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Improved Detection Methods for Selected Vibrio Species in the Marine Aquatic Environment

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IMPROVED DETECTION METHODS FOR SELECTED *VIBRIO* SPECIES IN THE MARINE AQUATIC ENVIRONMENT

by

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ABSTRACT

IMPROVED DETECTION METHODS FOR SELECTED *VIBRIO* SPECIES IN THE MARINE AQUATIC ENVIRONMENT

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Vibrio vulnificus, *Vibrio cholerae*, and *Vibrio parahaemolyticus* are the three most commonly encountered human pathogens in the *Vibrio* genus. They are frequent agents of foodborne illness contracted from seafood. The work presented here focuses on three aspects of detection of these bacteria. First is a novel agar formulation designed to isolate and directly enumerate *V. vulnificus* from oyster tissue by using x-gal, a chromogenic analog to lactose, as a carbon source and agent of differential morphological colony growth. Second is a field study on the relative abundance of the three main pathogenic Vibrios following the opening of the Bonnet Carre spillway to prevent the Mississippi River from flooding New Orleans. This influx of fresh water into the Mississippi sound had a significant impact on the salinity and enabled *V. cholerae* growth to dominate over the other two pathogenic Vibrios during the time the spillway was open. The third and last chapter of work presented here is the development and validation of a novel method for enumeration of *V. parahaemolyticus* in the viable but nonculturable state. This dormant state prevents the bacteria from being enumerated by standard methods. The work presented here allows enumeration of the bacteria from environmental waters in total, and when coupled with other methods, allows enumeration of the VBNC portion of the population. The work

presented in this thesis serves to further characterize these bacteria and presents novel methods for their detection that may be used to mitigate their risk to human health.

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CHAPTER I

INTRODUCTION

Bacterial infections are a major source of human disease. There are an estimated 48 million illnesses, 128,000 hospitalizations, and 3000 deaths in the U.S. annually from food-borne bacterial infections (7). With antibiotic resistance increasing, one effective way to limit these diseases is to focus on prevention. While this may be difficult with community and nosocomial acquired infections, foodborne and recreational water infection risk can be mitigated by appropriate risk assessment plans, including thorough and accurate detection of pathogenic bacteria, particularly those of environmental origin (67, 69). When dealing with environmental pathogens, rapid and reliable methods for enumeration and isolation of bacterial pathogens is an important component of any risk assessment model (67).

Marine bacteria are a common source of infection, and there are at least 30 marine genera known to be etiologic agents for both animal and human disease (67). Consumption of raw or undercooked seafood is the most common method of transmission for these marine pathogens to humans; for this reason, gastroenteritis is the most frequently contracted illness, followed by wound and skin or ear infections (67). Most of these cases are self-limiting, though underlying conditions can be exacerbating factors contributing to serious and potentially life threatening complications. Some of these factors include diabetes and liver disease (19, 57, 64). The infectious dose required to cause disease varies from one pathogen to the next and even within a species, depending on

the virulence factors present in the strain and risk factors associated with the host.

My thesis research focuses on one genus of the marine microbiota, the *Vibrio* bacteria. Vibrios, members of the *Gammaproteobacteria* class, are gram-negative, straight or curved rods that are motile by means of polar flagella. All are halophiles with varying levels of salt tolerance ranging from that of full strength sea water (salinity ~35) to fresh water thus they are endemic to the marine environment. They have not been shown to be directly associated with any type of pollution though their growth can be stimulated by allochthonous nutrients. Rather their densities are primarily dependent on sea surface temperature, and to a lesser degree salinity and chlorophyll levels (42). There are currently 90 members of the genus that have been described, and at least 12 have been established as human pathogens (19, 27). The three most common agents of human disease, which are the main focus of this research, are *Vibrio vulnificus*, *Vibrio cholerae,* and *Vibrio parahaemolyticus*. There has been a great deal of research focused on understanding these organisms and the risk they pose to human health through contact with sea water and especially through the ingestion of seafood. According to The Cholera and Other *Vibrio* Illness Surveillance (COVIS) system, in 2009, excluding toxigenic *V. cholerae*, there were 825 *Vibrio* illnesses (vibriosis) reported to the CDC with 30% (251 cases) of the total occurring in Gulf states (11). The most frequently contracted *Vibrio* in Gulf states was *V. vulnificus* (27%), followed by *V. parahaemolyticus* (21%), *V. alginolyticus* (19%), and lastly non-toxigenic *V. cholerae* (7%) (11).

It is widely considered that these statistics are a significant underrepresentation of the actual incidence of disease caused by these bacteria, as a large number of *Vibrio* infections go unreported (11, 19). This is in part because the most common illness acquired due to *Vibrio* infection is a self-limiting diarrhea that many people do not see a physician to treat. A second reason is that many hospital and clinical labs do not routinely screen stool specimens on the typical *Vibrio* isolation medium, thiosulfate-citrate-bile salts-sucrose (TCBS) agar, unless they are specifically requested to do so (19). This highlights the need to develop accurate methods of enumerating these potential pathogens in the environment so that the general public can be made aware of environmental risk and thereby minimize or prevent infections and disease.

Current Detection Methods

There are several cellular aspects than can be utilized as targets for detection of marine bacteria. The oldest and simplest is the culture based approach, where samples are collected in the field and transported to the lab to cultivate the bacteria in artificial media. These methods rely on biochemical reactions and use a combination of carbon and nitrogen sources to selectively grow and identify bacteria. Alternate methods which do not require cultivation of the bacteria include fluorescent antibody staining (FAB), polymerase chain reaction (PCR), and fluorescence *in situ* hybridization (FISH) techniques. These methods do not use cellular metabolism as distinguishing characteristics and in most cases do not require the cell to be viable. FAB methods target a surface antigen with a specific antibody tagged with a fluorescent molecule. Samples are

either concentrated on a filter or an aliquot is taken which is then fixed with a preservative to preserve cellular structure. The sample is incubated with the labeled antibody, immobilized on a microscope slide, and the labeled cells are visualized with a fluorescence, most commonly epifluorescent, microscope. PCR, one of the most widely used techniques, involves the use of gene specific primers to amplify a section of DNA. It is particularly powerful in detection of rare pathogenicity factors when coupled with an enrichment step to increase the number of pathogenic cells in the sample. FISH methods also target genetic components of the cell through the use of a fluorophore labeled oligonucleotide. Cells are fixed and the membrane is permeabilized to enable the probe to get inside and bind to the target sequence. This technique allows individual cells to be visualized as with FAB staining and individual genes to be enumerated as with PCR amplification.

Culture Based Detection Methods

Culture based detection methods are widely used in microbiology labs, particularly in clinical settings. This is because with the right combination of growth media and growth conditions, results can be distinctive and these methods do not require highly specialized equipment to produce reliable and reproducible results. There are different types of artificial media available for bacterial cultivation: general growth, selective, and differential. Each of these can be in broth or agar form.

