additional gene coding for transcriptional regulator (*hepA*) and the *nqrA* gene, which is involved in Na<sup>+</sup> homeostasis, were detected in 89% of the isolates screened.

When pooling the percentage of genes detected in the isolates, we identified genotype/phenotype trend whereby a larger fraction of isolates testing positive for ASR genes displayed ASR positive phenotypes than did those with a smaller percentage of the ASR genes (**Fig.1**). The two clinical isolates, N16961 and VcO1, tested positive for all ASR genes; both isolates (100%) expressed and ASR positive phenotype. Greater than 80% of the genes were detected in 13 isolates (28%). 61% of these isolates were capable of ASR. Sixteen isolates (35%) tested positive for 70-80% of genes, 63% of these isolates mounting an ASR. 8 isolates (17%) tested positive for 60-70% of the genes; one of these isolates was capable of ASR. 3 isolates (7%) had 55% of the genes, 2 isolates

(4%) tested positive for 45% of genes and 2 isolates (4%) had <36% of the genes. Of the seven isolates which tested positive for < 50% of the genes, six isolates (86%) exhibited an ASR negative phenotype.

As the original hypothesis to be tested was that clinical strains of *V. cholerae* would test positive for a greater number of the ASR genes of interest than the environmental strains, we were interested to find that geographic origin of isolation does influence ASR capability of *V. cholerae* in a statistically significant manner (ANOVA,  $p < 0.001$ ). The organic acid challenge reveals that 50% of the clinical strains (2/4) had a demonstrable ASR. 40% of the isolates (7/19) from San Juan Creek mount an ASR. 50% of isolates (3/6) from San Diego Creek were shown capable of ASR. By contrast, 70% of isolates (5/7) from regions within Newport Bay and 100% (5/5) of isolates from the UC Irvin Crew dock demonstrated the capacity to survive exposure to lethal acidity presumably by way of ASR. These findings are consistent with previous work identifying *V. cholerae* pathogenic genotype and phenotype being influenced by environmental parameters (Keymer et al., 2007).

Regarding the regulatory factors and outer membrane porins involved in ASR regulation, *V. cholerae* N16961 and O1 were found to have all four of genes (ompU, ompW, toxT, toxR). 19 isolates (14%) were found to have 75% of these genes; 63% of these isolates were capable of adapting and surviving lethal acidity. 15 isolates (32%) contained at least two of these regulatory genes and seven of these isolates (47%) were capable of ASR. 7 isolates (15%) tested positive for the presence of one of these genes; one of these isolates survived lethal acidity. 2 of the isolates (4%) screened contained zero of these four regulatory agents; neither of these isolates were capable of surviving

lethal acidity. Of these regulatory factors, *ompW* and *toxR* were detected in 70% and 83% of all isolates, respectively. However, *ompU* and *toxT* were detected in 63% and only 6.5% of all isolates, respectively. While not strictly ASR factors, as their transcription is independent of acid exposure, these outer membrane porins and transcription regulators have previously been shown to be central to *V. cholerae* ASR (Li et al., 2000; Provenzano and Klose, 2000; Peterson, 2002; Reidl and Klose, 2002; Matson et al., 2007). As such, our findings suggest that while impairment of these genes may also impair an isolates response to stress, it is not a strict determinant of the isolates ability to respond.

The fact that ASR genotype was not a determinant of ASR positive phenotype was initially suprising in that the subset of ASR genes was selected due to previous work demonstrating that mutations within these genes greatly reduced *V. cholerae* N16961 ASR capability in the infant mouse model (Merrell and Camilli, 2002). Further, the transcriptional regulators (*toxT*, *toxR*) have been shown to be important regulators of ASR among other virulence pathways in *V. cholerae* and other enteric pathogens (Miller and Mekalanos, 1992; Cotter and DiRita, 2000; Merrell et al., 2001). The identification of ASR positive phenotypes independent of the full suite of ASR genes may be the result of additional factors governing ASR. Genes coding for the toxin co-regulated pili (TCP) have been shown imperative in *V. cholerae* survival in humans and animal models (Herrington et al., 1988) and mutations in these genes have been shown to interfere with *V. cholerae* ASR (Merrell and Camilli, 2002). Such genes were not tested in the present study. Additional regulatory factors such as the transcriptional regulators *rpoS* and *dksA* have been shown to govern stress response pathways to an extent that mutations attenuate



colonization of enteric pathogens in the infant mouse model (Turner et al., 1998; Merrell et al., 2000). Indeed, the alternate sigma factor *rpoS* has been demonstrated as conserved among  $\gamma$ -proteobacteria (Hengge-Aronis, 2000; Venturi, 2003) and has been shown to moderate the response of *Vibrios* to various forms of stress including extreme acidity (Yildiz and Schoolnik, 1998; Lin et al., 2002; Hulsmann et al., 2003; Joelsson et al., 2007). In a study by Bhagwat (2005), environmental *E. coli* isolates were rescued from acid sensitivity by insertion of a plasmid carrying the *rpoS* gene. And, in one of the most comprehensive studies to date on *E. coli* ASR, mutations in the *rpoS* gene resulted in acid sensitivity in nearly 20% of isolates tested (Waterman and Small, 1996). Additional outer membrane proteins (e.g., *ompT*) have been shown to influence survival of *V. cholerae* in the infant mouse model (Provenzano and Klose, 2000) and factors such as *dnaK*, which is a heat shock protein (HSP; Lindquist and Craig, 1988), play a significant role in regulation of stress and virulence (Young and Elliot, 1989; Sahu et al., 1994). The presence of such factors in the isolates screened in this study may be instrumental in the observed ASR phenotype and warrant further investigation.

Of all isolates screened for the presence of the virulence factors (*ctxA*, *ctxB*), only N16961 and O1, tested positive for the presence of both. In addition, these clinical strains were the only isolates testing positive for the alpha subunit of the cholera toxin (*ctxA*), which is responsible for eliciting the symptoms of Cholera (Riedl and Klose, 2002). The B subunit of the cholera toxin (*ctxB*), which binds the epithelial cells in the mammalian small intestine, was detected in 36 isolates (78%). 9 isolates (20%) were negative for the detection of both *ctxA* and *ctxB*. The reduced detection of *ctxA* may be due to the observation that *ctxA* and *ctxB* are detected in *V. cholerae* in 2 and 3 different

sequence types, respectively (Kaper et al., 1982; Nair et al., 2005; Hassan et al., 2010). Further, the *ctxB* sequences have been shown to vary significantly among environmental *V. cholerae* isolates but are effectively detected using the PCR primer set employed in this study (Jiang et al., 2000).

There remains the possibility that lack of primer specificity may contribute to our findings. It has been observed that many *V. cholerae* genes differ significantly in sequence composition which would hinder our ability to detect them using one primer set. As such, it is possible that these isolates may possess the genes targeted (i.e., *toxT*) in sequences undetectable by our primers and these genes may be instrumental in the observed ASR positive phenotype despite the detected ASR negative genotype. Nevertheless, the initial aim of this study was to detect the ASR genes as they have been described in *V. cholerae* N16961, the only *V. cholerae* isolate to date in which ASR had been investigated. Future studies should employ multiple primer sets to detect genes present with varied sequences but involved in *V. cholerae* ASR nonetheless.

### **ERIC-PCR Genome Fingerprint**

The ability of these *V. cholerae* isolates to adapt and survive exposure to lethal acidity was not attributed to ASR genotype using the five ASR genes and the four regulatory factors as has been previously suggested (Provenzano et al., 2000; Merrell et al., 2001; Merrell and Camilli, 2002). Molecular characterization studies have identified genomic fingerprint distinctions of virulent *V. cholerae* serovars that had previously been considered identical based on sequencing individual genes (Popovic et al., 1995; Choudbury et al., 1994). As such, we applied the enterobacterial repetitive intergenic

consensus (ERIC) sequence technique to the clinical and environmental *V. cholerae* isolates to determine if there was a correlation between genomic fingerprinting and observed ASR phenotypes. DNA fingerprints of these isolates reveal variability in genomic banding pattern similarities among ASR positive isolates with the dendrogram revealing 4 major clusters (**Fig.2**). While the clinical isolates used in the study cluster together, as did the isolates with shared origins and ASR capability, there is a distribution of ASR positive and negative populations throughout the dendrogram such that there is no discrete clustering of ASR positive isolates observed. This suggests that ASR phenotype is not exclusively due to a shared genotype observable through ERIC-PCR despite this techniques demonstration as excellent for use in bacterial classification and molecular typing (Dijkshoorn et al., 1996; Janssen and Dijkshoorn, 1996). Indeed it has been reported that *V. cholerae* isolates with nearly identical ERIC-PCR banding patterns differ with respect to virulence (Jiang et al, 2003).

### ***In Silico* Analysis and Multi-Locus Sequence Typing (MLST)**

To characterize sequence similarities of the ASR genes used during PCR screening, *in silico* analysis was conducted on 43 whole genome *V. cholerae* sequences and eight additional *Vibrio* genomes available at the time of access through the National Center for Biotechnology Information (March 2012; NCBI). Our results suggests a higher degree of ASR sequence similarity among clinical *V. cholerae* genomes or genomes isolated from environments where Cholera is endemic (**Table 4**). Stastical analysis reveals an association of ASR gene sequence similarity and genome origin (Pearson's chi-squared; 4,  $N=192.7$ ;  $p<0.001$ ). 54% of these clinical genomes or genomes originating in Cholera endemic regions, showed 100% sequence similarity to the *V. cholerae* N16961 query for

all ASR gene sequences. By comparison, 0% of environmentally derived genomes, genomes with undocumented origins or additional *Vibrio* genomes displayed complete sequence similarity of all seven ASR genes surveyed as compared to the query. The reduced sequence similarities observed for these ASR genes among environmental *V. cholerae* genomes and additional *Vibrio* genomes is of epidemiological interest in that, despite the limited sequence similarities observed in the present study, the waterborne pathogens *V. vulnificus* and *V. parahaemolyticus* have both been demonstrated as capable of robust ASR (Alvarez et al., 2003; Bang and Drake, 2005; Rhee et al., 2006; Chiang et al., 2012). It is likely that the sequence conservation of genes involved in ASR contributes to the infectious dose of pathogenic *Vibrio* species as ability to respond to lethal acidity within the mammalian GI tract has been linked to the concentration of cells necessary to cause disease (reviewed in Merrell and Camilli, 2002).

Of the genes surveyed, ompU was observed as having the greatest sequence variability among all surveyed genomes with 54% of all *V. cholerae* genomes used having <100% similarity to the query sequence. Additionally, 20% of genomes surveyed displayed ompU sequence similarities <75% of the query. Substantial sequence variability was also observed for recO and hepA. 42% of the *V. cholerae* genomes displayed recO similarities <95% compared to the query. 63% of the additional *Vibrio* genomes were shown to have sequence similarities <75% the query for all ASR genes. The sequence similarities for hepA and recO were nearly identical in *V. cholerae* genomes with 42% of genomes having similarities <95% identical to the query. Among the *V. cholerae* genomes, there was little variability in sequence similarity for the remaining ASR genes.

The sequence variability observed for *recO*, *hepA* and *ompU* is likely due to the function of these genes within the cell. Variability in genes coding for DNA repair proteins, such as *recO*, has been shown vary greatly which confers enhanced bacterial success in response to stress (Foster, 1991). The gene expression of DNA repair mechanism which functions with high fidelity, such as *recO*, has been shown to decrease in response to environmental stress and DNA repair mechanisms with reduced fidelity increase in expression. As such, it is plausible that the onset of stress induces genetic mutations (damage DNA) which, when unrepaired, may result in increased success of the progeny. Alternate regulatory factors such as *hepA* are commonly employed among opportunistic pathogens in response to various forms of environmental stress and have been shown to vary among these populations (Qi et al., 1996; Nonaka et al., 2006). Finally, as an outer membrane porin, *ompU* likely experiences sequence selection pressures in response to the different environmental conditions the cell encounters. And, while not an ASR gene *per se*, as its expression has not been cited as increasing exclusively as a result to exposure to acidity, the cells ASR capability is reliant on operation of *ompU* in ASR regulatory cascades (Merrell et al., 2001).

MLST analysis of the *V. cholerae* genomes reveals a clustering of nearly a quarter of the complete genomes. However there is no distinct clustering according to clinical designation or origin of genome isolation (**Fig. 3**). All four major categories of isolation origin are distributed throughout the dendrogram and there is no distinct clustering based on geographic origin of isolation. However, approximately 1/3 of the genomes used for *in silico* analysis (HC and HE series of genomes) were isolated at approximately the same

time from locations within Haiti and the high degree of sequence similarity in these genomes has been reported (Huang et al.; 2010).

## CONCLUSION

The aim of the present study was to explore whether clinical or environmental *V. cholerae* isolates were more capable of an ASR. Our findings suggest that ASR is an adaptive feature present in clinical and environmental *V. cholerae* isolates. For while two of the four clinical strains, *V. cholerae* N16961 and O1, were the only isolates to test positive for 100% of this subset of ASR genes, and both were capable of a robust ASR, a majority of the ASR genes were also detected in the environmental isolates and half of all the environmental isolates displayed an ASR positive phenotype. A majority of *V. cholerae* isolates tested positive for the regulatory factor (toxR) and outer membrane porins (ompW, ompU) for which they were screened. However, detection of these genes in the isolates screened was not a determinant of an ASR positive phenotype, nor was the absence of these genes. We argue that additional genes may be present in these isolates which facilitate the cells ability to respond to lethal acidity. Further, we suspect that sequence variation in the genes of interest among these isolates may interfere with detection of some significant regulatory factors (i.e., toxT) instrumental in *V. cholerae* ASR. The observed increase in the sequences of ASR genes among clinical genomes and genomes isolated from Cholera endemic regions may contribute to the observed infectious dose of these isolates which to our knowledge has not been reported. These findings are significant in that despite not testing positive for the ctxA toxigenic gene, the ability of these cells to potentially survive extreme acidity in the mammalian host may elicit an immune response and Cholera-like symptoms as is consistent elsewhere.

Further, transit through the mammalian host has been shown to increase virulence of *V. cholerae*. The ability of these cells to survive extreme acidity may therefore be an important factor guiding future virulence of these environmental isolates. As such, observation of ASR positive phenotypes in environmentally derived *V. cholerae* has potentially valuable implications in the ecology and evolution of infectious disease.

**Table 4.1.** Proposed function of genes involved in acid stress response (ASR) screened for during molecular genotyping of *V. cholerae* isolates used in this study. Possible role in ASR was proposed by Merrell and Camilli (2002) based on mutagenic investigations of these genes.

Gene	Common Name	Possible Role in ASR
hepA	HepA	Transcriptional Regulation
mrsA	Phosphoglucomutase	Maintenance of cell wall/LPS structure/phosphomannomutase
nqrA	NADH:ubiquinone subunit	Na <sup>+</sup> homeostasis
recO	RecO	DNA repair
cpdA	cAMP phosphodiesterase	Regulation of cAMP levels/transcriptional regulation
ompU	OmpU	outer membrane porin, environmental sensing, regulation of ASR
ompW	OmpW	outer membrane porin, environmental sensing, regulation of ASR
toxT	ToxT	Transcription Regulation
toxR	ToxR	Transcription Regulation
ctxA	Cholera toxin A	virulence factor, intestinal colonization, symptoms of Cholera
ctxB	Cholera toxin B	virulence factor; intestinal colonization



**Table 4.2.** Details of primers used to target and amplify ASR genes during PCR screening of *V. cholerae* isolates. For each primer set, the top sequence is the forward primer and the bottom sequence is the reverse.