General growth media contains the required nutrients for specific bacteria to grow and, as a general rule, does not contain ingredients which will inhibit

growth. This does not mean all bacteria will grow equally well on all types of general growth medium. As different classes of bacteria have different requirements a growth media for one class will contain ingredients that will inhibit a different class from growing. An example is halophilic verses non-halophilic bacteria; halophiles require salt in their media which will inhibit the growth of non-halophiles. These are considered general growth/non-selective medias because they contain nutrients, minerals and salts that are intended for halophilic bacteria as a whole.

Selective media also contain the necessary components for microbial growth but they also contain inhibitory compounds, omit a necessary component required by a portion of the population, or have been altered to reduce the growth of undesired microbes. This can be accomplished by the addition of components such as antibiotics or inhibitory chemicals like bile salts or specific dyes (47, 59, 68). It can also be performed by changing the availability of certain growth components such as the enriching with a single carbon source specific to the desired bacteria, an increase or decrease in the salinity, altering the pH, or a combination of these (68).

Differential media are selective media that contains components that cause distinctive growth or growth responses of specific bacteria. Some differential media include blood to detect hemolytic activity, selective sugars combined with indicator dyes to detect pH changes, or chemicals designed to detect specific enzymatic activity through color changes (68). Often a

combination of these media types is used in order to isolate and phenotypically identify an organism.

A significant obstacle with detection by culture based methods is that a large portion of the marine microbial population is not culturable with currently established methods (50). At any given time there is a portion of the normally culturable population which will not grow under artificial conditions (15, 56). This is due to the cells entering dormant phase known as the viable but non-culturable (VBNC) state. This is believed to be a result of activation of cellular mechanisms designed to help non-spore forming organisms withstand periods of nonfavorable environmental conditions (15, 56). This implies that current culture based detection methods are underestimating the actual density of the microbial population.

A second problem with culture based methods, particularly when dealing with the diverse populations found in the marine environment, is that such tests are time consuming and often subjective. The first step in phenotypic identification is isolation in pure culture. Some selective and differential media are very good at isolating the desired bacteria, but these media take time to cultivate the cells to a dense enough culture to enable an observable reaction. Also, phenotypic tests can produce variable results from different strains within a species, or even from cultured cells which have undergone mutations or gene expression changes caused by repeated passage or long term storage. This causes phenotypic identification to be cumbersome, time consuming and subjective. For these reasons non-culture based forms of detection based on

cellular characteristics (FAB) or genetic components (PCR, FISH) are often used as confirmatory methods or sometimes in lieu of culture entirely.

Fluorescent Antibody Staining

Fluorescent antibody staining techniques involve either direct or indirect labeling reactions with a fluorophore followed by visualization with an epifluorescent microscope. Direct fluorescent antibody (DFA) staining is a one-step reaction where the fluorophore is bound directly to the antibody which binds to the surface antigen on the target cell. In indirect fluorescent antibody (IFA) staining, a two-step method is employed where the cellular antigen first reacts with an unlabeled specific primary antibody and second a fluorophore labeled secondary antibody binds to the bound primary antibody. The secondary antibody is often a general antibody that reacts to the animal the primary was produced in. For example, a primary antibody that is grown in a rabbit can have a secondary antibody grown in a goat producing a reaction complex of cellular surface antigen to rabbit primary antibody to fluorophore labeled goat anti-rabbit secondary antibody. DFA and IFA techniques can be very sensitive and relatively easy assays to do and there is a wide array of commercially available antibodies that can be purchased for these assays. They are very useful in the clinical laboratory for diagnostic purposes or in research labs where the target cell is very specific (13, 38). Complications to this method arise when dealing with typing bacteria that require pre-treatment before the surface antigen is exposed and available for labeling (78). Another issue when using FAB visualization for enumeration of environmental bacteria is the sometimes vast

variability of the target antigens. Some species contain many different strains with various surface antigens making it difficult if not impossible to find a single surface antigen with which to target for enumeration (26). While this is a powerful tool for use in many diverse applications, its limitations prevented it from being a viable method for the work presented here.

Polymerase Chain Reaction

A very common and widely used method for enumeration of genes in environmental samples is the polymerase chain reaction. PCR involves the use of primers which flank a genetic region of interest and amplification of this region with DNA polymerase via either end point or real-time reactions. End point reactions are analyzed by separating the products through gel electrophoresis and staining with an intercalating dye to identify a fragment of appropriate size. Real-time assays involve either the addition of a specific fluorophore labeled probe that binds between the primers or incorporation of an intercalating fluorophore during the amplification reaction. Real-time reactions can be quantitative (qPCR) through the addition of a standard curve. The fluorescence detected in the samples is compared to that of the standard curve to quantitate the number of gene copies in each sample. PCR methods for environmental samples can be particularly useful when attempting to enumerate rare genes by employing an enrichment step. Drawbacks to this, however, include the result of relative rather than truly quantitative results and a concern that enrichment of environmental samples may cause a selection bias of different genotypes (72). Another encountered problem with direct PCR of environmental samples is the

inhibition of the reaction by naturally occurring marine chemicals (31, 61). To overcome this, the reaction may either contain a compound intended to overcome the inhibition or the DNA/RNA may be extracted prior to amplification (31).

Fluorescence in situ Hybridization

Another common method used to identify and enumerate bacteria is fluorescence *in situ* hybridization (FISH). This, like DFA and IFA, involves the use of fluorophore labeling followed by microscopic visualization. The difference here is the target is inside the cell rather than on its surface. A fluorophore is bound to a short oligonucleotide sequence to create a probe which is specific to a DNA or RNA sequence of interest. A widely used target for this type of method is ribosomal RNA. A hybridization reaction is run at a temperature sufficient to prevent non-specific binding of nucleotides. The probes each bind to only one genetic location which makes rRNA a powerful target due to its abundance within the cell resulting in a natural amplification of fluorescence. A significant limitation to the use of rRNA however, is the conserved nature of this genetic component within a genus and the requirement of a relatively short sequence for probe generation. It is a very useful tool for identifying and enumerating bacteria in a mixed environmental sample to the genus level, but when attempting to identify to the species level an alternate target is often required. For this reason many different FISH methods have been developed involving various targets, and each has its own method for signal amplification to enable visualization of the fluorophore with fewer targets for the probe to bind to.

My research focuses on the use of current methods as well as the development of novel techniques for detection and enumeration of three important *Vibrio* pathogens, *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus*. Chapter two focuses on a novel agar formulation for *V. vulnificus* that exploits a unique biochemical aspect of this species in order to directly enumerate this pathogen in oyster tissue. Chapter three focuses on the Mississippi Sound densities of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* using species specific PCR in response to a major salinity shift after the opening of the Bonnet Carre Spillway. Chapter four focuses on the development and validation of a novel method for the detection of *V. parahaemolyticus* that has entered the VBNC state using Recognition of Individual Gene – Fluorescence *In Situ* Hybridization (RING-FISH).

CHAPTER II

A NOVEL AGAR FORMULATION FOR ISOLATION AND DIRECT ENUMERATION OF VIBRIO VULNIFICUS FROM OYSTER TISSUE

Abstract

A new selective and differential medium, *Vibrio vulnificus* X-Gal (VVX), was developed for direct enumeration of *Vibrio vulnificus (Vv)* from oyster samples. This agar utilizes cellobiose and lactose as carbon sources, and the antibiotics colistin and polymyxin B as selective agents. Hydrolysis of 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (x-gal), used in the agar as a lactose analog, produces an insoluble blue dye that makes lactose positive colonies easily distinguishable from any non-lactose fermenting bacteria. Various bacterial species were spot plated onto thiosulfate-citrate-bile salts-sucrose agar (TCBS), and CHROMagar Vibrio, two vibrio-specific selective agars, non-selective agar, and VVX to compare selectivity of VVX to other widely used media. A *V. vulnificus* pure culture was serially diluted on VVX and non-selective agar to determine the VVX percent recovery. Water and oyster samples were spread plated on VVX agar and allowed to incubate for 16-18 hours at 33°C. Blue and white colonies from VVX agar were picked and screened by end point PCR for the *Vv* hemolysin *vvhA*. VVX agar showed a significant improvement over TCBS and CHROMagar at preventing non-target growth. There was an 87.5% recovery compared to non-selective plating and a 98% positivity rate of blue colonies picked from oyster tissue plating. The findings suggest this new

agar is a fast, distinctive, and accurate method for enumeration of *V. vulnificus* from the environment.

Introduction

Vibrio vulnificus represents a significant health threat to certain populations causing gastroenteritis, wound infections that may become necrotic, septicemia, necrotizing fasciitis, and death (64). Such infections are often contracted by the consumption of raw or undercooked seafood, especially oysters (17, 51, 75). Due to public health risk, it is important to have a rapid and reliable method for isolation and enumeration of *V. vulnificus* densities. The current standard method is a two-pronged approach involving both most probable number (MPN) sample enrichment, and direct plating of environmental samples, mainly water, oyster homogenate, and sediment, onto agar plates followed by overnight incubation and transfer of the colonies to filter paper discs (24). The discs are then hybridized with an alkaline phosphatase conjugated gene probe for the *V. vulnificus* cytolysin gene *(vvh)* (76). The enriched samples are screened via polymerase chain reaction (PCR) for presence/absence of the *vvh* gene followed by comparison of the results to a MPN table to determine the density of the gene present in the parent sample (35, 73). A few important drawbacks to these methods include the potential for PCR reaction inhibition in the complex oyster matrix, the time required to obtain the results, and the wide confidence intervals inherent in the MPN. There are other differential and selective agars that have been developed for *V. vulnificus* but some of these agars use highly stringent growing conditions, contain many specialized

ingredients making them expensive and difficult to prepare and/or they promote the growth of other species that mimic *V. vulnificus* morphology causing difficult to interpret results (12, 47, 48, 76).

V. vulnificus, originally referred to as the lactose-positive *Vibrio*, was first reported in 1976, and it was this fermentation characteristic which distinguished it from *V. parahaemolyticus* and *V. alginolyticus* (37). The agar formulation presented here makes use of that unique trait by including lactose and 5-bromo-4chloro-3-indolyl-beta-D-galactopyranoside (x-gal), a chromogenic analog to lactose, as carbon sources for the bacteria. The lack of available carbon sources other marine bacteria can utilize, combined with the presence of inhibitory antibiotics to restrict non-target growth, allow for growth conditions at a temperature (33°C) near optimum for the growth of *V. vulnificus* (57). Building on previous studies this new agar formulation, hereafter referred to as VVX, has shown improved selectivity over commonly used agars without the need of stringent growth conditions that may limit recoverability.

Here, I report the preparation and validation of the novel agar formulation for detection and enumeration of *V. vulnificus* in oyster tissue samples. With a high selectivity and sensitivity this new selective, differential agar potentially will be a benefit to assessment of the risk this bacteria poses to foodborne illnesses.

Materials and Methods

Bacterial Cultures

Control strains used in initial recoverability and selectivity tests and quality control for each batch prepared were maintained on 2-mL T1N3 slants [10g

tryptone (EMD Chemicals, Darmstadt, Germany), 30 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), 20 g agar (Alfa Aesar, Heysham, Lancaster), 1 L deionized water]. The three main *V. vulnificus* cultures 99-645, BUF 7211, and ATL 7-1503 (FDA, A. DePaola) were maintained on T1N3 agar plates and passed every 2-3 weeks to fresh plates.

Preparation of the VVX agar

Based on widely used *Vibrio* media recipes and the scientific literature, various ingredients were tested for potential use in VVX agar. VVX agar is prepared in three solutions. Solution one contains the nitrogen source, salts and agar dissolved in 900 mL deionized water. The pH is adjusted and the base is boiled to dissolve the agar prior to autoclaving for 15 minutes at 121°C for formulations without Sodium Deoxycholate. Solution two contains x-gal solution either purchased as a ready-to-use stock at either 50 or 20 mg/mL or prepared from powder by adding 5mL N'N'-dimethylformamide (Fisher Scientific, Fair Lawn, NJ) to 1g powdered x-gal (G-Biosciences, St. Louis, MO) to yield a 200-mg/mL solution. Solution three contains the antibiotics, sugars, and, in some formulations, sodium deoxycholate. Solution three was filtered using a Millipore 0.2-µm filter cup with collection bottle (Millipore, Billerica, MA) and added with solution two after tempering solution one to ~50°C.

Formulation A1 contained 20 g bacto-peptone (Becton Dixon, Franklin Lakes, NJ) 10 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), 5 g magnesium chloride heptahydrate (EMD Chemicals, Darmstadt, Germany), 5 g potassium chloride (Mallinckrodt Baker Inc., Phillipsburg, NJ), 15 g agar (Alfa

Aesar, Heysham, Lancaster), 100 mg x-gal (Promega, Madison, WI), 5 g D-cellobiose (MP Biomedicals, Solon, OH), 0.1 g lactose (Mallinckrodt Baker Inc., Phillipsburg, NJ), $1x10^6$ U of colistin sodium metanesulfate (Sigma Aldrich, St. Louis MO), and $4x10^5$ U of polymyxin B (USB Company, Cleveland, OH) in 1 L of deionized water at pH 8.5.

Formulation A2 contained an addition of 1 g/L of sodium deoxycholate (Alfa Aesar, Heysham, Lancaster) to formulation A1 with a drop in pH to 8.2.

Formulation B1 contained a reduction to 0.5 g/L sodium deoxycholate (Alfa Aesar, Heysham, Lancaster) with no other changes.