Target	Sequence (5' - 3')	Amplicon Size (bp)	Tm <sup>a</sup> (°C)	References
16S	GTACACACCGCCCGT TACCTTGTTACGACTT	100	43.3 57.4	Frank et al., 2008
recO	GGTTTGCCGCCAAGATACGGCT GCGCGGAAAACGCTCCAACC	326	59.9 55.3	Merrell and Camilli, 2002
hepA	CGCAGGATAAGTTGTTTG GTTGCAGCGTTTCAGGTA	306	55.3 57.6	Merrell and Camilli, 2002
cpdA	ACCCGAGTCTTATCAACG GACGACATCCCAAAAACG	361	57.6 57.6	Merrell and Camilli, 2002
mrsA	ACCGGACCAATGCCAACG AAGCTTCAAACCAGATAG	306	62.2 53.1	Merrell and Camilli, 2002
nqrA	ATGCGATCAAAACTCAGC GATGTGGGCCGTCAAAGA	300	55.3 59.9	Merrell and Camilli, 2002
ITS	GTCGTAACAAGGTAGCCGTA GCCAAGGCATCCACC	Variable <sup>b</sup>	54.1 53.8	Cardinale et al., 2004
ompU	ACGCTGACCGGAATCAACCAAAG GCGGAAGTTTGGCTTGAAGTAG	869	62.7 62.7	Kumar et al., 2009
ompW	GGTAGCCTTGGTGATATTGGTG TAGCAGCAAGTCCCCATGAGT	208	62.7 62.6	Goel et al., 2010
toxT	ACTGTATAGCAAAGCATATTCAGAGA CAGTGATACAATCGAAAATAGGATC	1,115	59.9 59.7	Mukhopadhyay et al., 2001
toxR	CCTTCGATCCCCTAAGCAATAC AGGGTTAGCAACGATGCGTAAG	779	62.7 62.7	Kumar et al., 2009
ctxA	GATACACATAATAGAATTAAGGATG GGTTGCTTCTCATCATCGAACCAC	461	56.4 64.6	Olsvik et al., 1993
ctxB	GCCGGGTTGTGGGAATGCTCCAAG GCCGGGTTGTGGGAATGCTCCAAG	536	69.7 66.2	Goel et al., 2007
ERIC	ATGTAAGCTCCTGGGGATTAC AAGTAAGTGACTGGGTGAGCG	Variable <sup>b</sup>	62.7 62.6	Rivera et al., 1995

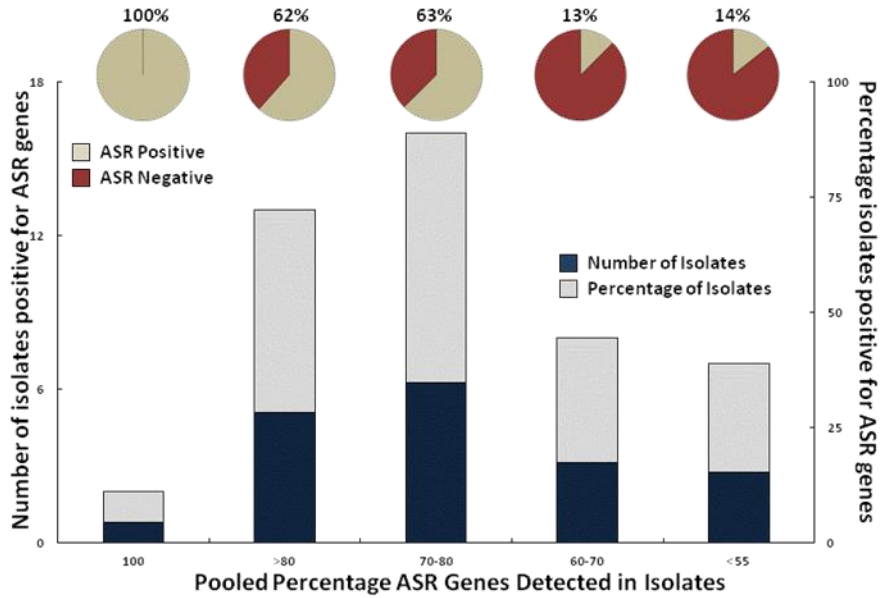
<sup>a</sup> Melting temperature  
<sup>b</sup> Amplicon size varies according to strain

**Table 4.3.** ASR genotype and phenotype of clinical and environmental *V. cholerae* isolates used for molecular screening of ASR factors and the ability of the isolates to mount ASR. Environmental strains are primarily uncharacterized isolates collected from aquatic ecosystems throughout California. ASR genotype is reported as + = presence, - = absence for gene of interest. Phenotype is reported as + = positive, - = negative for ability to mount ASR.

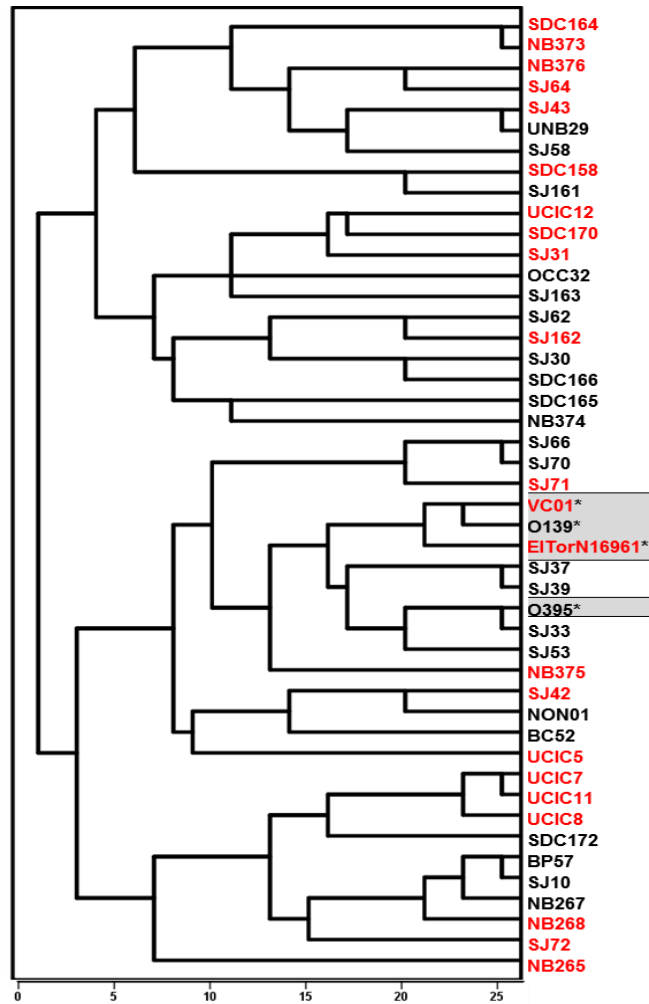
Origin of Isolation	Strain Designation	Targeted Genes of Interest									ASR		
		ASR Genes					Virulence		Regulatory Factors				
	Clinical	<i>recO</i>	<i>hepA</i>	<i>cpdA</i>	<i>mrsa</i>	<i>nqrA</i>	<i>ctxA</i>	<i>ctxB</i>	<i>ompU</i>	<i>ompW</i>	<i>toxT</i>	<i>toxR</i>	
	El Tor N16961	+	+	+	+	+	+	+	+	+	+	+	+
	VCO1	+	+	+	+	+	+	+	+	+	+	+	+
	O139	-	+	-	-	-	-	-	-	-	-	-	-
	O395	+	+	+	+	-	-	-	-	-	+	+	-
	Environmental												
	NON-O1	+	+	+	-	-	-	-	+	-	-	-	-
<b>Big Caynon</b>	BC52	+	+	+	-	-	-	+	+	-	-	+	-
<b>Bolboa Pier</b>	BP57	+	+	+	+	+	-	-	+	+	-	+	-
<b>San Juan Creek</b>	SJ10	+	+	-	+	+	-	+	-	+	-	+	-
	SJ30	-	+	+	+	+	-	+	+	-	-	+	-
	SJ31	+	+	+	+	+	-	+	+	-	-	+	+
	SJ33	+	+	+	+	+	-	+	+	-	-	+	-
	SJ37	+	+	+	+	+	-	+	+	+	-	-	-
	SJ39	+	+	+	+	+	-	+	+	+	-	+	-
	SJ42	+	+	+	+	+	-	+	+	+	-	+	+
	SJ43	+	+	+	+	+	-	+	+	+	-	+	+
	SJ53	-	+	+	+	+	-	+	-	+	-	+	-
	SJ58	+	+	+	+	+	-	-	+	+	-	+	-
	SJ62	+	+	+	+	+	-	+	-	-	-	+	-
	SJ64	+	+	+	+	+	-	+	+	+	-	+	+
	SJ66	+	+	+	+	+	-	+	+	+	-	+	-
	SJ70	+	+	+	+	+	-	+	+	+	-	+	-
	SJ71	+	+	+	+	+	-	+	+	+	-	+	+
	SJ72	+	+	-	+	-	-	+	+	+	-	+	+
	SJ161	+	+	+	+	+	-	+	+	+	-	+	-
	SJ162	+	+	+	+	+	-	+	-	+	-	+	+
	SJ163	+	+	+	+	+	-	+	-	-	-	+	-
<b>UC Irvine Crew Dock</b>	UCIC5	+	+	+	+	+	-	+	-	+	-	+	+
	UCIC7	+	+	+	+	+	-	-	+	+	-	+	+
	UCIC8	+	-	+	+	+	-	+	+	+	-	+	+
	UCIC11	+	+	+	+	+	-	+	+	+	-	+	+
	UCIC12	+	+	+	+	+	-	+	-	+	-	+	+
<b>Upper Newport Bay</b>	UNB29	+	+	+	+	+	-	+	-	+	-	-	-
<b>San Diego Creek<sup>a</sup></b>	01-158	+	+	+	+	+	-	+	-	+	-	+	+
	02-164	+	+	+	+	+	-	+	+	+	-	+	+
	02-165	+	+	+	+	+	-	-	-	-	-	-	-
	02-166	+	+	+	+	+	-	+	+	-	-	-	-
	03-170	+	-	+	+	+	-	+	-	-	-	+	+
	03-172	+	-	-	+	+	-	+	-	-	-	+	-
<b>Newport Beach<sup>*</sup></b>	265	+	-	+	+	+	-	+	+	+	-	+	+
	267	+	+	+	+	+	-	+	+	+	-	+	-
	268	+	+	+	+	+	-	+	+	+	-	+	+
	373	+	+	+	+	+	-	+	+	+	-	+	+
	374	+	+	+	+	+	-	+	-	+	-	+	-
	375	+	+	+	+	+	-	+	-	+	-	+	+
	376	+	+	+	+	+	-	+	+	+	-	+	+
<b>Orange Coast<sup>*</sup></b>	OCC-32	-	-	+	+	+	-	-	-	-	-	-	-

<sup>a</sup>Strains isolated from three separate sites within San Diego Creek

<sup>\*</sup>Exact location not defined



**Figure 4.1. Detection of ASR factors among clinical and environmental *V. cholerae* isolates screened by PCR and percentage of isolates expressing ASR positive phenotypes.** Bar graph represents the number and percentage of *V. cholerae* isolates testing positive for ASR genes of interest. Genotype pools were created based on percentage of genes detected in each isolate. Pie graphs represent the ASR phenotype of isolates from each ASR genotype pool.



**Figure 4.2. ERIC-PCR genomic fingerprinting of clinical and environmental *V. cholerae* isolates used in ASR genotype/phenotype screening.** Isolates in red express and ASR positive phenotype. Isolates with an ASR negative phenotype are depicted in black. Clinical isolates are indicated with astrices and are inside grey shadow boxes.

**Table 4.4.** *In silico* analysis of ASR gene sequence similarities in *Vibrio* genomes from varied origins of isolation. ASR gene sequences were compared to the El Tor *V. cholerae* N16961 query to determine sequence similarities. Genomes are categorized according to origin of isolation. Heat map displays the percent similarity as high sequence similarity (dark) to low sequence similarity (light).

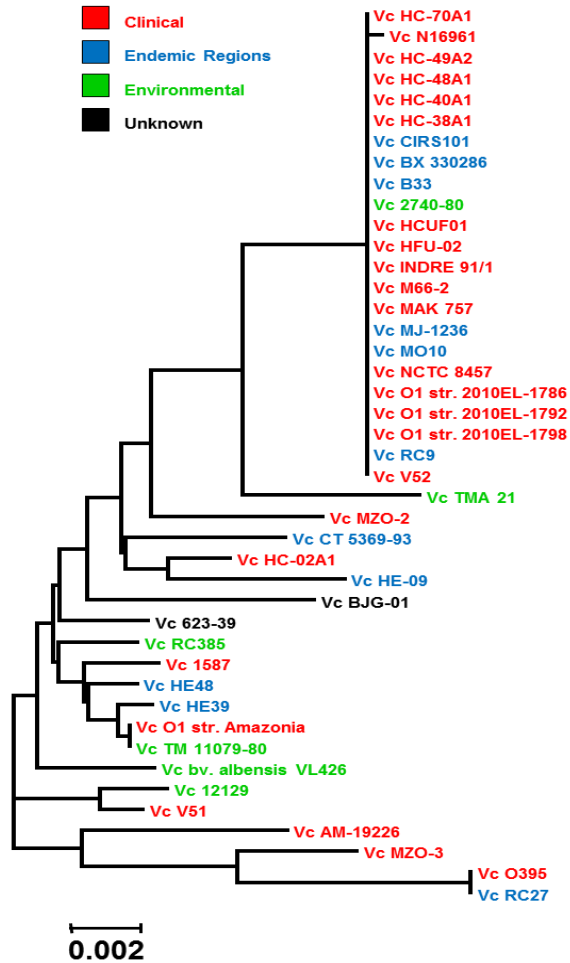
Clinical Genomes*	Country	TaxID <sup>†</sup>	ASR Genes Compared via <i>In Silico</i> Analysis							Similarity (%)
			<i>recO</i>	<i>hepA</i>	<i>cpdA</i>	<i>mrsA</i>	<i>nqrA</i>	<i>ompU</i>	<i>ompW</i>	
Vc N16961 (query)	Bangladesh	243277								100
Vc INDRE 91/1	Mexico	675808								99
Vc M66-2	Indonesia	579112								96-98
Vc O1 STR. 2010EL-1786	Haiti	914149								90-95
Vc O1 STR. 2010EL-1792	Haiti	925769								80-89
Vc O1 STR. 2010EL-1798	Haiti	925768								70-79
Vc HC-38A1	Haiti	991959								60-69
Vc HC-40A1	Haiti	991961								<59
Vc HC-48A1	Haiti	991965								N/A
Vc HC-49A2	Haiti	991935								
Vc HC-70A1	Haiti	991984								
Vc HFU-02	Haiti	991989								
Vc MAK 757	India	412967								
Vc O395	India	345073								
Vc NCTC8457	Saudi Arabia	417339								
Vc MZO-3	Bangladesh	412883								
Vc MZO-2	Bangladesh	417398								
Vc V51	USA	345075			**			**		
Vc V52	Sudan	345076								
Vc 1587	Peru	412966								
Vc AM-19226	Bangladesh	404974								
Vc HC-02A1	Haiti	991948								
Vc HCUF01	Haiti	991930								
Vc O1 STR. AMAZONIA	Brazil	1055325								
<b>Genomes from Environments within Endemic Regions</b>										
Vc CIRS101	Bangladesh	661513								
Vc MJ-1236	Bangladesh	593588								
Vc MO10	Bangladesh	345072								
Vc BX 330286	Australia	593587								
Vc RC9	Kenya	593589								
Vc B33	Mozambique	417400								
Vc RC 27	Indonesia	675807								
Vc CT 5369-93	Brazil	675809								
Vc HE-09	Haiti	991998								
Vc HE39	Haiti	991945								
Vc HE48	Haiti	991947								
<b>Environmental Genomes</b>										
Vc TMA 21	Brazil	593590								
Vc RC 385	USA	345074								
Vc TM 11079-80	Brazil	593586								
Vc BV. ALBENSIS VL426	Germany	593585								
Vc 2740-80	Gulf Coast	412614								
Vc 12129	Gulf Coast	592313								
<b>Genome Origin Not Documented***</b>										
Vc 623-39	-	417397								
Vc BJG-01	-	991997								
<b>Additional Vibrio Genomes</b>										
<i>V. furnissii</i> NCTC 11218	-	903510								
<i>V. harveyi</i> ATCC BAA-1116	-	338187								
<i>V. parahaemolyticus</i> RIMD	Japan	223926								
<i>V. sp.</i> Ex25	-	150340								
<i>V. splendidus</i> LGP32	-	575788								
<i>V. vulnificus</i> CMCP6	-	216895								
<i>V. vulnificus</i> MO6-24/O	-	914127								
<i>V. vulnificus</i> YJ016	Taiwan	196600								

\*Genomes isolated from Cholera patients

\*\*Sequence not located

\*\*\*Genome origin not reported in PATRIC

†Taxonomy ID in NCBI database



**Fig. 4.3. Neighbor-joining phylogenetic tree of concatenated 16S, *recA* and *rpoA* gene sequences used for Multi Locus Sequence Typing (MLST) of *V. cholerae* genomes investigated during *in silico* analysis.** Color coding indicates origin of genome isolation as designated in NCBI. Genomes were isolated from patients with Cholera (red), from environmental regions where Cholera was endemic at the time of genome isolation (blue) or from environments where Cholera was not endemic at the time of isolation (green). Origin of isolation was not reported for two of the genomes used in this analysis (black) nor was the origin described in supporting literature.

## CHAPTER 5

### ACID STRESS PRESPONSE ELICITATION OF *VIBRIO CHOLERA*E IN ENVIRONMENTAL SWAMP WATER

## ABSTRACT

*Vibrio cholerae*, the causative agent of the diarrheal disease Cholera, is commonly isolated from aquatic ecosystems. Acid stress response (ASR) is an adaptive feature that is vital to the ability of this opportunistic enteric pathogen to survive the mammalian host GI tract and colonize the small intestine. ASR has been investigated in the context of human infection in laboratory settings. However, environmental elicitation of this response remains largely unexplored. In the present study we report on the population growth dynamics of the clinical *V. cholerae* N16961 strain cultivated in environmental swamp water samples (CSW). Additionally, we investigate the impact of residence in swamp water on elicitation of *V. cholerae* ASR by monitoring population survival upon exposure to extreme acidity and measuring expression variability of five genes (*recO*, *mrsA*, *nprA*, *cpdA* and *hepA*) previously identified as central to *V. cholerae* ASR. Our findings suggest that *V. cholerae* N16961 is capable of robust growth in environmental swamp water samples at rates similar to the bacterial community autochthonous to CSW. Moreover, pre-conditioning in *in situ* swamp water facilitates enhanced cell survival upon exposure to extreme acidity. Molecular analysis suggests pre-conditioning cells in swamp water elicits ASR as gene expression observed in this study are similar to those previously described. To our knowledge, this is the first study demonstrating environmental elicitation of ASR in *V. cholerae* N16961.



## INTRODUCTION

*Vibrio cholerae* is a historically significant, environmentally ubiquitous bacterium with the capacity to cause the diarrheal disease, Cholera. Accounts of Cholera are in the historical record as early as 400BC and there have been eight Cholera pandemics date. In the 19<sup>th</sup> Century, *V. cholerae* was identified as the causative agent of this disease however the ecology of this bacterium was poorly understood (Barau, 1991; Riedle and Klohe, 2002). While Cholera is essentially eradicated in the developed world, current estimates suggest it is annually responsible for hundreds of thousands of infections, tens of thousands of deaths, more than \$1Billion (US) and untold human suffering, primarily in elderly, adolescent and the immuno-compromised populations within developing counties (WHO, 2002).

*Vibrio cholerae* is a motile, gram-negative, curved rod in the family Vibrionaceae. The diversity of *V. cholerae* is determined by serotyping based on epitope variation in O-antigen lipopolysaccharide (LPS) composition. There are currently 50 known species of *Vibrio* and more than 200 serotypes of *V. cholerae*; the vast majorities of which are non-toxicogenic, environmentally ubiquitous and beneficial in biogeochemical cycles (Purdy et al, 2005). Historically, strains carrying the O1 antigen were considered clinically relevant and non-O1 strains were not (Yamai et al., 1997; Jiang et al., 2000). However, strains such as O139, have emerged which are capable of causing Cholera at epidemic proportions and are hypothesized to have environmental origins (Albert et al., 1993; Nair et al., 1994; Ramamurthy et al., 1993; Jiang 2000).

Outbreaks of Cholera have been shown to be seasonal and correlated with regional weather and algal blooms (Huq et al., 1990; Epstein, 1993; Islam et al., 1994; Colwell, 1996; Pascual et al., 2000; Collwell and Huq, 2001; Kirschner et al., 2008). *V. cholerae* is an autochthonous constituent of bacterial communities within aquatic ecosystems where it exists in free-living forms and associated with copepods, zooplankton and algae (West and Lee, 1982; Worden et al., 2006; Lipp et al., 2002). It has been suggested that these particle associations are important in acquisition of virulence genes and may enhance *V. cholerae* survival in the environment (Nalin et al., 1979; Blokesch and Schoolnik, 2007; Hsieh et al., 2007; Pruzzo et al., 2008).

Early investigations into effect of physicochemical parameters on *V. cholerae* population dynamics suggest that temperature and salinity are strong determinants of growth and environmental distribution (Kaper et al., 1979; Singleton et al., 1982; Colwell, 1996; Huq et al., 2005). This bacterium is commonly detected in coastal and brackish ecosystems and population growth in artificial seawater experiments has been reported (Singleton et al., 1986; Jiang et al., 2002; Binztein et al., 2004; Louis et al., 2003; Mourino-Perez et al., 2003; Worden et al., 2006). Environmental detection studies have cited the preference of *V. cholerae* for higher salinities which were thought to influence optimal growth and distribution of this opportunistic pathogen (Singleton et al., 1986; Louis et al., 2003; Huq et al., 2005). Laboratory studies have assessed *V. cholerae* growth in nutrient amended artificial seawater at salinities as low as 0.5% NaCl (Singleton et al., 1982). While freshwater systems have been shown to foster *V. cholerae* populations (Shapiro et al., 1999), very few studies to date have assessed population growth dynamics in environmental freshwater, thus prolonging the conclusion that *V.*

*cholerae* is a marine bacterium. In fact, several studies have successfully isolated El Tor strains of this bacterium from aquatic ecosystems with salinities ranging from 0.2-14 from California, the Chesapeake Bay and Asian and African sample sites (Jiang et al., 2001; Nair et al., 2006; Goel et al., 2008). Recently, Vital et al. (2007) assessed growth in environmental freshwater using laboratory microcosms and the clinical El Tor strain of *V. cholerae* N16961 as the model organism. And, while *V. cholerae* growth has been assessed in water from alkaline lakes (Kirschner et al, 2008), and studies have been conducted on bacterial growth in acidified freshwater systems (Percent et al., 2008), assessment of *V. cholerae* growth in acidified environmental water samples remains largely unexplored.

It was discovered at the turn of the millennium that *V. cholerae* is capable of mounting an acid stress response (ASR; Merrell et al., 2001; Merrell and Camilli, 2000; Merrell and Camilli, 2002) which provides insight into how this ubiquitous aquatic bacterium is capable of causing pandemic level disease. ASR is a dynamic adaptive feature that facilitates enhanced population survival during transit through the variable and extreme acidity of the mammalian GI tract. This phenomenon involves rapid induction of biochemical and physiological modifications when cells are pre-conditioned in a sub-lethal acidity (pH 4-6) and subsequently exposed to extreme acidity (pH 1-3). Western blot analysis of protein synthesis during ASR in the clinical *V. cholerae* N16961 strain reveals variation in the regulation of more than 110 proteins, with upregulation of >60 proteins and downregulation of >50 proteins (Merrell and Camilli, 1999, 2002).

In addition to protecting cells from extreme acidity, ASR activates auxiliary adaptive responses such as virulence, biofilm formation, chemotaxis and antibiotic

resistance and may be elicited upon exposure to various growth media and environmental cues (Rowbury, 1995; Foster, 1999; Merrell et al., 2001; Polon et al., 2003; Koutsoumanis et al., 2003; Maurer et al., 2005; Butler et al., 2006; Hayes et al., 2006). Further, ASR has been shown to provide cross-protection to other forms of stress (Leyer and Johnson, 1992; Rallu et al., 2000; Frees et al., 2003; Xie et al., 2004). ASR has been characterized in a range of model organisms including gram-positive, industrially relevant, primary pathogens and Archaea (van de Guchte et al., 2002; Merrell et al., 2003; Cotter and Hill, 2003; Beales, 2004; Xie et al., 2004; Ciaramella et al., 2005; Padan et al., 2005; Baker-Austin and Dopson, 2007; Mols et al., 2010). Further, this response has been characterized in foodborne pathogens such as *Escherichia coli* and *Salmonella enterica*, where it has been investigated with regards to survival during fermentation and food preservation practices (Capozzi et al., 2009; Arnold et al., 2001; Polen et al., 2003; Kirkpatrick et al., 2001). And, while there are limited studies investigating the ASR of environmentally derived *E. coli* (Bagwhat, 2005), the role of environmental systems in elicitation of ASR remains largely unexplored.

In the present study, we investigate population growth dynamics of *V. cholerae* cultivated in *in situ* environmental swamp water. Additionally, we assess the impact that cultivation in environmental swamp water rich in organic acids has on eliciting the ASR of *V. cholerae* N16961 by monitoring population survival upon exposure to extreme acidity and expression variability of ASR genes. The five ASR genes used in this study (*recO*, *cpdA*, *mrsA*, *hepA*, *nqrA*) have previously been demonstrated as central to *V. cholerae* ASR (Merrell and Camilli, 2002) with mutations in these genes significantly reducing the population survival of *V. cholerae* N16961 in extreme acidity. We are

testing the hypotheses that a) *V. cholerae* N16961 is capable of prolonged growth in environmental swamp water and that b) pre-conditioning *V. cholerae* in environmental swamp water elicits ASR. The results of this study will serve to improve understanding of the environmental distribution of *V. cholerae* and the role of these aquatic ecosystems in pre-conditioning this clinically relevant opportunistic enteric pathogen to extreme acidity.

## MATERIALS AND METHODS

**Sampling site and collection.** Congaree swamp water was collected from the Congaree National Park (33°46'12.1"N 080°37'51.2"W) near Columbia, SC. Water samples were collected aseptically from the water column, by attaching 0.5L wide mouth high density polyethylene (HDPE) carboys to an aluminum extension pole and transferring to 4L polyethylene carboys; care was taken to ensure no gaseous head space in carboy. Samples were transported to the laboratory at *in situ* temperatures. On the day of sampling, CSW pH was measured with an *in situ* pH 5.5 ± 0.3 (Pinpoint pH monitor; American Marine Inc.). An *in situ* salinity 3 was measured via refractometer. Prior to measuring salinity, water samples were centrifuged (10,000g; 5min) to remove any suspended solids that may alter salinity measurements.

For the organic acid challenge and growth assays, approximately 2L of the swamp water was filtered through 0.2µm polycarbonate filters and filtrate was stored at 4°C. The dissolved organic carbon (DOC) concentrations of the CSW samples were analyzed using high temperature oxidation with a Shimadzu TOC-V with an inline chemiluminescent nitrogen detector (Davis and Benner, 2005). CSW was found to have DOC 900µM.

**Swamp water growth assay strains and inoculum preparation.** Source plates of the clinical *El Tor* O1 strain *Vibrio cholerae* N16961 were maintained for the growth assays and organic acid challenge assays by cultivation from frozen stock onto Luria Broth Agar (LBA; 1% tryptone, 0.5% yeast extract, 0.3% NaCl, 1.5% agar, 1L DiH<sub>2</sub>O) and incubating overnight at 27°C. To prepare inocula for the Congaree Swamp Water (CSW) growth experiments, a loopful of the resulting growth was aseptically transferred from the fresh source plate into 45mL of 0.2µm filtered CSW, amended with 0.1% (wt/vol) tryptone and incubated overnight at 27°C. To prepare the CSW autochthonous bacterial community (ACB) culture, 0.8µm filtered CSW was amended with 0.1% (wt/vol) tryptone and incubated overnight at 27°C. Cell densities of this growth were enumerated via epifluorescence microscopy on a Nikon (etc.). Cultures were prepared for enumeration by transferring 500µL of respective cultures into 1.5mL of isotonic, sterile water. These samples were prepared for microscopy by fixing with borate buffered formalin (BBF; 1% FC) and staining with the fluorescent nucleic acid stain SYBRGreen™ (1x FC) and incubating in the dark for 15min. Microscopy counts were conducted on a Nikon E600 series epifluorescent microscope by counting a minimum of 20 grids under oil immersion as per Kirschman et al., 1982. Total bacterial abundance was calculated using the equation  $a = x * 1/v * cf$  where a=abundance, x=average number of bacterial cells per field, v=volume of sample filtered, cf=conversion factor for (filter surface area)/ocular grid surface area).

**Growth assessment.** After enumerating cells densities in overnight *V. cholerae* N16961 and CSW ACB cultures, inocula were prepared by harvesting cells via centrifugation (10,000g x 13min; 25°C). The supernatant was discarded and the pellets were

resuspended in 0.2µm filtered CSW. The process was repeated 3 times to minimize nutrient transfer into microcosms.

Microcosms were constructed by transferring 20mL of 0.2µm filtered CSW (pre-warmed to 20°C) into 22mL scintillation vials capped with autoclaved, non-absorbent cotton stoppers. 0.2µm HT Tuffryn® Membrane, Acrodisc® Syringe Filters (Pall) and sterile 50mL syringes were used to add the sterile CSW to the microcosms. Microcosms were inoculated with initial concentrations of  $1 \times 10^4$  cells mL<sup>-1</sup> *V. cholerae* N16961 and  $1 \times 10^6$  cells mL<sup>-1</sup> CSW ACBs to reflect environmentally relevant concentrations.

Subsamples were aseptically transferred from each microcosm in duplicate at three time points over the course of each 24hr cycle for total cell and viable colony counts. At termination of each 24hr cycle, 10% total volume from respective microcosms was transferred to fresh CSW microcosms. Microcosms were prepared in duplicate for *V. cholerae* N16961 and the CSW ACBs during each growth cycle. Un-inoculated, sterile 0.2µm filtered CSW microcosms were maintained and subsampled for the duration of experiment. Total bacterial counts were assessed by diluting duplicate samples 1:10 in sterile CSW from duplicate microcosms, fixed (BBF; 2% FC), stained with SYBRGreen™ (1X FC) and incubated in the dark for 15min prior to counting.

Subsamples were filtered through 0.2µm polycarbonate filters (Watmann) previously stained black with Irgalin black (Hobbie et al., 1977). Total counts were conducted on the Nikon Eclipse E600 series as described above. For viable counts, 100µl subsamples were plated from duplicate microcosm on LBA and incubated 48hrs at 20°C prior to counting colony forming units (CFUs).

**Organic Acid Challenge.** Fresh cultures of *V. cholerae* N16961 were prepared by transferring a loopful of fresh bacterial biomass to sterile Luria Broth (LB; 1% tryptone, 0.5% yeast extract, 3g NaCl, 1L DI water) and incubated overnight at 30°C with aeration. (*Note:* NaCl concentrations were adjusted for LB to reflect salinity of CSW and maintain isotonicity). Cells from the overnight cultures were harvested the following day via centrifugation (10,000g, 10min) of 5mL samples. For organic acid challenge, adaptation and shock broths were prepared as previously described (Merrell et al., 1999; Merrell and Camilli, 2002). Briefly, two LB broths were prepared for acid adaptation and acid shock by amending with HCl to a pH of 5.7 and 4.5, respectively. A 10x organic acid cocktail (87mM acetic acid, 25mM butyric acid and 37mM propionic acid) was prepared by addition of organic acids to LB. The acid adaptation broth was amended with 0.75x organic acid cocktail (6.5mM acetic acid, 1.9mM butyric acid, 2.8mM propionic acid; final pH 4.5); the acid shock broth was amended with 1x organic acid cocktail (8.7mM acetic acid, 2.5mM butyric acid, 3.7mM propionic acid; final pH 3.5).

Cell pellets were resuspended in one of four adaptation treatments prior to acid shock (**Fig. 1**). For organic acid adaptation, cells were resuspended in LB adaptation broth (pH 4.5). Cells were inorganic acid adapted by resuspension in LB amended with 16N HCl (pH 5.7). Unadapted samples were prepared by resuspension of cell pellets in unamended LB (pH 7). To assess the impact of environmental water samples on adaptation to extreme acidity, *V. cholerae* N16961 cells were resuspended in either *in situ* Congaree swamp water (CSW; pH 5.7) or CSW amended with organic acids (6.5mM acetic acid, 1.9mM butyric acid, 2.8mM propionic acid; final pH 4.5). During the adaptation phase of the organic acid challenge assay, samples incubated at 30°C with



aeration for 1.5 hours. After adaptation, cells were plated in duplicate across a three factor dilution range (i.e.,  $10^2$ - $10^0$  cells  $\text{mL}^{-1}$ ;  $n=6$  per treatment, time point) on LBA to assess densities prior to acid shock ( $T_0$ ). All plating involved sub-sampling 100 $\mu\text{l}$  samples. Cells were pelleted via centrifugation (10,000g, 10min) prior to exposure to extreme acidity. Cells from respective adaptation treatments were resuspended in acid shock broth (see above) and incubated at 30°C with aeration for 60min. 100 $\mu\text{l}$  subsamples of respective acid shock treatments were plated as previously described at the 30min and 60min interval on LBA. Plates incubated overnight at 30°C and CFUs were counted the following day. Cells from respective treatments were retained by pelleting (10,000g; 10min) and then storing them in the RNA stabilizing agent, *RNAlater*<sup>TM</sup> at -20°C for less than 1 week prior to molecular analysis.