Formulation C1 contained per liter 5 g yeast extract (Acros Organics, Morris Plains, NJ), 10 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), 5 g magnesium chloride heptahydrate (EMD Chemicals, Darmstadt, Germany), 5 g potassium chloride (Mallinckrodt Baker Inc., Phillipsburg, NJ), 15 g agar (Alfa Aesar, Heysham, Lancaster), 5 g of D-cellobiose (MP Biomedicals, Solon, OH), 0.1 g of lactose (Mallinckrodt Baker Inc., Phillipsburg, NJ), $1X10^6$ U colistin sodium metanesulfate (Sigma Aldrich, St. Louis MO), $4X10^5$ U polymyxin B (USB Company, Cleveland, OH) and 1 g of sodium deoxycholate (Alfa Aesar, Heysham, Lancaster) at pH 8.2.

Formulation D1 contained an increase in sodium chloride (Fisher Scientific, Fair Lawn, NJ) over formulation C1 to 20 g/L with no other changes.

Formulation E contained an increase in sodium chloride (Fisher Scientific, Fair Lawn, NJ) over formulation C1 to 15 g/L and a reduction in sodium deoxycholate (Alfa Aesar, Heysham, Lancaster) to 0.75 g/L.

Formulation B2 contained 4 g yeast extract (Acros Organics, Morris Plains, NJ), 10 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), 4 g magnesium chloride heptahydrate (EMD Chemicals, Darmstadt, Germany), 4 g potassium chloride (Mallinckrodt Baker Inc., Phillipsburg, NJ), 15 g agar (Alfa Aesar, Heysham, Lancaster), 5 g of D-cellobiose (MP Biomedicals, Solon, OH), 0.1 g lactose (Mallinckrodt Baker Inc., Phillipsburg, NJ), $1X10^5$ U colistin sodium metanesulfate (Sigma Aldrich, St. Louis MO), $1X10^5$ U polymyxin B (USB Company, Cleveland, OH) at pH 8.5.

Formulation C2 differed from B2 only by increasing to 1 g/L D-cellobiose (MP Biomedicals, Solon, OH).

Formulation D2 contained a substitution of potassium phosphate (Fisher Scientific, Fair Lawn, NJ) for potassium chloride in formulation B2.

Formulation E2 contained an addition of 0.1 g/L sodium deoxycholate (Alfa Aesar, Heysham, Lancaster) to formulation B1.

Sensitivity Testing and Percent Recovery

Formulations A, B2, C2, D2 and E were tested for recovery of *V. vulnificus* from pure culture. A 1-µL loop of heavy BUF 7211 growth was added to 25 mL of 10X alkaline peptone water (APW)(100 g Bacto-Peptone (Becton Dixon, Franklin Lakes, NJ), 10 g sodium chloride (Fisher Scientific, Fair Lawn, NJ) in 1 L distilled water) and allowed to grow overnight at room temperature (~25°C) Five 1:10 dilutions were made with phosphate buffered saline (PBS)(0.58 g Sodium dihydrate phosphate (Fisher Scientific, Fair Lawn, NJ), 2.5 g sodium monohydrate phosphate (Fisher Scientific, Fair Lawn, NJ), 8.5 g sodium chloride

(Fisher Scientific, Fair Lawn, NJ), in 1L DI water) of this stock culture. The dilutions were spot plated by adding a 10-µL drop of each well mixed dilution in series around all VVX formulations.

The chosen formulation was tested for percent recovery compared to T1N3 agar. The lowest dilution from the sensitivity testing was diluted 1:2 eight times and spot plated as above on VVX and T1N3. The plates were incubated overnight at 33°C and CFU/mL was determined for the 1:64 and 1:256 dilutions. The CFU/mL for these two dilutions was averaged and the percent recovery of VVX to T1N3 was calculated.

Specificity Testing

A total of 26 different bacterial isolates (Table 1) were tested for their ability to grow and/or produce blue colonies on VVX agar.

Table 1

Isolates Tested for VVX Specificity

Isolate Name	Type
V. alginolyticus D-04-1	Clinical
V. alginolyticus 2010-001	Environmental
V. alginolyticus AQHL-007	Shrimp Hemolymph
V. alginolyticus	Environmental
V. campbellii BAA-1116	ATCC
V. cholerae 14035	ATCC
V. cholerae 11623-TT	ATCC

Table 1 (continued).

V. cholerae 2010-003	Environmental
V. cholerae 2010-014	Environmental
V. fischeri ES114	Squid
V. fischeri VLS2	Squid
V. harveyi RS	Red Snapper
V. harveyi C	Crab
V. mimicus	Crab
V. splendidus AF1012	Environmental
V. vulnificus 99-645	FDA
V. vulnificus BUF 7211	FDA
V. vulnificus ATL 7-1503	FDA
V. vulnificus 2009-002	Environmental
V. vulnificus 2010-006	Environmental
V. vulnificus	Crab
Enterobacter aerogenes	ATCC
Enterococcus faecalis	ATCC
Escherichia coli	ATCC
Photobacterium damsel sub. *damselae*	Red Snapper
Shewanella algae	Environmental
Staphylococcus aureus	Clinical

Many *V. vulnificus* strains, *E. coli*, *E. aerogenes*, and *E. faecalis* were tested for growth by streaking across a VVX plate with a sterile toothpick. All other isolates were plated using a 48-prong replicator. Three 8-well rows of a sterile 96-well plate were filled with 150 µL of 10X APW followed by inoculation of a 1-µL loop of growth from the corresponding isolate slant. The inoculated plate was incubated overnight at 33°C and checked for turbidity. The replicator was flame sterilized and cooled with sterile PBS prior to, and between each inoculated plate. Formulation B2 and C2 VVX agars were stamp plated along with T1N3 to verify the viability of the cultures, and CHROMagar™ Vibrio (CHROMagar, Paris, France) and TCBS (Becton Dixon and Co, Sparks, MD) as comparisons to other widely used differential media.

Sea water, sediment, and oyster samples were collected from various sites to validate the use of VVX with environmental samples. Water and sediment were collected in sterile containers. The water was vigorously agitated (25 times in 7 seconds) and 1-mL aliquots were plated; sediment was mixed with an equal weight of PBS and 0.01 and 0.05 g were plated; oysters were washed, shucked, and blended with an equal volume of PBS and 0.01- and 0.1-g aliquots were plated on T1N3 and VVX agar plates. Plates were incubated at 33°C for 16-18 hours. VVX plates were inspected for blue colonies and colonies were lifted from T1N3 plates following the procedure of Nordstrom et al. for subsequent probing with *vvh* direct plating/colony hybridization probe (53). Briefly, colonies were transferred to Whatman 541 filter paper discs (GE Lifesciences, Piscataway, NJ), cells were lysed with 1 mL lysis buffer [0.5 M

sodium hydroxide (Fisher Scientific, Fair Lawn, NJ), 1.5 M sodium chloride (Fisher Scientific, Fair Lawn, NJ)], heat fixed by microwaving on high until dried, treated with 2 M ammonium acetate (Fisher Scientific, Fair Lawn, NJ) and washed with 1X standard saline citrate solution [8.77 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), and 4.41 g sodium citrate dehydrate (Fisher Scientific, Fair Lawn, NJ)]. Filter discs were treated with proteinase K, (Promega, Madison, WI) hybridized with an alkaline phosphatase labeled probe (DNA Technology, Risskov, Denmark), and visualized with nitro blue tetrazolium chloride 5-Bromo-4chloro-3-indoyl phosphate, toluidine salt (NBT/BCIP) substrate (Roche Diagnostics, Indianapolis, IN) as previously described by Cook *et al.* (18).