## RT-PCR

Subsamples of *V. cholerae* were retained from respective organic acid challenge assays, cell densities were enumerated via epifluorescence microscopy as described above. RNA was extracted from  $1 \times 10^7$  *V. cholerae* cells using the MoBio Ultra Clean Mini-RNA (Carlsbad, CA, USA) extraction kit and following the manufacturer's instructions. Once RNA was extracted, complimentary DNA (cDNA) was synthesized according to the ProtoScript<sup>®</sup> M-MuLV Taq RT-PCR kit available through New England BioLab (Ipswich, MA, USA) using 10 $\mu\text{L}$  of RNA template. Samples were heated to 42°C for one hour using the random dT23VN primers provided at a concentration of 100pmol per reaction. Enzymes were deactivated by heating to 80°C for 5minutes. Following the synthesis reaction, all five ASR genes (*recO*, *mrsA*, *nprA*, *cpdA*, *hepA*; **Table 2**) were amplified using primer sets of Merrell and Camilli (2002; **Table 3**) under the following

thermocycling parameters: initial denaturation at 95°C for 1 minute; 30 cycles of additional denaturation at 94°C for 30sec, annealing at 50°C for 20sec, elongation at 68°C for 20sec and a final extension phase at 68°C for 7min. Following amplification, PCR products were confirmed via gel electrophoresis of 5µL of product in a 1.5% agarose gel run for 30min in Sodium Boric Acid (SBA) at 125V. Gel was stained in 0.5 µg mL<sup>-1</sup> Ethidium Bromide (EtBr), visualized in a UVP Bioimaging Systems and digital image analysis was conducted using LabWorks imaging software. PCR product amounts were quantified using the imaging software and are reported here as the ratio of gene product normalized to the same gene product expressed in the same number of cells cultivated in LB pH7.

### **Statistical Analysis**

Statistical analysis was conducted using IBM SPSS version 20. Results of the Congaree swamp water growth dynamics study, organic acid challenge assays, gene expression and growth rate comparisons were analyzed separately with univariate analysis of variance (ANOVA) and an  $\alpha=0.05$ . When appropriate, post-hoc analysis was conducted with the Ryan-Einot-Gabriel-Welsch Range (R-E-G-W-Q) test. Percent population survival results were arc sin transformed to satisfy assumptions of ANOVA.

## **RESULTS AND DISCUSSION**

### **Bacterial Growth in Congaree Swamp Water (CSW)**

Based on the total cell counts (**Fig.2A**), it is evident that the autochthonous bacterial community (ACB) isolated from the CSW and *V. cholerae* N16961 (VcN) are capable of growth in *in situ* swamp water with both demonstrating stable growth over four cycles in

the microcosms. *V. cholerae* is capable of prolonged growth reaching densities in excess of the known minimum infectious dose ( $1 \times 10^4$  cells  $\text{mL}^{-1}$ ) based on total counts and viable counts. Viable cell counts suggest a similar response of *V. cholerae* to the CSW with pulse-like growth observed in cycles 1 and 2 (**Fig.2B**). The rapid increase in CFU counts observed for VcN and CSW ACBs during cycle one may be an artifact of transfer out of the 0.1% tryptone amended CSW used for cultivation of initial inoculum. As such, cycle one serves to acclimate the cells to lower nutrient concentrations of the CSW. While the CSW ACBs were able to reach high densities during the first cycle, growth upon transfer to the subsequent cycles was less rapid. By comparison, *V. cholerae* CFU counts suggest rapid growth in the first two cycles of transfer to the CSW microcosms. Variability of VcN during cycle three may be the result of transitional morphologies. *V. cholerae* has been shown to rapidly transition between rod and coccus as a function of cell density, temperature and nutrient availability which subsequently effects culturability (Carroll et al., 2001; Chaiyanan et al., 2007). Transition from rod to cocci morphology has been observed as occurring within 3 hours of exposure to nutrient limited culture conditions (Krebbs and Taylor, 2011). Cycle four demonstrates the prolonged population stability of *V. cholerae* in the CSW as this cycle lasted for 72 hours.

Growth rate comparisons of VcN and the CSW ACBs (**Fig.2C**) suggests that VcN is capable of growth at rates greater than that observed for the ACBs (ANOVA,  $p < 0.001$ ). One probable factor influencing the observed differences in growth between the *V. cholerae* and CSW ACBs involves bacterial competition and polymicrobial interactions that inherently exists in mixed bacterial communities from marine (Long and Azam, 2001) and freshwater (Romani et al., 2006) systems and likely occurred within the

ACB microcosm. Future investigations of *V. cholerae* growth in environmental swamp water would benefit by investigating the growth of *V. cholerae* within a mixed community of the CSW ACBs.

While there have been microcosm studies assessing the growth of *V. cholerae* in various salt amended water (Singleton et al., 1982) these studies did not assess growth in the lower freshwater ranges (i.e., salinity <5) observed in this study. However, their findings did suggest reduced growth and culturability at lower salinities thus propagating the notion that *V. cholerae* is a marine bacterium. The duration of growth and the calculated growth rates for *V. cholerae* in CSW is similar to that observed in microcosm studies using seawater amended with photosynthate derived dissolved organic carbon (DOC) such to simulate algal bloom conditions (Moreno-Perez et al., 2006). The ecological implications of this are significant in that *V. cholerae* growth following algal blooms is thought to be an important regulatory factor in Cholera outbreaks (Huq and Colwell, 1996; Lobitz et al., 2000). Growth rates for *V. cholerae* in CSW ( $0.47 \pm 0.03 \text{ h}^{-1}$ ) was observed as more than twice the maximum growth rates observed for *V. cholerae* growing in fresh lake water ( $0.22 \pm 0.008 \text{ h}^{-1}$ ) but approximately the half the  $\mu_{\text{max}}$  observed in lake water amended with 0.5% NaCl ( $0.84 \pm 0.001 \text{ h}^{-1}$ ) when the growth rates are calculated as per Vital et al. (2007). Studies comparing *V. cholerae* growth in alkaline lakes of the Neusiedler See in Austria demonstrated microscopically derived growth rates comparable to the ACBs therein (Kirschner et al., 2008). Microscopically derived growth rates in the present study are consistent with the findings of Kirschner and colleagues who observed growth rates of *V. cholerae* being inversely proportional to the concentration of humic organic matter in the batch cultures. In addition to limits in

freshwater growth studies of *V. cholerae*, studies assessing growth of this waterborne pathogen in sub-lethal pH conditions are lacking and warrant attention. For example, in aquatic systems along the Rio Grande in Texas, pH was shown to be a significant parameter in growth and environmental survival of the opportunistic enteric pathogen *Salmonella enterica* (Nutt et al., 2003).

Current predictive models rely on physicochemical parameters to determine environmental distribution of select waterborne opportunistic pathogens. Projects investigating *V. cholerae* in the Chesapeake Bay, MD have resulted in generation of a ‘now-casting’ system predicting, with proven reliability, the distribution within this estuarine system (Constantine de Magny et al., 2008). Interestingly, this model predicts highest incidence of *V. cholerae* in locations where freshwater tributaries feed the estuary. Similar predictive models have proven effective and are currently employed for assessing distribution of other *Vibrio*’s in the Chesapeake Bay (Jacobs et al., 2010). Despite these exceptions, the environmental distribution of *V. cholerae* within freshwater systems remains under explored. The findings of this work compliment the previous findings and provide evidence contrary to this common conception.

### **Population survival**

The results of the organic acid challenge demonstrate that *V. cholerae* experiences elevated population survival when pre-conditioned in organic acid (**Fig.3**). Statistical analysis reveals that *V. cholerae* populations survival during organic acid challenge is influenced by adaptation treatment (ANOVA;  $p < 0.001$ ). Of greatest interest in this study, and the primary purpose in conducting the experiment, is the observation that *V. cholerae* N16961 populations adapted in *in situ* and organic acid amended CSW (pH 5.7

and 4.5, respectively) survive exposure to extreme acidity at levels statistically indistinguishable from those observed from each other and the organic acid adapted cultures. This is supported by R-E-G-W-Q post-hoc statistical analysis which reveals two subsets based on acid adaptation treatment; the unadapted and inorganic acid adapted cultures represent one sub-group. The adapted cells form a sub-group with *V. cholera* adapted in the *in situ* and organic acid amended CSW.

The organic acid adapted cells demonstrate a robust response upon transfer to extreme acidity with greater than 10% of the population surviving 1 hour of extreme acid shock as compared to the unadapted cells which were observed as having a population survival several orders of magnitude lower. These findings are consistent with those reported elsewhere (Merrell et al., 1999; Merrell and Camilli, 2002). The inorganic acid adapted populations experienced rapid decline in percent population survival after 30 minutes of acid shock but stabilized to improved viability counts at T<sub>60</sub>. This is consistent with the literature which demonstrates inorganic ASR as being slower than organic ASR (Merrell and Camilli, 1999). Future studies should investigate longer adaptation phases on ASR elicitation as the magnitude of ASR has previously been shown to be effected by incubation duration in *E. coli* isolates (Brudzinski and Harrison, 1998). The response of certain bacterial pathogens (i.e., *E. coli* O157:H7) to fermentation bi-products such as propionic acid has been documented (Russell and Diez-Gonzalez, 1997) as select pathogens are capable of prolonged growth in the presence of such acidity. However, this is the first study to our knowledge demonstrating elicitation of ASR with cells pre-conditioned in environmental swamp water. The findings reported here have application in understanding pathogenicity and aquatic ecology of related

bacterial species as another prominent marine pathogen, *V. vulnificus*, is also capable of a robust ASR (Jee Eun et al., 2004).

### **RT-PCR of ASR Genes**

Variability in mRNA expression of the five ASR genes reveals no statistically significant difference in gene expression due to adaptation treatment (ANOVA,  $p>0.05$ ). However, with the exception of *recO*, the expression variability for nearly all of these genes is consistent with trends observed elsewhere (**Fig. 4**; Merrell and Camilli, 2002). Four of the ASR genes (*recO*, *mrsA*, *nqrA*, *hepA*) demonstrated expression approaching or exceeding two-fold increases as compared to the expression of respective genes cultured in LB pH7. Additionally, *nqrA* displayed elevated expression as compared to the other four genes at levels marginally below statistical significance (ANOVA,  $p=0.058$ ).

While the molecular analysis conducted in the study does not shed new light *per se* on the molecular mechanisms involved in *V. cholerae* ASR, the expression patterns observed in this study are higher than those previously reported (Merrell and Camilli, 2002). We attribute this to cultivation in different broths and water samples as well as advancements in molecular reagents in the decade since the initial report. In the current study, *recO* expression was shown to be slightly elevated when normalized to expression in LB pH7 however previous investigations using RNA protection assays demonstrated a reduction in the expression of this gene (Merrell and Camilli, 2002). The moderately increased levels of *recO* in the present study may also be due to the fact that the culture broth used in this study had a lower salinity (Sal. 3) than did the culture broth used by Merrell and Camilli (Sal.10). Additionally, the temperature used in the Merrell and

Camilli RNA protection assay was 37°C while that used here was 30°C. These findings would suggest that elevated salinity and temperature may influence expression of this ASR factor suggesting that additional environmental parameters may influence *V. cholerae* ASR.

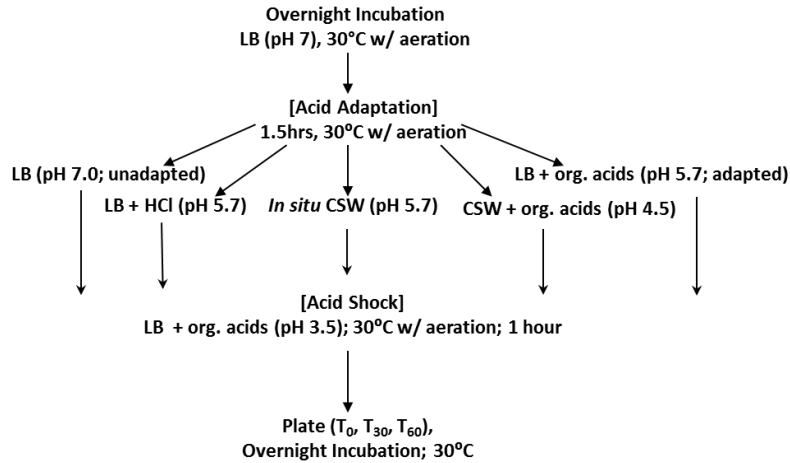
The induction of acid shock proteins has been shown to be a critical factor in the ASR capacity of enteric pathogens (Foster, 2000; Merrell et al., 2001). Transcriptomic analysis of expression variability in *V. cholerae* have revealed large categories of genes in response to varying acidic conditions (Zhu and Mekalanos, 2003; Schild et al., 2007). Further, activation of similar genes have been observed in *Vibrio alginolyticus* during colonization of diseased fish kidneys (Dean et al., 2004) signifying the linkage between ASR and pathogenicity for multiple pathogen types. As such, further metatranscriptomic analysis of *V. cholerae* cultivated in environmental swamp water may provide insights into the role of select environments in the activation of biochemical pathways involved in stress response and pathogenicity.

## CONCLUSION

The findings of this study highlight the ecological implications of *V. cholerae* growth in environmental swamp water and the potential risk to human health that this represents. Of greatest significance in this report is the enhanced survival of *V. cholerae* to organic acid shock after a short interim in environmental swamp water. It would be valuable in future studies to investigate the duration of *V. cholerae* growth in this and similar freshwater systems. Presumably these water ways experience seasonal variability in DOC values and other influential physicochemical parameters (i.e., pH, salinity) which may contribute to variability in growth dynamics and ASR elicitation in *V. cholerae*.



Finally, while the adaptation and shock durations used in this study were selected based on previously described methods, investigations into the durational response of *V. cholerae* in acid adaptation and shock broth warrants attention to more closely approximate the temporal residence of this pathogen in the mammalian host. Contamination of freshwater remains a significant mechanism implicated in the spread of Cholera. As such, the findings of the *V. cholerae* population dynamics in the freshwater samples used in this study may have significant ecological and clinical implications. These works may aid in identification of environmental ‘hotspots’ of bacterial pathogenicity which would aid in the development of improved predictive models and guide efforts in disease prevention and mitigation.



**Figure 5.1. *V. cholerae* N16961 culture treatments during organic acid challenge.** Overnight cultures were acid adapted for 1.5hr in organic and/or inorganic acid amended Luria broth (LB), and either *in situ* or organic acid amended Congaree Swamp Water (CSW) prior to resuspension in shock broth. Cultures were sub-sampled over a 1hr time course during acid shock. Plates incubated overnight prior to quantifying population survival. Organic acid cocktail concentrations for adaptation and shock were Adapt (CSW and LB): 6.5mM acetic acid, 1.9mM butyric acid; 2.8mM propionic acid; Shock: (8.7mM acetic acid, 2.5mM butyric acid; 3.7mM propionic acid).

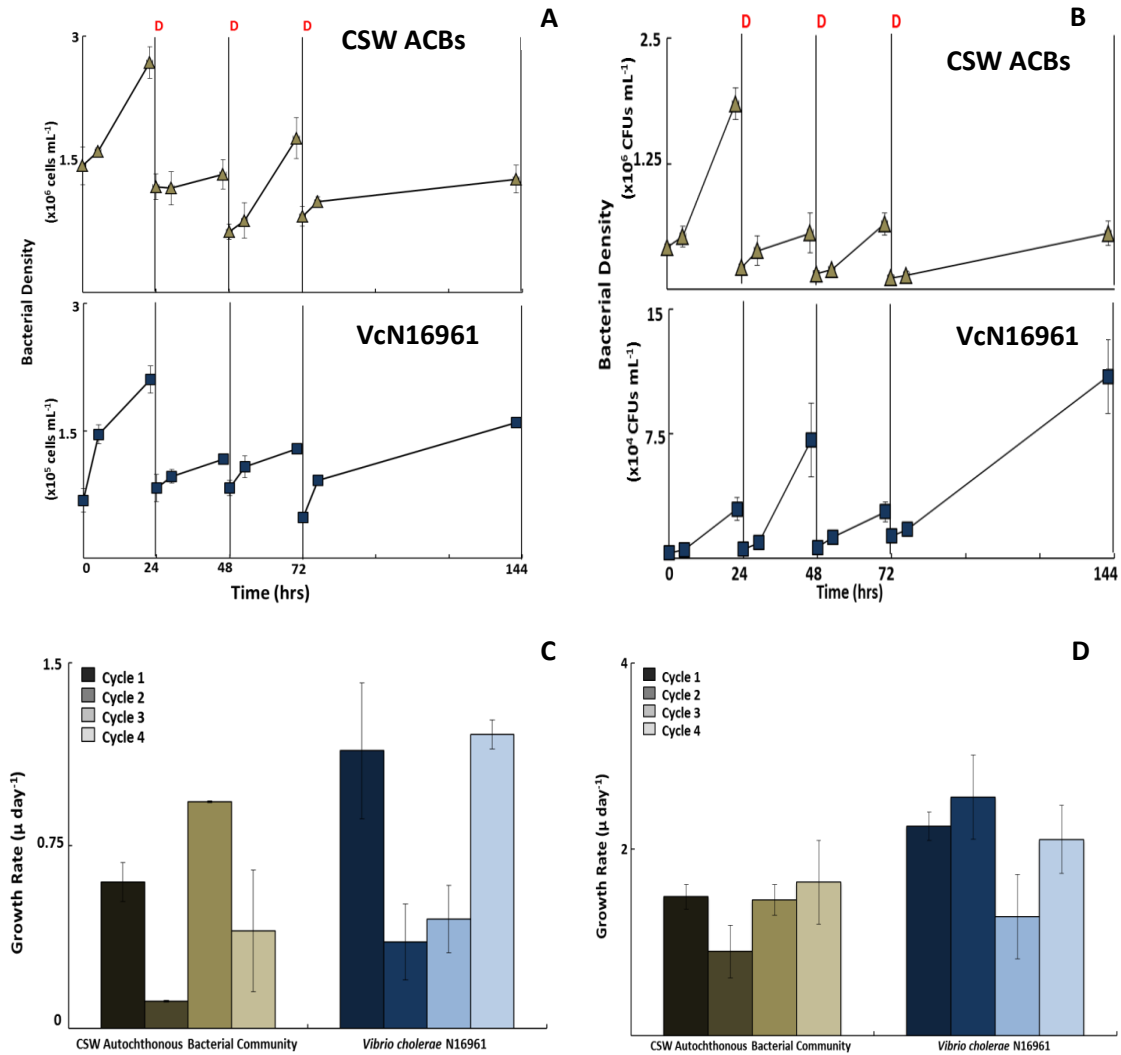
**Table 5.1.** Proposed function of genes involved in acid stress response (ASR) which were targeted for gene expression analysis. Variation in the expression of these genes was assessed for *V. cholerae* N16961 pre-conditioned in different adaptation treatments during organic acid challenge.