Oysters were also collected and temperature abused for testing. The oysters were held at 33°C in raw sea water collected at the time of harvest for two hours followed by ambient storage (~22°C) overnight prior to shucking and plating as described above.

Isolated blue colonies on VVX agar were picked with a sterile toothpick and identified. Colonies were inoculated into a 96-well plate containing 100µL 10X APW, grown until turbid and stamped using a 48-prong flame sterilized replicator onto T1N3 plates. The plate was spun at 2250g for 5 minutes; cell pellets were washed with PBS to remove traces of broth, and then lysed in 30 µL deionized water. Cellular debris was pelleted by gentile centrifugation at 2250 g and lysate was screened for *vvhA* via PCR for identification as *V. vulnificus.* Colony PCR conditions were as follows, initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for

45 seconds, and a final extension of 72°C for two minutes. Products were screened on a 1.5% agarose gel for a 411-bp amplicon.

Results and Discussion

It has been established that ingestion of raw or undercooked oysters is a common source of *V. vulnificus* infection for susceptible individuals (17, 51, 57). For this reason it is important to have a rapid and reliable method of enumerating these potentially debilitating microbes to reduce the risk they may pose. The specificity and sensitivity of the VVX agar formulation presented here in detecting *V. vulnificus* through simple and distinctive colony morphology, has shown that this new formulation can be a significant benefit to the rapid assessment of *V. vulnificus* densities in oyster tissue.

The original formulation for VVX agar with bacto-peptone as the base was very successful at recovery of *V. vulnificus* but testing revealed that many colonies exhibiting the blue colony morphology were not *V. vulnificus*. At this point sodium deoxycholate was added to the formulation in an attempt to remove the undesired colonies; it was added at a concentration found in other Gram-negative and *Vibrio* specific agars. This addition proved to cause significant inhibition of *V. vulnificus* recovery. The switch to yeast extract was driven by a notation in Bergey's manual stating that *V. harveyi* is able to utilize lactose in peptone-type media (28). The greatest recovery coupled with the best selectivity occurred in formulations B2 and C2; therefore, only these were subjected to environmental sample testing.

VVX agar shows a significant reduction non-target growth, with only one isolate tested showing a similar color change. This isolate, *V. mimicus*, did not produce good growth exhibiting a severely reduced colony size that, it is theorized, will be easily out-competed in mixed culture (Figure 1).

Table 2

Plate Map Showing the Arrangement and Selection of Isolates Tested on Stamp Plates for Selectivity

Row 1	Row 2	Row 3
V. harveyi 120	*S. algae*	*V. splendidus* AF1012
V. harveyi 151	*V. vulnificus* 2010-006	*P. damselae*
S. aureus	*V. vulnificus* 2009-002	*V. vulnificus* Crab
V. campbellii BAA-1116	*V. cholerae* 2010-003	*V. alginolyticus*
V. cholera 14035	*V. cholerae* 2010-014	*V. mimicus* Crab
V. cholerae 11623-TT	*V. alginolyticus* 2010-001	*V. harveyi* Red Snapper
V. fischeri ES114	*V. alginolyticus* D-04-1	*V. harveyi* Crab
V. fischeri VLS2	*V. alginolyticus* AQHL-007	

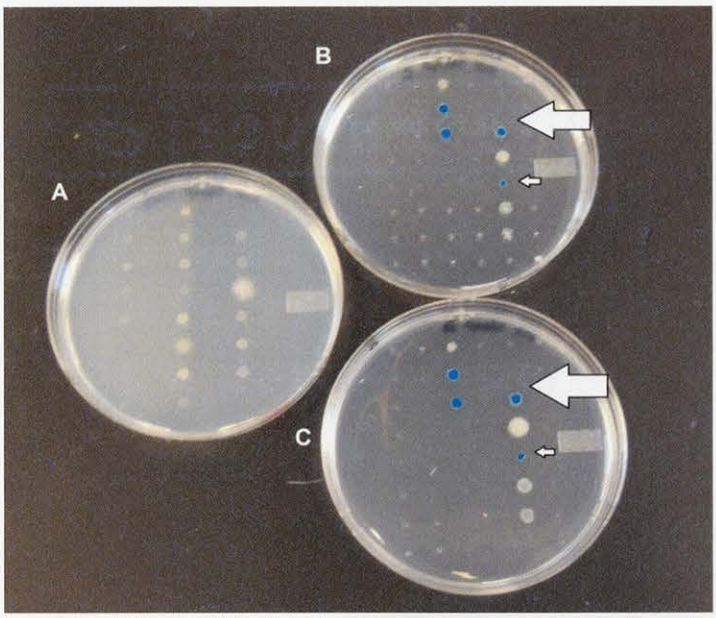

Figure 1. Stamped Plates for Selective Growth. A: T1N3 shows the viability of all organisms, B and C are VVX. The large arrow points to three tested *V. vulnificus* isolates, the smaller arrows point to *V. mimicus.*

VVX agar shows a marked reduction in non-target growth over other agars currently used to isolate and identify *V. vulnificus* in environmental samples (Figure 2).

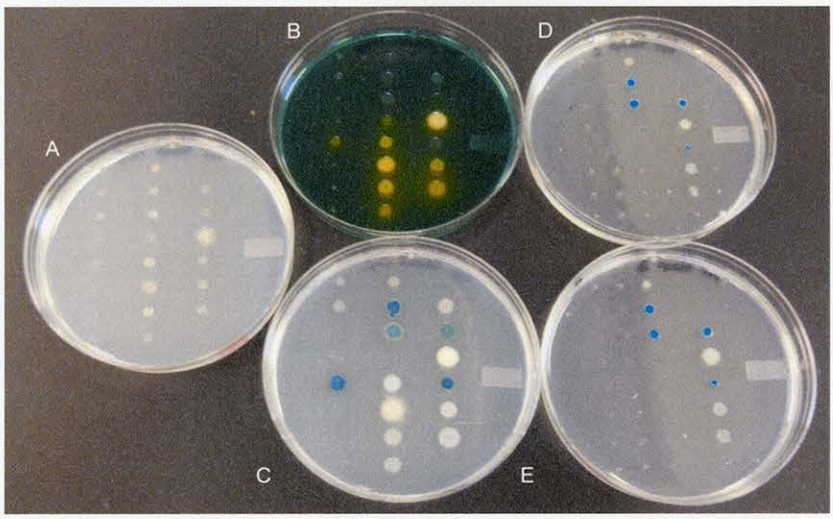

Figure 2. Stamped Plates Showing the Selectivity of VVX Agar Compared to Three Others. Plate A is T1N3, Plate B is TCBS, Plate C is ChromAgar, Plates D and E are VVX.