Gene	Common Name	Possible Role in ASR
hepA	HepA	Transcriptional Regulation
mrsA	Phosphoglucomutase	Maintenance of cell wall/LPS structure/phosphomannomutase
nqrA	NADH:ubiquinone subunit	Na <sup>+</sup> homeostasis
recO	RecO	DNA repair
cpdA	cAMP phosphodiesterase	Regulation of cAMP levels/transcriptional regulation

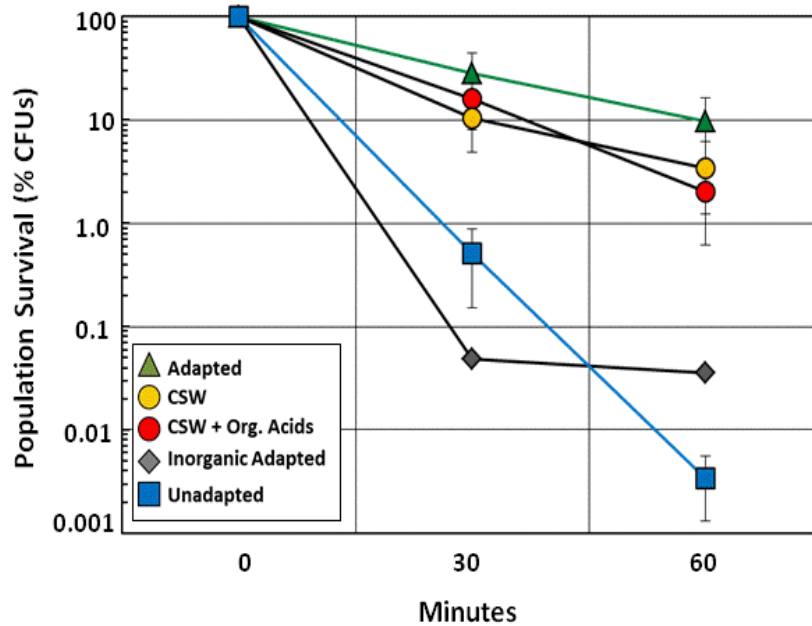
**Table 5.2.** Sequences of primers used for RT-PCR analysis of expression variation in genes active in *V. cholerae* N16961 ASR during organic acid challenge.

Target	Sequence (5' - 3')	Direction	Amplicon Size (bp)	Tm <sup>a</sup> (°C)	Reference
recO	GGTTTGCCGCCAAGATACGGCT	F	326	59.9	Merrell and Camilli, 2002
	GCGCGGAAAACGCTCCAACC	R		55.3	
hepA	CGCAGGATAAGTTGTTTG	F	306	55.3	Merrell and Camilli, 2002
	GTTGCAGCGTTTCAGGTA	R		57.6	
cpdA	ACCCGAGTCTTATCAACG	F	361	57.6	Merrell and Camilli, 2002
	GACGACATCCCAAAAACG	R		57.6	
mrsA	ACCGGACCAATGCCAACG	F	306	62.2	Merrell and Camilli, 2002
	AAGCTTCAAACCAGATAG	R		53.1	
nqrA	ATGCGATCAAAACTCAGC	F	300	55.3	Merrell and Camilli, 2002
	GATGTGGGCCGTCAAAGA	R		59.9	

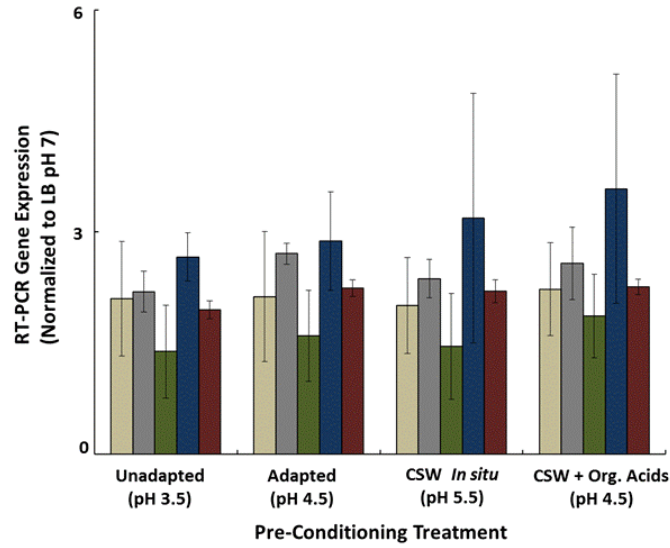
<sup>a</sup> Melting temperature  
<sup>b</sup> Amplicon size varies according to strain



**Figure 5.2. Growth of *V. cholerae* N16961 and the autochthonous bacterial community from environmental swamp water.** Bacterial densities of *V. cholerae* (VcN16961) and the bacterial community native to the Congaree swamp water (CSW ACBs) was assessed by microscopically for total counts (Fig.5.2A) and colony forming units (Fig.5.2B; CFUs) to assess the culturable community. Growth rates of *V. cholerae* N16961 and CSW ACBs were calculated as  $\mu = \ln(T_{24}/T_0)$  based on total counts (Fig.5.2C) and CFU counts (Fig.5.2D). Error bars represent standard deviation from replicate microcosms.



**Figure 5.3. Survival of *V. cholerae* N16961 populations during organic acid challenge** after acid adaptation in various laboratory and environmental treatments. Cells were acid adapted for 1.5hr in either LB + org. acids (pH4.5; adapted), *in situ* Congaree Swamp Water (CSW; pH 5.7), organic acid amended CSW (pH 4.5), LB + HCl (Inorganic adapted; pH 5.7) or LB (pH 7; unadapted) prior to exposure to lethal acidity (LB + organic acids; pH 3.5) for 1hr. Subsamples were plated during acid shock and the resulting CFU growth was used to calculate percent population survival. Error bars represent standard deviation of three separate experiments (n≥9).



**Figure 5.4. Expression variability of a subset of genes involved in *V. cholerae* N16961 ASR.** Gene expression of recO (□), mrsA (■), cpdA (■), nqrA (■) and hepA (■) was assessed via RT-PCR in  $1 \times 10^7$  cells sub-sampled during organic acid challenge. Expression from each treatment was normalized to respective genes in Luria Broth (LB) pH 7. Error bars represent standard deviations of triplicate samples.

## CHAPTER 6

# THE RESPONSE OF ENVIRONMENTAL BACTERIAL COMMUNITIES FROM PRISTINE AND CONTAMINATED SEDIMENT PARTITIONS TO EXTREME ACIDITY

## ABSTRACT

The success of opportunistic enteric pathogens is affected by their ability to survive the extreme acidity encountered in the mammalian host intestinal milieu. The acid stress response (ASR), which imparts this survival, has historically been investigated in the context of individual strains under laboratory conditions. In the present study, we explore the capacity of environmentally derived bacterial communities to respond to extreme acidity. Samples were collected from pristine estuarine sites, riverine systems and contaminated experimental mesocosms. Water and sediment samples were processed such the interstitial and attached bacterial communities were evaluated for their response to extreme acidity. Using culture based methods, and molecular and high-throughput sequencing techniques, we investigated ASR capabilities of these bacterial communities. Our results indicate that sediment associated bacterial communities are capable of robust ASR. Further, sediments contaminated with copper and mixed polyaromatic hydrocarbons select for bacterial communities more capable of ASR compared to bacterial communities from control sediments. These findings may aid in identification of environments harboring bacterial communities representing a risk to human health.



## INTRODUCTION

The global rise in foodborne infections and intestinal diseases over the past quarter century resulting from contaminated produce and improper irrigation has generated interest into bacterial community composition in various environments, environmental distribution of opportunistic pathogens and the role environments on stress conditioning and pathogenicity of infectious bacteria (Doyle and Erickson, 2008; Capozzi et al., 2009).

Vertical distribution of bacteria in the water column has important ecological implications in abundance and viability of pathogens in the environment. Particulate organic matter (POM), which can be separated from colloidal and dissolved organic matter by 0.2-0.45 $\mu$ m pore size filtration, provides sufficient stratum colonized by dense and complex bacterial communities with elevated metabolic levels (Crump et al., 1999). Further, particles and sediment associated bacterial communities from marine and freshwater systems have heightened antagonistic properties which impact the bacterial community structure (Thomashow et al., 1990; Long and Azam, 2001; Grossart et al., 2004; Gram et al., 2010). This particulate matter has been shown to be heavily colonized by fecal coliforms (George et al., 2004) which survive twice as long as free-living cells when particle associated (Craig et al., 2004; Garcia-Armisen and Servais, 2009).

Increased fecal coliform concentrations have been observed in the sediment as compared to the water column (Goyal et al., 1977; Gannon et al., 1983; Irvine and Pettibone, 1993; Crabill et al., 1999). Microcosm experiments suggest that sediment

associated coliforms remain culturable for 1-2 months longer than free-living constituents (Whitman and Nevers, 2003; Craig et al., 2005; Pote et al., 2009). Anderson et al. (2005) demonstrated the role of nutrient treatment (i.e., contamination, wastewater), distribution in the water column (i.e., free living, sediment associated) and *E. coli* strain type as key factors driving viability and survival. Additional studies confirm that sediments may represent a risk to patrons of recreational waters due to elevated pathogen abundances in marine and freshwater systems (LaLiberte and Grimes, 1982; Davies et al., 1995; An et al., 2002; Alm et al., 2003; Evason and Ambrose, 2006).

Nutrient input in aquatic ecosystems alters physicochemical profiles within aquatic ecosystems and the bacterial community composition (BCC). Algal blooms following nutrient loading events increase particulate matter which is colonized by resident bacteria and settles out of the water to seed the benthos (Smith, 2003). These actively respiring bacteria deplete dissolved oxygen (DO) concentrations creating hypoxic or anoxic zones in the sediment and lower water column which foster higher concentrations of clinically relevant pathogens (Gordon et al., 2008). Watershed runoff following rainfall events mobilizes fecal coliforms and anthropogenic waste into aquatic systems (Lipp et al. 2001; Noble et al., 2003) which is significant in the context of crop irrigation and frequencies in foodborne illness (Foucher et al., 2006).

Increased depth and changing oxygen availability in environmental sediments effect bacterial community composition therein (Ludemann et al., 2000). Anthropogenic wastes such as copper, lead and polyaromatic hydrocarbons create a selective force on bacterial communities of contaminated sediment (Colwell et al., 1990; Pennanen et al., 1996; White et al., 1997; Baath et al., 1998; Mendez et al., 2008; Gough et al., 2010;

Besaury et al., 2013). The bacterial communities in contaminated sediments employ multiple stress response pathways which enhances bacterial survival therein (White et al., 1997; Diaz-Ravina and Baath; 1996; Pennanen et al., 1996). Contamination induced acidification in these sediments make them suitable for investigating the response of bacterial communities to stress (Calmano et al., 1993; Mendez et al., 2008; Besaury et al., 2013).

Acid stress response (ASR) is a key adaptive feature that enhances cell survival upon exposure to extreme acidity (pH 1-4). ASR varies by organism and nutrient availability (Rowbury, 1995, Lin et al., 1996; Foster, 1999; Koutsoumanis et al., 2003) and may provide cross protection to other forms of environmental stress (Flahout et al., 1996; Rallu et al., 2000; Frees et al., 2003; DiAngelis and Gobetti, 2004; Xie et al., 2004). ASR is conserved among opportunistic pathogens where it may activate additional biochemical pathways involved in virulence, biofilm formation, chemotaxis and antibiotic resistance (Leyer and Johnson, 1992; Merrell and Camilli, 2002; Polen et al., 2003; Maurer et al., 2005; Butler et al., 2006; Hayes et al., 2006).

ASR has been investigated using model foodborne pathogens such as *Escherichia coli* and *Salmonella enterica* in response food processing and production techniques (reviewed in Swenson et al., 2013). Reports on ASR in an environmental context are lacking. One exception is the study by Bhagwat (2005) investigating the ASR capability of *E. coli* strains isolated from various international environments. However, this was a population level study. ASR capability of environmentally derived bacterial communities remains underexplored.

In the current study, we collected water and sediment from mudflats and oyster reefs in a pristine estuary, a tidally influenced blackwater tributary and experimental mesocosms with copper and polyaromatic hydrocarbon (PAH) contaminated sediment to evaluate the bacterial community response to extreme acidity. Sediments from sample sites were partitioned into attached and interstitial fractions to investigate the impact of sediment association on cell survival. We are testing the hypotheses that a) the bacterial community from sediment partitions responds more robustly to extreme acidity, and b) bacterial communities from contaminated sediments display a more robust ASR. This is accomplished by monitoring the survival of the culturable fraction of the community after exposure to acid shock. Additionally, the bacterial communities from the experimental mesocosms are assessed using molecular techniques to determine phylogenetic community structure before and after organic acid shock.

## MATERIALS AND METHODS

**Estuarine sample site and sampling.** Estuarine water and sediment samples were collected during low tide from Oyster Landing sampling station (33°20' 57.66 N, 79°11' 19.97 W) in the pristine estuary North Inlet-Winyah Bay National Estuarine Research Reserve (NERR) Belle W. Baruch Institute for Marine and Coastal Sciences near Georgetown, SC. Average water temperature and salinity at collection location were 23.9±7.1°C and 34.4±3.0 (data courtesy of NERR system). Seawater was aseptically collected in 4L polyethylene carboys and caps were secured under water to avoid presence of headspace. Sediment cores were collected in acid washed 5cm x 3.5cm coring tubes by placing core tubes vertically in exposed sediment during low tide and carefully removing cores to ensure the euphotic and anoxic partitions were not disrupted.

In addition to mudflat samples, sediment cores were also collected directly adjacent to a mature oyster reef and processed as described below. Sediment cores were capped on the bottom, stored in seawater and transported to the laboratory at *in situ* temperatures. Prior to processing sediment and water (within 5 days of sample collection), samples were stored at 20°C with a 12hour light/dark cycle.

**Sample preparation.** Seawater and sediment cores were partitioned within one week of collection as follows: Seawater samples were prepared as total seawater (TSW) which is comprised of the particle associated bacterial community. Seawater was also passed through a glass fiber filter (GFF; Watmann) with a pore size of approximately 0.7µm thus the sample represents the free-living bacterial community (FSW). Sediment samples were prepared by removing 0.1 grams of the top 3mm (Euphotic partition) and 0.1 grams from the anoxic region of the core (anoxic partition). These samples were then partitioned into the interstitial and sediment attached bacterial communities (Euph.INT, Euph.ATT, Anox.INT, Anox.ATT). This was accomplished by vigorously vortexing respective sediment samples for 5 minutes in 2mL of 0.2µm filtered freshly collected seawater. After vortexing, samples were briefly centrifuged (10,000g, 10seconds) to pellet sediment while leaving the interstitial and loosely associated bacterial community (IBC) from respective partitions in suspension. Post-centrifugation, supernatant was aspirated and transferred to respective sample tube. Sediment attached bacterial partitions were then resuspended in fresh 0.2µm filtered seawater. Sample processing is summarized below (**Fig.1**). To pellet the cells from respective water and sediment partitions, samples were centrifuged for 13,000g for 10min. Seawater supernatant was then removed and pellets were resuspended in respective laboratory broths. All partition

samples were prepared in duplicate and experiments were conducted on at least four separate occasions.

**Riverine sample site and sampling.** Water and sediment samples were collected from Sanpit Creek near Georgetown, SC (33°37.051'N 79°44.981'W). This is a tidally influenced, black water creek in the Boggy Swamp and Winyah Bay tributary. At the time of sampling, Sanpit Creek was measured to have an *in situ* pH  $5.5 \pm 0.3$  as measured by the handheld pH probe Pinpoint pH monitor (American Marine Inc.). Salinity (sal. 5) was measured via refractometer. Water samples from the Black River were centrifuged (10,000g; 1min) prior to measuring salinity to remove any suspended solids that may interfere with refractometry readings. Water samples were collected in 4L carboys as previous described. Approximately 500g sediment samples were collected in 0.5L high density polyethylene (HDPE) wide mouthed carboys attached to an extendable sampling pole at a depth of 1m. Samples were transported to the laboratory at *in situ* temperatures.

**Riverine sample preparation.** Water samples were processed to harvest the free-living and particle attached bacterial communities as described above. Due to lack of significant exposed sediment flat surface, it was not possible to distinguish euphotic and anoxic partitions within the sediment cores. As such, sediment samples originated within the creek bed and were partitioned into the interstitial and sediment associated bacterial communities as described above. To maintain isotonic conditions, sediment samples from Sanpit Creek were resuspended in 0.2 $\mu$ m filtered creek water (collected during sampling) during harvesting of the interstitial and sediment associated bacterial communities. This resulted in four sample partitions for the Black River sample site;

aquatic free-living (FRW) and particle attached (TRW) communities and the interstitial (Sed.INT) and sediment attached (Sed.ATT) bacterial communities.

**Mesocosm construction and sampling.** Mesocosms were constructed in triplicate at the Baruch Research Facility near Georgetown, SC with sediment bricks collected from Oyster Landing. Mesocosms consisted of sediment bricks in Tupperware tubs with seawater circulating on 6 hour on/off tidal cycle circulating twice daily. For treatment mesocosms, sediment bricks were overlaid with 4mm of sediment contaminated with Copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ;  $70\mu\text{g gram sediment}^{-1}$ ) and a mixed PAH's (pyrene, anthracene, phenanthrene;  $10\mu\text{g gram sediment}^{-1}$ ). Mesocosms remained at the Baruch Institute at *in situ* temperatures for experiment duration.

Sediment cores were collected in triplicate on three separate occasions from control and mixed Cu/PAH treatment mesocosms as described above but using 6.5cm x 2.5cm core tubes. Sediment cores and seawater samples were transported to the lab (USC) at *in situ* temperatures and stored at 20°C in a 12hour light/dark cycle until processing (< 5 days). Seawater used for circulation in mesocosms had previously been sand filtered (approximately 0.8µm filtered) and only this aquatic partition was examined during organic acid challenge assays. Sediment samples were partitioned into the euphotic interstitial and sediment attached (Euph.INT, Euph.ATT) and anoxic interstitial and sediment attached (Anox.INT, Anox.ATT) bacterial communities exactly as described for estuarine samples (see above).

**Grain Size Distribution of Sample Site Sediment Cores.** 5cm x 3.5cm sediment cores from respective sample sites were partitioned into the euphotic (top 2mm) and anoxic

(bottom 2mm) partitions and allowed to dry completely. The entire mass from respective partitions were separated using a Mini sieve microsieve set (Scienceware) by gently tapping the sieve so all sediment was distributed through the multi-sieve apparatus until sediment grains were partitioned into four categories according to grain size. The mass of grains of each size grouping was then measured and calculated as a percentage of the whole for each sediment partition from each sample site.

**Bacterial community acid challenge.** All experimentation was conducted on media that approximated the sample site *in situ* salinity. For all experiments except the Black River organic acid challenge, ZoBell broth (ZBB; 0.5% peptone, 0.1% yeast extract in 900mL filtered seawater + 100mL deionized water) and/or ZoBell agar (ZBA; 0.5% peptone, 0.1% yeast extract, 1.5% agar in 900mL filtered seawater + 100mL deionized water) were used for bacterial cultivation. To maintain isotonic conditions during freshwater experiments, Luria Broth (LB; 1% tryptone, 0.5% yeast extract, 0.4% NaCl in 1L DiH<sub>2</sub>O) and Luria broth agar (LBA; 1% tryptone, 0.1% yeast extract, 0.4% NaCl, 1.5% agar in 1L DiH<sub>2</sub>O) were modified from original protocol and used for the Black River organic acid challenges.

Parallel samples of the bacterial communities from each partition were either adapted prior to organic acid shock or were immediately exposed to extreme acidity (**Fig. 1**). The organic acid challenge broths consisted of the growth media (LB or ZBB) amended with organic acids. Briefly, two stocks of the respective broths were prepared for acid adaptation and acid shock by amending with HCl to pH 5.7 and 4.5, respectively. A 10x organic acid cocktail (87mM acetic acid, 25mM butyric acid and 37mM propionic acid) was prepared in broth (LB or ZBB). The acid adaptation broth was amended with



0.75x organic acid cocktail (6.5mM acetic acid, 1.9mM butyric acid, 2.8mM propionic acid; final pH 4.5) and the acid shock broth was amended with 1x organic acid cocktail (8.7mM acetic acid, 2.5mM butyric acid, 3.7mM propionic acid; final pH 3.5).

To assess the acid stress response of the bacterial community's from the water and sediment partitions, 100µl subsamples were plated in triplicate on respective agar plates prior to acid exposure. To assess the composition of the culturable bacterial community prior to organic acid shock, 100µl subsamples were plated at T<sub>0</sub>. Samples were then centrifuged (10,000g, 10min) and the pellets were resuspended in acid shock broth. All samples incubated in shock broth for 1hour at 20°C with aeration. Post-incubation, all samples were plated in triplicate on respective agar plates and incubated for 3 days at 20°C with 12hour light/dark cycle.

To assess the effect of acid adaptation on elicitation of ASR in the bacterial communities, pellets from respective water and sediment partitions were resuspended in acid adaptation broth (see above) and incubated at 20°C for 1.5 hours with aeration. After acid adaptation, 100µl subsamples were plated in triplicate. After acid adaptation, samples were centrifuged (10,000g, 10min) and the pellets were resuspended in acid shock broth (see above). Samples incubated in shock broth at 20°C for 1 hour. After this organic acid challenge, 100µl subsamples were plated in triplicate. All plates incubated at 20°C for 3 days in a 12hour light/dark cycle to ensure complete growth of all culturable bacteria. Post-incubation, colony forming units (CFUs) were enumerated and plates were retained for molecular analysis.

**Molecular analysis of culturable bacterial community.** To assess the composition of the culturable communities from experimental mesocosms, bacterial biomass was harvested from pre-shock ( $T_0$ ) and post-shock ( $T_{60}$ ) plates immediately following CFU counts. The community was harvested by resuspending biomass in sterile, 0.2 $\mu$ m filtered phosphate buffered saline (PBS, 1X). 2mL of PBS was transferred onto the surface of representative plates from each partition, sample site and time point. After addition of the PBS, the plates incubated at 20°C for 5 minutes with moderate disturbance to re-suspend the CFU biomass into a slurry. 1mL of this slurry was collected and transferred to 2mL microcentrifuge tubes. All samples were centrifuged (10,000g, 10min) to pellet biomass. Post-centrifugation, the supernatant was aspirated and the pellet was resuspended in 20 $\mu$ l of the commercially available cell lysing detergent, Lyse-N-Go™. Samples were then transferred to 200 $\mu$ l PCR tubes and cycled through thermal parameters provided by the manufacturer on Eppendorf thermal cyclers. The lysate from this step served as the DNA template for molecular analysis.

Capillary Automated Ribosomal Intergenic Spacer Analysis (ARISA) was conducted to analyze the DNA fingerprint of the culturable bacterial communities of samples before (pre-shock) and after (post-shock) organic acid challenge. The DNA concentrations of template were quantified on a Gemini EM Max (Molecular Devices; Sunnyvale, CA) using PicoGreen® according to manufacturers quantification protocol. A total of 10ng DNA was used as template during ARISA. The highly conserved ribosomal RNA (rRNA) gene was targeted for amplification with bacteria specific ITS primers (Cardinale et al., 2005) containing a fluorescent label for ARISA analysis. Samples were processed at the high-throughput genomic facility, EnGenCore in the

school of Public Health (USC). Thermal cycling parameters were as follows: 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, followed by a final extension step of 72°C for 10 min. ARISA results were analyzed to assess DNA fingerprint of bacterial communities harvested from experimental mesocosm.

**Bacterial community phylogenetic analysis.** Bands amplified during ARISA were used to determine bacterial community fingerprint in samples from water and sediment partitions before (pre-shock) and after (post-shock) exposure to extreme acidity. Banding patterns of each community were derived by comparing amplified ARISA products with molecular size standards present in each sample. Amplified bands represented operational taxonomic units (OTUs) in each sample. ARISA results were converted to binary fingerprints indicating presence/absence of OTUs in each sample. Binary bacterial community fingerprints were used to construct phylogenetic trees in SPSS v20 using Ward linkages of squared Euclidean distances.

#### **454 pyrosequencing**

Bacterial biomass was harvested from experimental mesocosm plates and retained for sequence analysis to determine identification of taxonomies within the pre- and post-shock samples from control and contaminated mesocosms. DNA template for sequence analysis was generated as previously described. To ensure sufficient template, DNA concentrations of all samples were determined with a Thermo Scientific NanoDrop 2000 spectrophotometer (Wilmington, DE, USA). 200 ng samples were prepared by pooling

the interstitial and sediment attached bacterial biomass from respective partitions and microcosms.

For pyrosequencing analysis, the conserved bacterial 16S primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') (Ludwig et al., 1993) and 338R (5'-GCTGCCTCCCGTAGGAGT-3') (Suzuki et al., 1996) were used for PCR amplification of the V1–V2 hypervariable regions of 16S rRNA genes. At its 5' end, the forward primer carried the 454-adaptor A and a sample specific 10 nucleotide sequence to barcode each sample, while the reverse primer only carried the B adaptor. PCR products were loaded into a Roche GS Flx system using vendor specified chemistries.

### **Sequence analysis tools**

Raw sequences derived from 454 pyrosequencing were processed through the RDP pyrosequencing pipeline (Cole et al., 2005). Initial processing included screening and removing of short and low quality reads. Sequences were sorted based on sequence tags and trimmed of primer and tag sequences. Derived high quality, trimmed reads were checked for artificial chimeric formations using the UCHIME algorithm (Edgar et al., 2011). The remaining sequences were taxonomically identified using the Classifier tool (Wang et al., 2007) at 50% confidence level, then aligned in preparation for clustering. Alignment was performed with the 16S Aligner tool which uses the secondary-structure aware INFERNAL aligner (Nawrocki et al., 2009). By applying the furthest neighbor approach using the Compleat Linkage Clustering application of the RDP pyrosequencing pipeline, aligned sequences were assigned to phylotype clusters of 97% identity (Stackebrandt and Goebel, 1999; Rossello-Mora and Amann, 2001; Huse, 2010).

Subsequently, diversity statistic data and rarefaction curves were obtained. The number of sequence reads per sample was normalized for number of rRNA genes according to taxonomy as select bacterial phyla have been observed expressing multiple rRNA gene copies within the genome (Lee et al., 2009). Sequence data is reported as classification percentage per sample.

## RESULTS AND DISCUSSION

### **Bacterial Community Response to Organic Acid Challenge**

Statistical analysis of CFU counts for pre- and post- shock culturable bacterial communities from various sample sites indicates that partition association within the environment is a statistically significant factor in bacterial community survival in response to organic acid shock (ANOVA,  $p < 0.001$ ). The response of the bacterial communities from the respective partitions and different sample sites demonstrated similar responses to extreme acidity. The sediment associated cells from the euphotic and anoxic regions of the sediment cores demonstrated a robust response during the organic acid challenge (**Table 6.2**). For all sample sites, sediment attachment facilitated enhanced survival to acid shock in both the unadapted and adapted samples.

The density of fecal coliform like bacteria was enumerated by plating on the Eosine-Methylene Blue (EMB) which selects for gram negative, short rods and differentiates those cells based on the ability to ferment lactose. EMB has been used historically as a means of detecting fecal coliform like bacteria (Gauthier and Archibald, 2001; Whitlock et al., 2002). In this experiment, approximately 10% of the post-shock bacterial communities grew on EMB and were observed as lactose fermenting, gram-

negative bacteria. This suggests that those cells capable of surviving extreme acidity are fecal coliform like bacteria and possibly opportunistic enteric pathogens. The findings of bacterial communities from sediments isolated from various environmental sources including oyster reefs, that are capable of ASR is significant in light of the high densities of fecal coliforms and fecal-coliform like bacteria that have been detected in sediments (Lipp et al., 2006; Nocker et al., 2004). An estimated 20-50% of fecal coliform communities adsorb to the surface of particulate matter and sediment grains in freshwater systems and nearly 90% of 0.45-10 $\mu$ m particles are colonized by fecal coliforms (George et al., 2004). Further, many waterborne enteric pathogens such as *Vibrio parahaemolyticus* and *Vibrio vulnificus*, have been detected in high densities in sediments and are commonly isolated from oysters where they represent a significant risk to human health (Zimmerman et al., 2007; Jones and Oliver, 2009).

In general, the interstitial and loosely associated bacterial communities did not demonstrate a robust response to extreme acidity. However, the interstitial community from the Black River displayed an elevated response to acid shock compared to the interstitial communities of other sample sites. It is plausible that this is due to the observed *in situ* pH of the Black River (pH 5.5) which may select for bacterial communities capable of ASR. Additionally, smaller grains (silt, clay; <0.06mm) in these samples represented a larger fraction of the sediment core. The cationic charges of these smaller sediments may bind bacterial cells or other grains with great affinity (Christiaan et al., 2008) thus enhancing retention time of the interstitial bacterial communities and facilitating increased exposure to anoxia and sub-lethal pH in the sediments.

Acid adapting the bacterial communities from the water and sediment partitions appeared to moderately improve the response to acid shock. However, acid adaptation was not a statistically significant determinant of bacterial community survival in response to extreme acidity (ANOVA,  $p > 0.05$ ). This is likely because when used exclusively, CFU counts are an insufficient means of completely assessing bacterial community response to acid shock as it does not account for taxonomic changes within the bacterial community.

### **Phylogenetic analysis**

The response of the bacterial community from the experimental mesocosms based on CFU counts resulted in survival trends similar to those observed for other environmental sites (**Fig.6.4**). CFU counts of the bacterial community attached to sediments within the anoxic region of treated mesocosm sediment appeared slightly lower for pre-shocked samples than observed elsewhere. Based on CFU counts, nearly the entire bacterial community (89%) was capable of surviving exposure to extreme acidity (**Table 6.2**). However, using CFU counts alone limits the ability to accurately assess of the impact of contamination on bacterial community structure and ASR capabilities.

Exposure to environmental contaminants has been shown to select for bacterial communities with robust stress responses (Pennanen et al., 1996; White et al., 1997; Baath et al., 1998; Mendez et al., 2008; Gough et al., 2010; Besaury et al., 2013). Thus, phylogenetic analysis was conducted on the culturable bacterial community from the experimental mesocosms harvested before and after exposure to organic acid challenge. ARISA reveals that the pre-shocked bacterial community from contaminated sediments more closely resemble the post-shock communities from the control and contaminated

mesocosms; the pre-shocked communities from control sediments form an outgroup (**Fig.6.5**). This suggests that the bacterial communities from the contaminated mesocosm experience less community structure alteration after exposure to extreme acidity than do the control mesocosm communities. Contamination in the treatment mesocosms may be selecting for bacterial communities capable of surviving contaminant exposure by activation of stress response pathways which subsequently facilitate survival to organic acid shock. These findings are consistent with previous observations (Diaz-Ravina and Baath; 1996; Pennanen et al., 1996; White et al., 1997).

### **Sequence assessment of bacterial communities in experimental mesocosms**

The biomass of the culturable bacterial community from the control and contaminated mesocosms were processed for sequence analysis to characterize the taxonomic changes in community composition as a function of contamination and organic acid exposure. Results indicate that the contaminated sediment is selecting for a greater proportion of Vibrionales than is observed in the control mesocosms (**Fig. 6.7**). However, the Vibrionales do occur in post-shock anoxic partition samples in high proportions despite not being detected in pre-shock samples. Both control and contaminated mesocosms experience a shift in abundance of Bacillales after exposure to post-shock suggesting that these bacteria are capable of ASR. Finally, Alteromonadales are detected in lower percentages in the pre-shock samples from the contaminated mesocosms as compared to control samples. However, a greater proportion of these are detected in the post-shock samples of the contaminated versus control sediments suggesting that exposure to contamination may be pre-conditioning these cells to survive extreme acidity by elicitation of stress response pathways. Interestingly, the findings of these more



representative Orders in the contaminated mesocosms are consistent with previous investigations in the sediment of anthropogenically impacted mangrove swamps where Vibrionales and Bacillales were shown to be the dominant phylotypes (Diaz et al., 2009). Further, Alteromonadales was detected as one of the most dominant phylotypes in estuarine water and sediment collected from the East China Sea which frequently experiences elevated anthropogenic input (Feng et al., 2009).