The recovery of serially diluted samples on VVX and T1N3 are shown in Table 3.

Table 3

Dilution Plate CFU Counts for Percent Recovery Determination

	VVX CFU/mL	T1N3 CFU/mL
1:64	1100	1400
1:256	300	200
Average	700	800

The calculated percent recovery of VVX agar from this data is 87.5%. The data from the 1:128 dilution was not included in this average due to an obvious dilution or pipetting error. The selectivity of both B2 and C2 formulations were relatively equal as was the sensitivity, though B2 showed a slightly improved recoverability. For this reason, formulation B2 was chosen for the remainder of the validation studies.

The main target sample for enumeration with VVX agar is oyster tissue as this is the most common source of human infection (57). For this reason only limited studies were completed with sediment and water samples. Sediment samples showed an unacceptable number of non-*V. vulnificus* colonies exhibiting typical morphology, and it is therefore determined that VVX is not suitable as a single-step identification method for this milieu. Water samples however, did show promising results. Due to reduced recovery of *V. vulnificus* when sea

surface temperatures are low, there were few colonies recovered from water with which to test selectivity. With the few that were recovered from raw seawater there was a 60% positive rate for typical morphology. It is expected that the low N number (5) that was available for water assessment skewed the results and with a higher density in the water column, the percentage of positive isolates would be greater. This preliminary work suggests that VVX agar has a high possibility for being a viable one-step method for water enumeration when densities are high in warm months.

Studies have shown that oysters kept at ambient temperature post-harvest have significantly higher levels of *V. vulnificus* than at the time of harvest (17). Based on this knowledge, and due to the fact that *V. vulnificus* levels were low at the time of year this validation work was being done, oysters were collected and temperature abused at a temperature common to northern Gulf coastal waters in the summer, increasing the level of recoverable *V. vulnificus.* The number of blue colonies increased from zero to one colony recovered from fresh harvest to more than 300 from abused oysters enabling a thorough examination of a large collection of isolates. Figure 3 shows the agarose gel from the *vvh* confirmation PCR of 95 blue colonies picked randomly from the oyster plates.

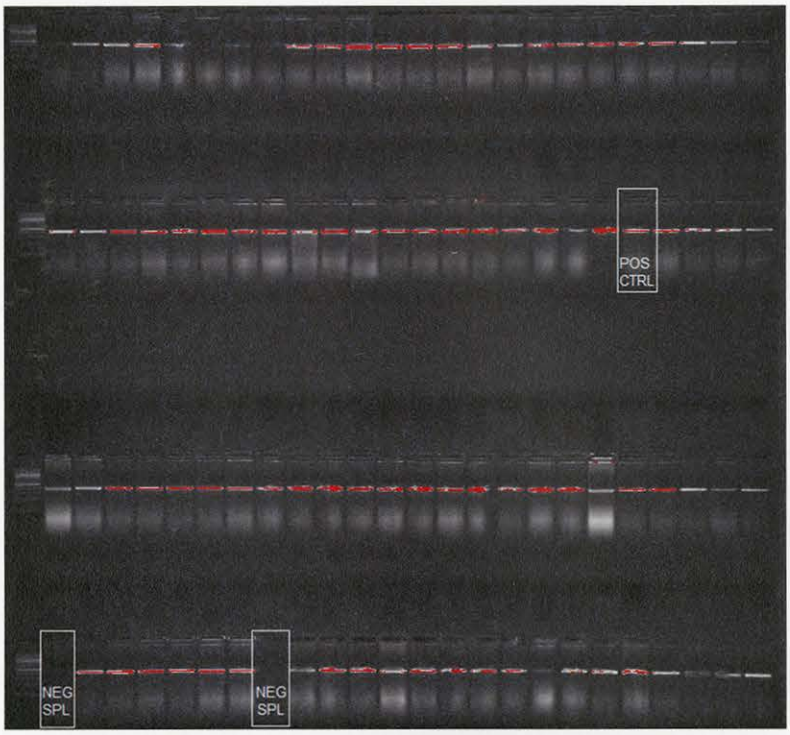

Figure 3. Agarose Gel Confirmation of Blue Colonies Picked From VVX Oyster Plates. The 411bp fragment is confirmation of the amplification of the *vvh V. vulnificus* gene. The positive control and two negative samples are indicated.

With only two out of 95 samples not producing the 411-bp *vvh* fragment, this shows 98% of the typical morphology sampled was confirmed as *V. vulnificus* from oyster homogenate.

The work presented here shows the validation of a novel agar formulation that appears to be efficacious for the detection and enumeration of *V. vulnificus* in oyster tissue. The results indicate it may also be useful for water enumeration when the density is high. The combination of high specificity and percent recovery, as compared to non-selective media, indicates that single step phenotypic identification of *V. vulnificus* growth from VVX agar is a reliable method for enumeration of the bacterial density in oyster tissue.

CHAPTER III

ABUNDANCE AND DISTRIBUTION OF VIBRIO CHOLERAE, VIBRIO PARAHAEMOLYTICUS, AND VIBRIO VULNIFICUS FOLLOWING A MAJOR FRESHWATER INTRUSION INTO THE MISSISSIPPI SOUND

Abstract

In response to the major influx of freshwater to the Mississippi Sound following the opening of the Bonnet Carre Spillway, water samples were collected from three sites along the Mississippi shoreline to assess the impact of altered salinity on three pathogenic Vibrio species. Salinity readings across the affected area during the sample period ranged from 1.4 to 12.9 (mean = 7.0). Analyses of the data collected showed a reduction in densities of *Vibrio parahaemolyticus* and *V. vulnificus* with a concurrent increase of *V. cholerae* numbers, with *V. cholerae* becoming the only *Vibrio* detected once readings dropped to 6. Follow-up samples taken in early 2012 after recovery of the salinity in the sound show that the relative densities of the three pathogenic Vibrios had reverted back to normal levels. This study shows that while the Spillway was open but a few weeks and the effects were time limited; the Mississippi River water had a profound, if temporary, effect on *Vibrio* ecology in Mississippi Sound.

Introduction

Vibrio parahaemolyticus, Vibrio vulnificus and *Vibrio cholerae* are three important human pathogens in the *Vibrio* genus. *V. parahaemolyticus* is often an agent of foodborne disease contracted through ingestion of contaminated, raw or

undercooked seafood, but it can also cause wound infection through exposure to seawater (19). *V. vulnificus* is also a foodborne pathogen contracted through ingestion of seafood and it causes a self-limiting diarrheal disease, though with complicating factors can proceed to septicemia and death. It also can cause necrotic wound infections through contact with sea water containing *V. vulnificus* (19, 57). *V. cholerae* is the etiologic agent for the disease Cholera, a well-established human pathogen, characterized by severe rice-water diarrhea, nausea, and vomiting which quickly leads to dehydration, and without rehydration therapy, death. While infections in the United States with *V. vulnificus* and *V. parahaemolyticus* can be relatively frequent, *V. cholerae* infections are rare despite the fact that *V. cholerae* is endemic to coastal waters (6, 11, 25). This lack of Cholera diseases is largely owing to the advent of modern water and sewage treatment systems eliminating potable water fecal contamination. The reduced infection rate of environmental and a foodborne *V. cholerae* may be due to the endemic bacterial population lacking the necessary genetic components required to cause disease. There are three predominant factors which determine the virulence of *V. cholerae* strains: the presence of the CTXΦ phage that produces the toxin, the presence of the toxin coregulated pilus (TCP) operon which enables colonization, and possession of the 01 or 0139 surface antigen, which are present in pandemic strains, as a part of their lipopolysaccharide (30, 60).