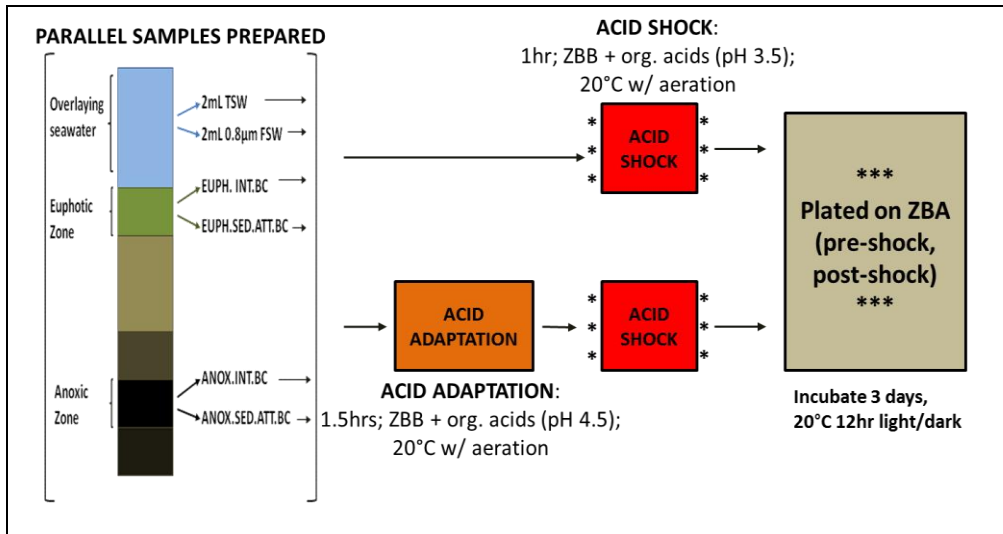
### CONCLUSION

The results of this project suggest that the sediment attached bacterial communities are capable of a robust response to extreme acidity. While the exact phenomenon driving the observed response is not completely delineated, sediment attachment in the euphotic and anoxic zone is a clear factor improving the community response to extreme acidity. It is difficult to conclude without further investigation if environmental sediment collected from the estuarine and riverine sites is protecting cells from extreme acidity or if these partitions are selecting for bacterial communities with greater ability to surviving acid shock. Future directions on such efforts should investigate the repeated response of the post-shock cultured bacterial community upon subsequent exposures to extreme acidity.

Molecular analysis suggests that contaminated sediment selects for bacterial communities responding to contaminant exposure and subsequently surviving extreme acidity. Bacterial community fingerprinting indicates that contaminated sediment communities are not experiencing substantial changes as a result of acid exposure as do the control sediment communities. Results of sequence analysis are consistent with previous efforts demonstrating certain phylotypes found in great proportions in contaminated sediment. Further, one of the phylotypes, Alteromonades, appears to be

expressing ASR in contaminated sediment which facilitates enhanced survival upon exposure to organic acids as compared to control sediments.

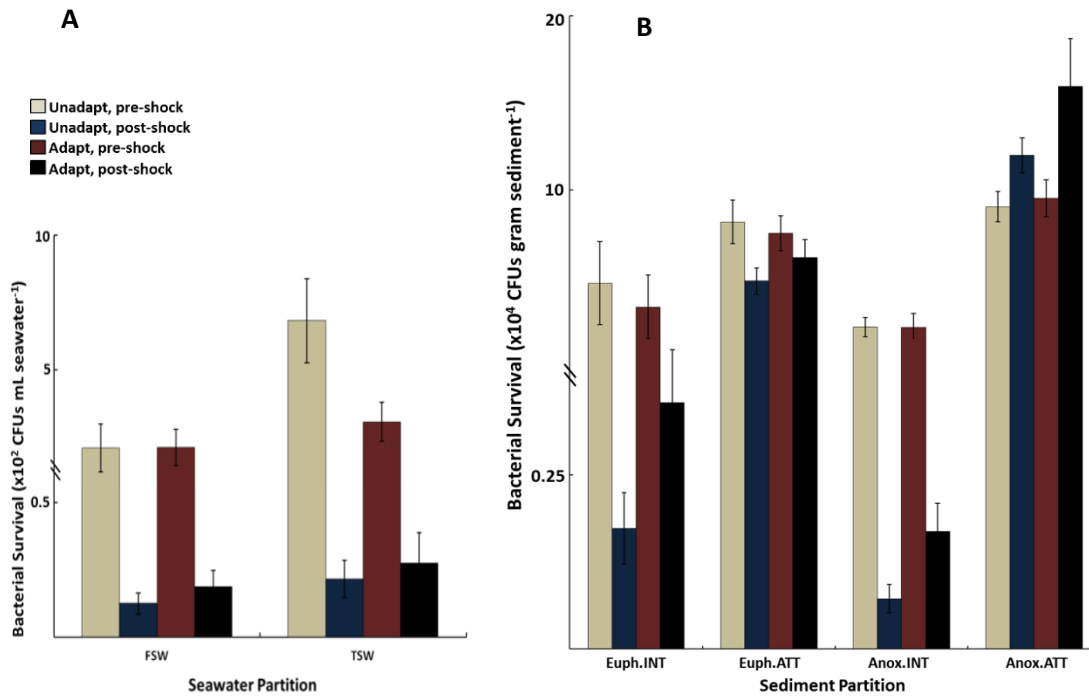
Sediment and particle attached bacterial communities have previously been shown to be very diverse with increased densities as compared to free-living communities. Sediment and particle associated communities have also been demonstrated to have an increased capacity to survive environmental stress. The findings of this work have important ecological implications in that rainfall events and disturbances mobilize sediment associated bacterial pathogens such as fecal coliforms into aquatic ecosystems used for recreation, irrigation and aquaculture. As such it is probable in the context of this work that rainfall events may be mobilizing bacterial communities from the sediment core that are expressing ASR and may therefore represent a risk to patrons of aquatic ecosystems.



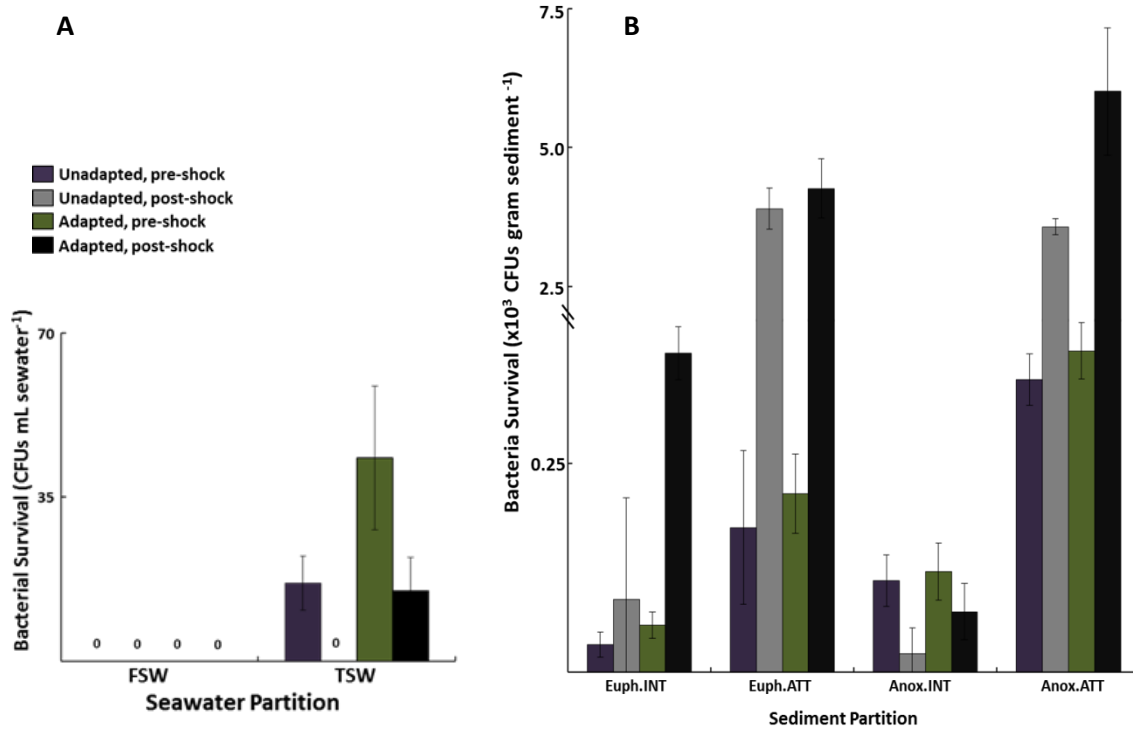
**Figure 6.1. Schematic representation of sample preparation for organic acid challenge.** Seawater was partitioned into the free-living and particle associated communities. Sediment from the euphotic and anoxic sediment zones were partitioned into the interstitial and sediment associated bacterial communities. For all but the mesocosm experiments, samples were prepared in parallel to determine effect of adaptation on bacterial community composition prior to acid shock. Samples from the mesocosm experiment did not include the FSW partition and all samples were exposed directly to lethal acidity without an adaptation phase. TSW: total seawater, FSW: 0.8µm filtered seawater, EUPH.INT.BC: interstitial and loosely associated bacterial community from euphotic partition of sediment core, EUPH.ATT.BC: bacterial community attached to sediment from euphotic partition, ANOX.INT.BC: interstitial and loosely attached bacterial community from anoxic partition of sediment core, ANOX.ATT.BC: bacterial community attached to sediment from anoxic partition. ZBB: ZoBell broth. Actrices (\*) indicate where sub-samples were plated before and after acid shock.

**Table 6.1.** Culture conditions and organic acid cocktail concentrations used in acid adaptation and shock treatments during organic acid challenge. ZBB: ZoBell broth.

<b>Acid Adaptation</b>	1.5hrs, 20°C w/ aeration ZBB + organic acids (6.5mM acetic acid, 1.9mM butyric acid; 2.8mM propionic acid) – pH 4.5
<b>Organic Acid Shock</b>	1hr, 20°C w/ aeration ZBB + organic acids (8.7mM acetic acid, 2.5mM butyric acid; 3.7mM propionic acid) – pH 3.5



**Figure 6.2. Estuarine bacterial community response to organic acid shock when cultured on the general purpose media, Luria broth agar (LBA).** The response of acid adapted and unadapted culturable bacterial communities from estuarine seawater (A) and sediment (B) partitions when cultured on general purpose media, LBA before (pre-shock) and after (post-shock) exposure to lethal acidity. Error bars represent standard deviation from four separate experiments (n=12).

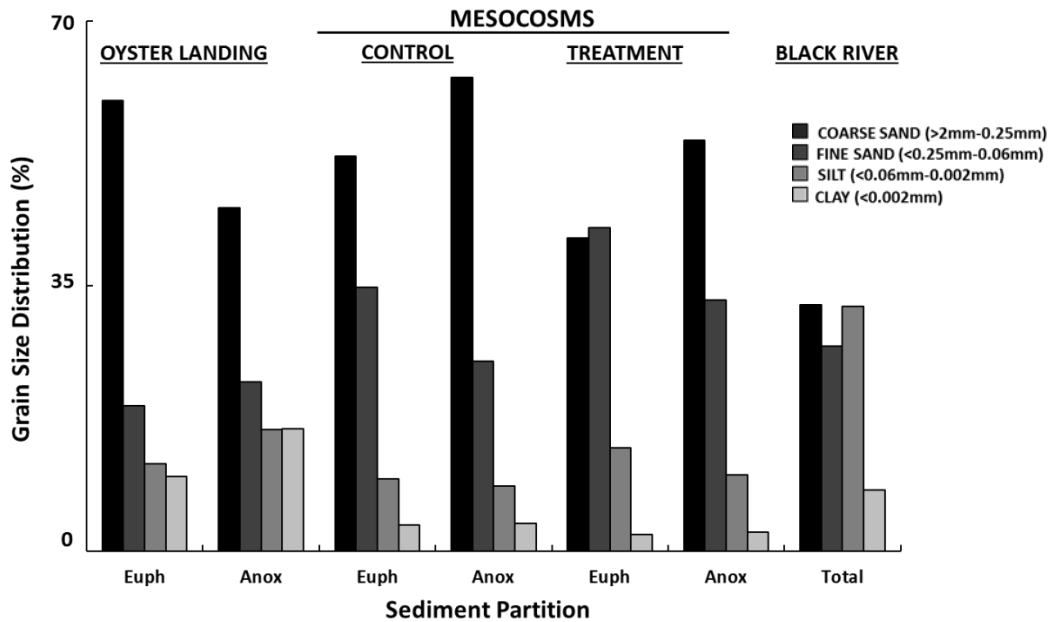


**Figure 6.3. Estuarine bacterial community response organic acid shock when cultured on the selective/differential media, Eosine methylene blue (EMB).** The response of acid adapted and unadapted culturable bacterial communities from the same sample site and partitions as in **Fig.6.2**. These estuarine seawater (A) and sediment (B) partitions were cultured on EMB which selects for gram-negative, lactose fermenting fecal coliform-like bacteria. Samples were plated before and after exposure to lethal acidity (pre-shock and post-shock respectively). 0 = no growth. Error bars represent standard deviation of triplicate samples (n=3).

**Table 6.2.** Response and survival of bacterial communities from partitions within various environmental sample sites cultured before (pre-shock) and after (post-shock) organic acid challenge. Samples from respective partitions were either acid adapted prior to organic acid challenge (adapt) or were transferred directly into acid shock without an adaptation phase (unadapt). Samples were taken from an estuarine mudflat, an estuarine oyster reef, a blackwater river as well as control and treatment experimental mesocosms. Survival % represents the fraction of the culturable community surviving organic acid challenge (post-shock/pre-shock\*100). CFUs: colony forming units, LBA: Luria broth agar, EMB: eosine methylene blue agar. Values represent mean culturable bacterial community  $\pm$  standard deviation of replicate samples. For each sample  $n \geq 9$  except for Estuary EMB ( $n=3$ ).

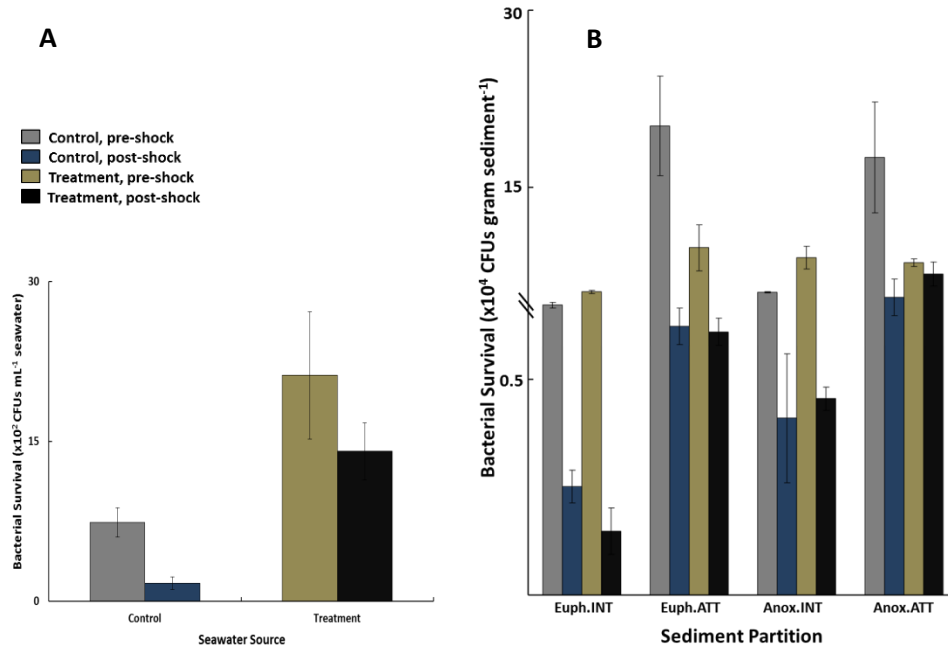
SAMPLE SITE	PARTITION	UNADAPT			ADAPT		
		Bacterial Survival (CFUs)*		SURVIVAL (%)	Bacterial Survival (CFUs)*		SURVIVAL (%)
		PRE-SHOCK	POST-SHOCK		PRE-SHOCK	POST-SHOCK	
<b>ESTUARY (LBA)</b>							
	FSW	2.1 $\pm$ 0.9	0.2 $\pm$ 0.0	7	2.1 $\pm$ 0.7	0.2 $\pm$ 0.1	9
	TSW	6.8 $\pm$ 1.6	0.2 $\pm$ 0.1	3	3.0 $\pm$ 0.7	0.3 $\pm$ 0.1	9
	EUPH.INT	4.6 $\pm$ 2.3	0.2 $\pm$ 0.1	4	3.3 $\pm$ 1.8	0.4 $\pm$ 0.1	11
	EUPH.ATT	8.2 $\pm$ 1.2	4.8 $\pm$ 0.8	58	7.5 $\pm$ 1.0	6.0 $\pm$ 1.0	82
	ANOX.INT	2.1 $\pm$ 0.6	0.1 $\pm$ 0.0	3	2.1 $\pm$ 0.8	0.2 $\pm$ 0.0	8
	ANOX.ATT	9.0 $\pm$ 0.8	12 $\pm$ 1.0	133	9.5 $\pm$ 1.1	16 $\pm$ 2.8	168
<b>ESTUARY (EMB)</b>							
	FSW	0 $\pm$ 0.0	0.0 $\pm$ 0.0	0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0
	TSW	17 $\pm$ 5.8	0.03 $\pm$ 0.0	0	43 $\pm$ 15	15 $\pm$ 7.1	35
	EUPH.INT	0.4 $\pm$ 0.2	1.0 $\pm$ 1.5	267	0.7 $\pm$ 0.2	4.6 $\pm$ 0.4	680
	EUPH.ATT	2.1 $\pm$ 1.1	39 $\pm$ 3.7	1875	2.6 $\pm$ 0.6	42.7 $\pm$ 5.4	1663
	ANOX.INT	1.3 $\pm$ 0.4	0.3 $\pm$ 0.4	20	1.4 $\pm$ 0.4	0.9 $\pm$ 0.4	60
	ANOX.ATT	4.2 $\pm$ 0.4	35 $\pm$ 1.5	850	4.6 $\pm$ 0.4	60 $\pm$ 11	1300
<b>OYSTER REEF</b>							
	FSW	50 $\pm$ 0.6	0.1 $\pm$ 0.1	0	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1	60
	TSW	59 $\pm$ 1.2	0.5 $\pm$ 0.2	1	1.9 $\pm$ 0.4	0.6 $\pm$ 0.1	32
	EUPH.INT	3.6 $\pm$ 0.1	0.1 $\pm$ 0.0	2	1.2 $\pm$ 0.4	0.1 $\pm$ 0.0	5
	EUPH.ATT	5.1 $\pm$ 0.9	3.2 $\pm$ 0.4	62	3.8 $\pm$ 0.8	5.5 $\pm$ 0.5	144
	ANOX.INT	2.7 $\pm$ 1.0	0.1 $\pm$ 0.0	4	2.6 $\pm$ 0.7	0.02 $\pm$ 0.0	1
	ANOX.ATT	5.3 $\pm$ 0.3	3.5 $\pm$ 0.6	67	7.4 $\pm$ 1.7	7.0 $\pm$ 1.2	95
<b>BLACK RIVER</b>							
	FRW	0.11 $\pm$ 0.0	0.01 $\pm$ 0	9	0.04 $\pm$ 0.0	0.01 $\pm$ 0.0	25
	TRW	1.2 $\pm$ 0.0	0.1 $\pm$ 0.0	7	0.07 $\pm$ 0.0	0.1 $\pm$ 0.0	71
	SED.INT	4.2 $\pm$ 0.0	0.2 $\pm$ 0.0	5	14 $\pm$ 5.2	1.7 $\pm$ 0.5	12
	SED.ATT	3.1 $\pm$ 1.1	2.7 $\pm$ 1.1	88	1.9 $\pm$ 0.0	2.0 $\pm$ 0.3	105
<b>EXPERIMENTAL MESOCOSMS</b>							
		CONTROL			TREATMENT		
		PRE-SHOCK	POST-SHOCK	SURVIVAL (%)	PRE-SHOCK	POST-SHOCK	SURVIVAL (%)
	TSW	0.8 $\pm$ 0.4	0.2 $\pm$ 0.1	22	1.9 $\pm$ 1.0	1.4 $\pm$ 0.7	73
	EUPH.INT	5 $\pm$ 0.3	0.3 $\pm$ 0.04	5	6.1 $\pm$ 0.1	0.1 $\pm$ 0.1	2
	EUPH.ATT	20 $\pm$ 4.2	3.2 $\pm$ 1.6	16	10 $\pm$ 2.0	2.7 $\pm$ 1.2	27
	ANOX.INT	6.1 $\pm$ 0.1	0.4 $\pm$ 0.2	7	9.0 $\pm$ 1.0	0.5 $\pm$ 0.0	5
	ANOX.ATT	18 $\pm$ 4.7	5.6 $\pm$ 1.5	31	8.6 $\pm$ 0.3	7.6 $\pm$ 1.0	89

\*Bacterial density on eosine methylene blue (EMB) reported as CFUs mL seawater<sup>-1</sup> and x10<sup>3</sup> CFUs gram sediment<sup>-1</sup>  
 \*Density for all other sample sites cultured on LBA and reported as x10<sup>2</sup> CFUs mL seawater<sup>-1</sup> and x10<sup>4</sup> CFUs gram sediment<sup>-1</sup>

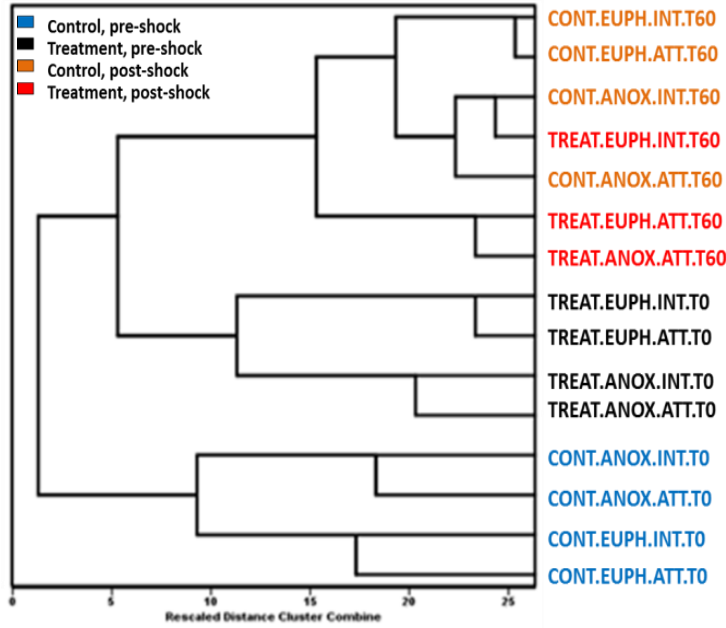


**Figure 6.4. Grain size distribution of sediment partitions from estuarine, riverine and experimental mesocosm sediment used in this study.** Sediment samples were fractionated according to grain size in a Mini Sieve Micro Sieve set (Scienceware®) by capturing sediment grains on screens with known mesh sizes. The mass of sediment captured on each screen was weighed and reported as percent of total sediment mass. Grain size analysis of euphotic and anoxic sediment was conducted for all environmental and experimental sites sampled.

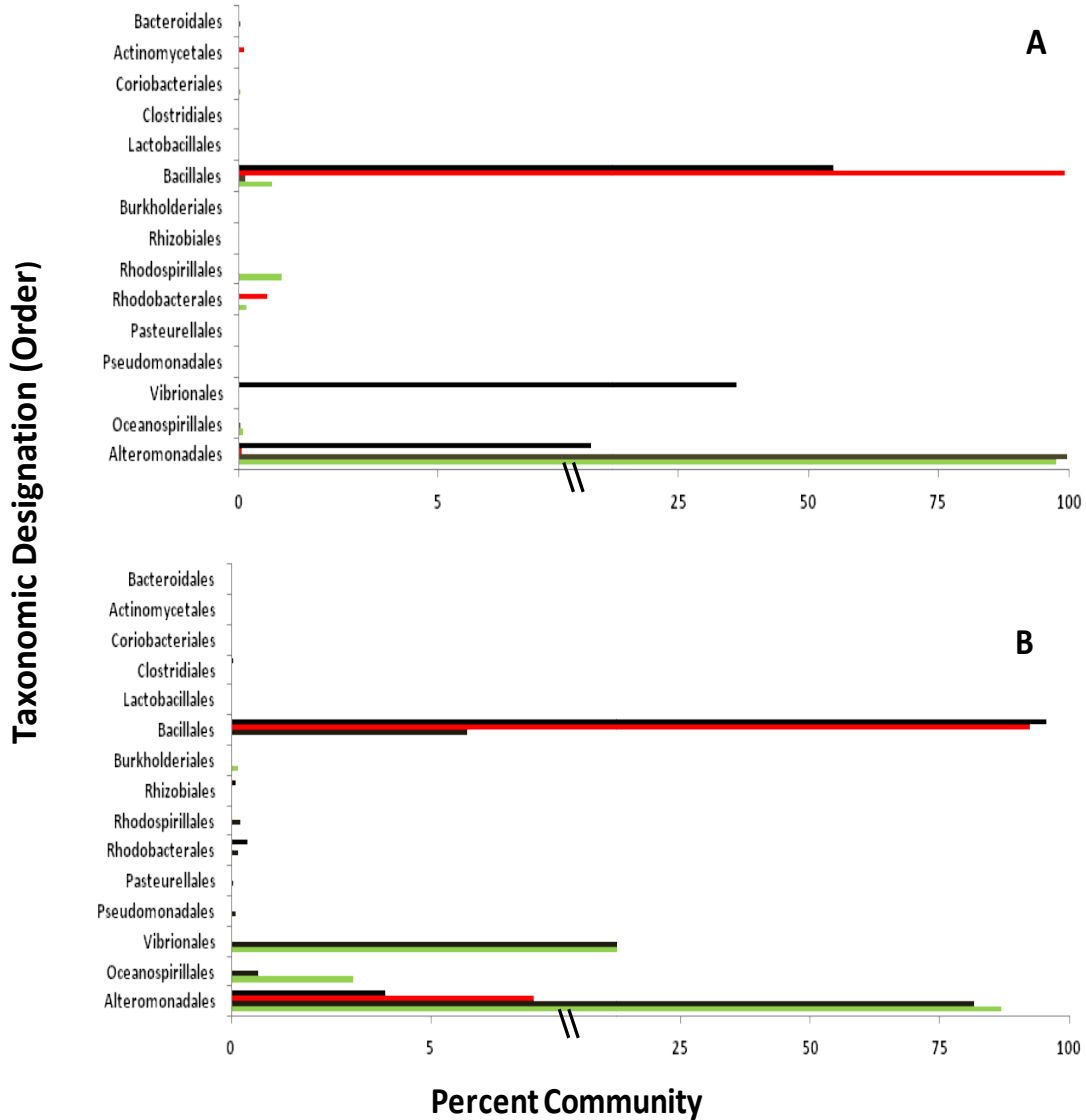




**Figure 6.5. Response of bacterial communities harvested from control and contaminated experimental mesocosms.** Mesocosms were constructed using control sediments and sediment continuously treated with copper sulfate ( $70\mu\text{g gram sediment}^{-1}$ ) and mixed polyaromatic hydrocarbons ( $10\mu\text{g gram sediment}^{-1}$ ). The bacterial communities autochthonous to the overlaying seawater (A) and sediment partitions (B) from control and treated mesocosms were plated before (pre-shock) and after (post-shock) exposure to lethal acidity. CFU counts from the partitions were enumerated after incubation. Error bars represent standard deviation of three separate experiments (n=9).



**Figure 6.6. Automated ribosomal intergenic spacer analysis (ARISA) of culturable bacterial community** from experimental mesocosms surviving organic acid challenge. Phylogenetic tree was constructed using Wards linkage of Euclidean distances in SPSS v20. Labels represent cultured bacterial communities from control and treatment mesocosms before and after exposure to extreme acidity.



**Figure 6.7. Bacterial community taxonomic designation of control (A) and contaminated (B) experimental mesocosms as determined by sequence analysis. Bacterial communities were harvested and sequenced using the Roche-454 after organic acid challenge for pre-shock euphotic sediment (■), pre-shock anoxic sediment (■), post-shock euphotic sediment (■) and post-shock anoxic sediment (■).**

## CONCLUSION

*Vibrio cholerae* is a ubiquitously distributed opportunistic enteric pathogen that is commonly isolated from marine, brackish and freshwater environments. In recent years the perspective of genotype as a determinant of pathogenic phenotype has been altered with the discovery of *V. cholerae* strains that do not possess classical toxin genes having the capacity to cause Cholera-like symptoms. And, the influence of environmental factors on the ability of *V. cholerae* to survive exposure to extreme acidity and subsequently cause harm in the mammalian host is underexplored. Finally, the response of environmentally derived bacterial communities to extreme acidity, and the influence of the environment on eliciting this response, has not been well characterized.

The goal of this dissertation research was to explore ways in which the environment influences virulence in bacterial populations and communities by elicitation of the acid stress response (ASR). Early dissertation research investigated the conservation of acid stress response in populations of *V. cholerae* isolated from environmental and clinical origins. These findings suggest that nearly half of the isolates used in the study display an ASR positive phenotype which was not determined by presence/absence of the subset of ASR genes used during screening. The findings that environmental *V. cholerae* isolates were capable of ASR suggests that this response may be important to the environmental survival of this bacterium. The finding that ASR gene sequences were more highly conserved among clinically derived *V. cholerae* genomes

and genomes isolated from Cholera endemic regions may influence the infective dose of this bacterium such that fewer cells from these regions would be required to cause disease. Future work on this effort would benefit by investigating a broader suite of genes involved in ASR as has been observed in other enteric pathogens. Of great value would be identification of genes involved in global regulation of ASR and stress response pathways that may be more specific to environmental versus clinical strains. It would be of additional interest to assess the response of these *V. cholerae* isolates to various forms of environmental stress (i.e., UV, antibiotic, oxidative, salinity) to determine what fraction of isolates used in this work are capable of broader and perhaps overlapping stress responses.

The observation that the clinical strain of *V. cholerae* N16961 is capable of prolonged growth at densities exceeding reported minimum infectious doses has value in understanding the environmental distribution and aquatic ecology of this bacterium. Moreover, the observation that brief cultivation in environmental swamp water samples significantly improved the survival of *V. cholerae* in extreme acidity as confirmed in population survival assays and gene expression assessment has significant ecological implications for this opportunistic pathogen. Future projects should investigate seasonality of the aquatic conditions eliciting ASR in *V. cholerae*. Further, it would be valuable to assess elicitation of ASR among multiple clinical and environmental *V. cholerae* populations cultivated in these aquatic conditions.

Sediment associated bacterial communities were shown to be capable of robust response to extreme acidity which may be important in understanding the ecology of infectious diseases. The finding that contaminated sediments appear to select for

bacterial communities with a robust response to extreme acidity has ecotoxicological implications as many environments in developing countries where enteric disease is prevalent often endure high levels of anthropogenic contamination in aquatic and sediment systems. Future work on this should evaluate the response of the cultured bacteria that survived organic acid shock for their individual ability to express ASR. Further, phylogenetic evaluation of the bacterial community composition in response to acid shock warrants further attention.

A robust response to acid stress confers enhanced pathogenicity in many opportunistic pathogens and investigating the environmental elicitation of ASR is important in the context of human health. Advances in techniques and renewed interest in microbial ecology are providing new opportunities for understanding the activities of bacterial populations and communities within various environmental niches. In some environments, bacteria have been investigated on the very small (i.e., micro-, milli-) scale where their activities have been shown significant in processes at the very large (i.e., regional and global) scale. Subsequently, understanding the role of individual bacteria in large scale processes is providing changing paradigms. One overarching goal of this dissertation was to explore the contribution of the environment on bacterial activity and the subsequent risk that this activity presents to human health. The findings of this dissertation may shed new light on the contribution of select environments in eliciting ASR in clinical and environmental populations of known opportunistic pathogens.

The role of select water and sediment systems in elicitation of ASR within select environments may represent a significant linkage in understanding the ecology of infectious disease. For example, it has been demonstrated that Cholera epidemics follow

regional weather systems (i.e., rainfall events, monsoons) which has in the past been attributed to physicochemical parameters (i.e., salinity) effecting regulation of virulence factors. It is also probable that rainfall events mobilize sediment grains that are heavily colonized with bacterial communities capable of ASR into aquatic systems used for human consumption. Additionally, rainfall events create temporary wetlands such as swamps and small ponds which are also used for consumption. This dissertation demonstrated that these systems effectively elicit ASR in known bacterial pathogens and that these pathogens are capable of robust growth in these systems. As such, the aquatic ecosystems that follow seasonal rainfall events may harbor dense bacterial communities capable of ASR that when consumed, represent a potential risk to human health. As such, the effect of rainfall events on transport and fostering of bacterial pathogens should be investigated with scrutiny as these may be alternate mechanisms in the spread of human disease.

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