In order for *V. cholerae* to cause Cholera it must possess the CTXΦ phage. Presence of this phage along with the *toxR* gene which regulates its

expression, and the toxin coregulated pilus (*tcpA*), essential for colonization of the intestine, allow for the production of the cholera toxin endotoxin which causes the hallmark diarrheal symptoms (21, 36). These two genes are clustered in the larger of the two chromosomes in *V. cholerae* which facilitates horizontal gene transfer between toxigenic and non-toxigenic strains (21, 50).

There are more than 150 serogroups of *V. cholerae* that have been reported and, until 1992, all pandemic agents possessed the 01 surface antigen as part of their lipopolysaccharide layer (2). In 1992, a new serogroup, 0139, emerged as the cause of outbreaks in India and Bangladesh (2, 50). Not all 01 or 0139 strains of *V. cholerae* contain *ctx* and not all strains that are *ctx*+ are members of these serotypes, but the prevalence of these serotypes with pandemics makes them important markers for disease (29).

V. cholerae possess a rare ability in the *Vibrio* genus in that it has a wide salinity tolerance from <1-25 (16). Among the Vibrios, only *V. mimicus* shares this ability to withstand and grow at such low salt concentrations (28). This characteristic became noteworthy to the Gulf Coast ecology recently when, in May 2011, the U.S. Army Corps of Engineers opened the doors in the Bonnet Carre Spillway to prevent the Mississippi River from flooding New Orleans. The spillway, opened on May 11th, released an estimated 21.8x10^{9} cubic meters of freshwater into Lake Pontchartrain which migrated through Lake Borgne and into the Mississippi Sound. This large volume of water is roughly equivalent to 3.3 Lake Ponchatrains (Erick M. Swenson, unpublished data).

Figure 4. Google Earth View of the Freshwater Flow Path. Water from the Bonnet Carre Spillway flowed into Lake Pontchartrain, through Lake Borgne, and into the Mississippi Sound. Copyright: Google Earth, 2012

Figure 5. 2008 View of the Opening of the Bonnet Carre Spillway. Copyright: NASA Earth Observatory (earthobservatory.nasa.gov)

Based on the knowledge that *V. cholerae* is endemic to the Gulf Coast and reports that it has the ability to out compete other *Vibrio* spp. in low salinity environments, a study was conducted to assess the impact of the major salinity

shift on the abundance and distribution of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (4).

Materials and Methods

Sample Collection and Preparation

Over a span of six weeks, from late May to late June 2011, and a similar length of time from late January to mid-March, 2012, water samples were taken from three sites along the Mississippi coast (Figure 3).

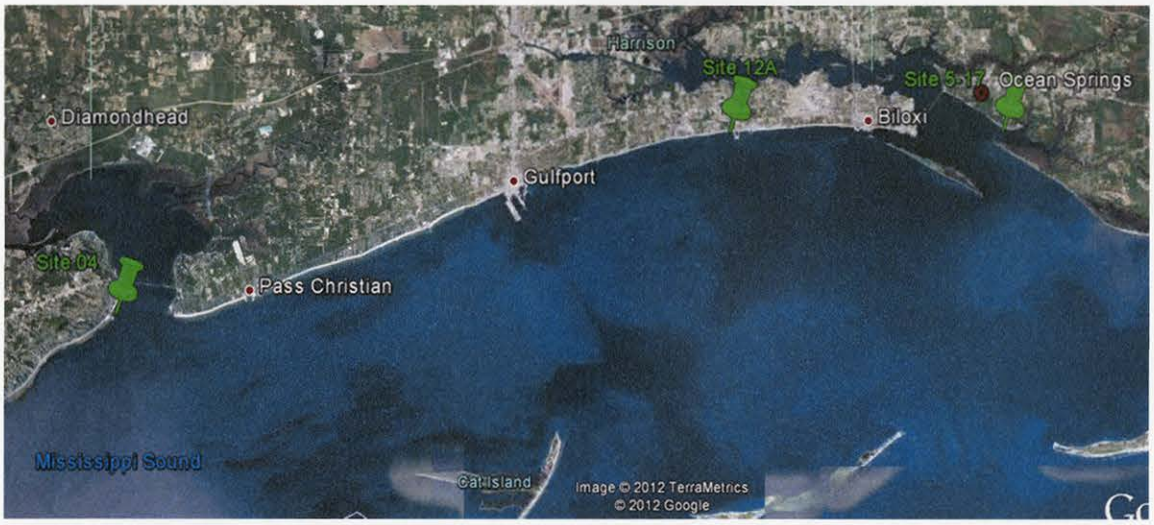

Figure 6. Google Earth Map Showing the Location of the Three Sample Sites. Copyright: Google Earth, 2012

Grab samples were obtained by wading out knee deep (~0.5m) with minimal sediment disturbance, and collecting approximately 6 L of surface water *in a 10-L sterile carboy; salinity and temperature measurements were taken with* a YSI 600XLM sonde. Samples were placed on ice and transported to the lab where they were processed immediately.

In the lab, water was vigorously agitated by shaking (25 times in 7 seconds) followed by removal of 1-L, 100-mL, 10-mL, 1-mL, and 100-µL aliquots. To each aliquot, 111mL, 11mL, 1.1mL, 111µL, and 11µL of 10X alkaline peptone

water (1 L deionized water, 100 g Bacto peptone; BD, Difco, Becton Dixon and Co. Sparks, MD) were added, respectively. The enriched aliquots were mixed and incubated for 16-18 hours at 42°C.

Isolate collection and PCR lysate preparation

After incubation, the samples were removed from the incubator with minimal or no disruption of the surface pellicle. From the initial samples taken during the fresh water intrusion, a calibrated 10-µL loop was used to sample the pellicle of each sample. Pellicle samples were streak plated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (BD, Difco, Becton Dixon and Co. Sparks, MD) plates. The plates were incubated overnight at 42°C. The enrichment samples were then mixed thoroughly and 1-mL aliquots were removed to labeled microcentrifuge tubes and immersed in boiling water for 10 minutes. The lysate was immediately placed on ice for a minimum of 5 minutes and then stored at -20°C.

Colony identification

After 24 hours, the TCBS plates were removed and checked for typical yellow colonies. Isolated colonies were picked with a sterile toothpick and placed into a 96-well plate containing 200 µL 1% tryptone broth (EMD, Darmstadt, Germany). The well plate was incubated overnight at 33°C. After incubation, wells were checked for turbidity and then stamped onto duplicate T1N1 ([10g tryptone (EMD Chemicals, Darmstadt, Germany), 10g sodium chloride (Fisher Scientific, Fair Lawn, NJ), 20g agar (Alfa Aesar, Heysham, Lancaster), 1 L

deionized water] plates using a 48-prong replicator. These plates were incubated over night at 33°C.

Colonies on the stamped plates were subjected to the string test and to oxidase and catalase biochemical tests to determine if they were putative *V. cholerae*. The oxidase test was performed using BD oxidase reagent droppers (Becton Dixon and Co. Sparks, MD), following the manufacturer's instructions. The catalase test was performed by placing one drop (transfer pipet) of hydrogen peroxide into the wells containing turbid growth. Visualization of bubbles in the well was indicative of a positive result. The string test was performed by placing some of each colony into a drop of sodium deoxycholate (0.5%, w/v) on a glass slide. Visualization of an unbroken string extending up from the droplet to a sterile toothpick after rubbing the end of the toothpick in the droplet was indicative of a positive result. Five isolates from each dilution exhibiting positive results from all three tests were stored on T1N3 slants [10 g tryptone (EMD Chemicals, Darmstadt, Germany), 30 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), 20 g agar (Alfa Aesar, Heysham, Lancaster), 1 L DI water] for further testing.

MPN density determination by PCR

All boiled lysate samples were removed from the freezer, thawed completely, mixed and centrifuged for 2 minutes at 2500 x g. The samples were then analyzed by PCR for the gene coding for a *V. cholerae* outer membrane protein (*ompW*), the thermolabile hemolysin gene (*tlh*) in *V. parahaemolyticus*, and the V. vulnificus hemolysin/cytolysin gene (*vvhA*). *V. cholerae* was detected

following the procedure outlined by Nandi *et al.* (52). *V. parahaemolyticus* was detected using *tlh* primers (54) at a concentration of 0.1 µM with 1 U NEB taq (New England Biolabs, Ipswich MA), 10X NEB ThermPol buffer containing 2 mM $MgCl_2$, 0.25 mM dNTPs (Promega, Madison, WI), and 2-µL template in a volume of 20µL, with the following conditions; initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and elongation at 72°C for 45 seconds, and a final elongation at 72°C for 2 minutes. *V. vulnificus* was detected using *vvhA* primers (73) and identical reaction conditions as used with tlh. All PCR products were run on 1.5% agarose gels containing 0.1% ethidium bromide for visualization of a 304-bp fragment. Using a most probable number (MPN) calculator, the density of each *Vibrio* was determined for each sample.

Characterization of V. cholerae isolates

Ten isolates from each sample date were grown overnight in 1 mL of marine broth (HiMedia Lab, Pvt. Ltd. India). The cells were pelleted by gentle centrifugation at 3,000 g for 10 minutes. The broth was removed and the cells were lysed by addition of 200 µL of sterile DI water followed by immersion in boiling water for 10 minutes. The lysate was iced and then cellular debris was pelleted as above with MPN samples. The samples were screened for *ompW* as above for confirmation of ID as *V. cholerae*. The samples were then tested for presence of the cholera toxin gene (*ctxA*) by PCR with the following conditions, initial denaturation at 94°C for 5minutes followed by 35 cycles of 94°C for 60

seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and a final elongation at 72°C for 7 minutes.

Results and Discussion

As shown in Table 3, temperature readings in the 2011 samples for the three sites varied from a low of 27.4°C to a high of 33.3°C, a gradient of less than 6°C. Salinity readings had a range of 1.4 to 12.9, a gradient of more than 11 with the most significant impact at site 4, the western most site, and the least impacted at the eastern most site, 5-17.

Table 4

2011 Temperature and Salinity Readings, along with MPN/mL Densities for V. cholerae (Vc), V. vulnificus (Vv), and V. parahaemolyticus (Vp) Associated With Each Sample Collection Date

Date	Site	Temp°C	Salinity	MPN/mL Vc	MPN/mL Vv	MPN/mL Vp
5/26/2011	12a	27.4	7.2	2.4000	2.4000	0.1900
5/26/2011	4	26.9	3.2	2.4000	0.0000	0.0000
6/1/2011	5-17	30	9	0.2300	0.0091	0.0940
6/1/2011	12a	31.4	6.1	0.2300	0.0009	0.0000
6/2/2011	4	31.6	1.7	3.0000	0.0000	0.0000
6/7/2011	5-17	29	9.6	0.0280	0.0230	0.0230
6/7/2011	12a	33.3	6.2	3.0000	0.0230	0.0009
6/8/2011	4	31	1.4	3.0000	0.0000	0.0000
6/14/2011	5-17	32.7	10.2	2.4000	0.0230	0.2300
6/16/2011	12a	31.3	9.6	2.4000	0.0090	0.0023

Table 4 (continued).

6/16/2011	12a	31.3	9.6	2.4000	0.0090	0.0023
6/21/2011	4	28.5	3.7	3.0000	0.0000	0.0000
6/21/2011	5-17	29.2	11.3	3.0000	2.4000	0.0000

Table 5

2012 Temperature and Salinity Readings, along with MPN/mL Densities for V. cholerae (Vc), V. vulnificus (Vv), and V. parahaemolyticus (Vp) Associated With Each Sample Collection Date.

Date	Site	Temp°C	Salinity	MPN/mL Vc	MPN/mL Vv	MPN/mL Vp
1/19/2012	4	14.14	21.48	0.0023	0	2.4
1/24/2012	12b	18.59	19.84	0.0023	0	2.4
1/31/2012	5-17	14.6	18.7	0.23	0.23	0.23
1/31/2012	12b	13.42	21.41	0.023	0.0023	0.23
2/3/2012	4	17.58	18.65	0	0.0023	0.23
2/8/2012	5-17	16.7	16.6	0.23	0.023	0.023
2/20/2012	12b	17.34	18.21	0.094	2.4	0.023
2/21/2012	5-17	15.8	5.8	0	0.023	0
2/23/2012	4	18.99	7.77	0.0023	0.23	0.094
2/28/2012	4	16.55	9.13	0.094	0.023	0.094
2/29/2012	5-17	18.8	10	0.023	0.0023	0.023
3/7/2012	12b	19.66	15.48	0.094	0.023	0.023

Table 5 (continued).

3/7/2012	5-17	19	17.7	0.23	0.023	2.4
3/13/2012	5-17	20	9.3	0.23	0.23	2.4

MPN calculations for samples taken in 2011 during the time period the spillway was open ranged from non-detect to a maximum of 3 MPN/mL. Table 3a shows this distribution of data.

Figure 7 shows the regression analysis of the three *Vibrio* species' densities, as a function of salinity, and Figure 8 shows the percent of the total each *Vibrio* comprised at the different salinity readings.

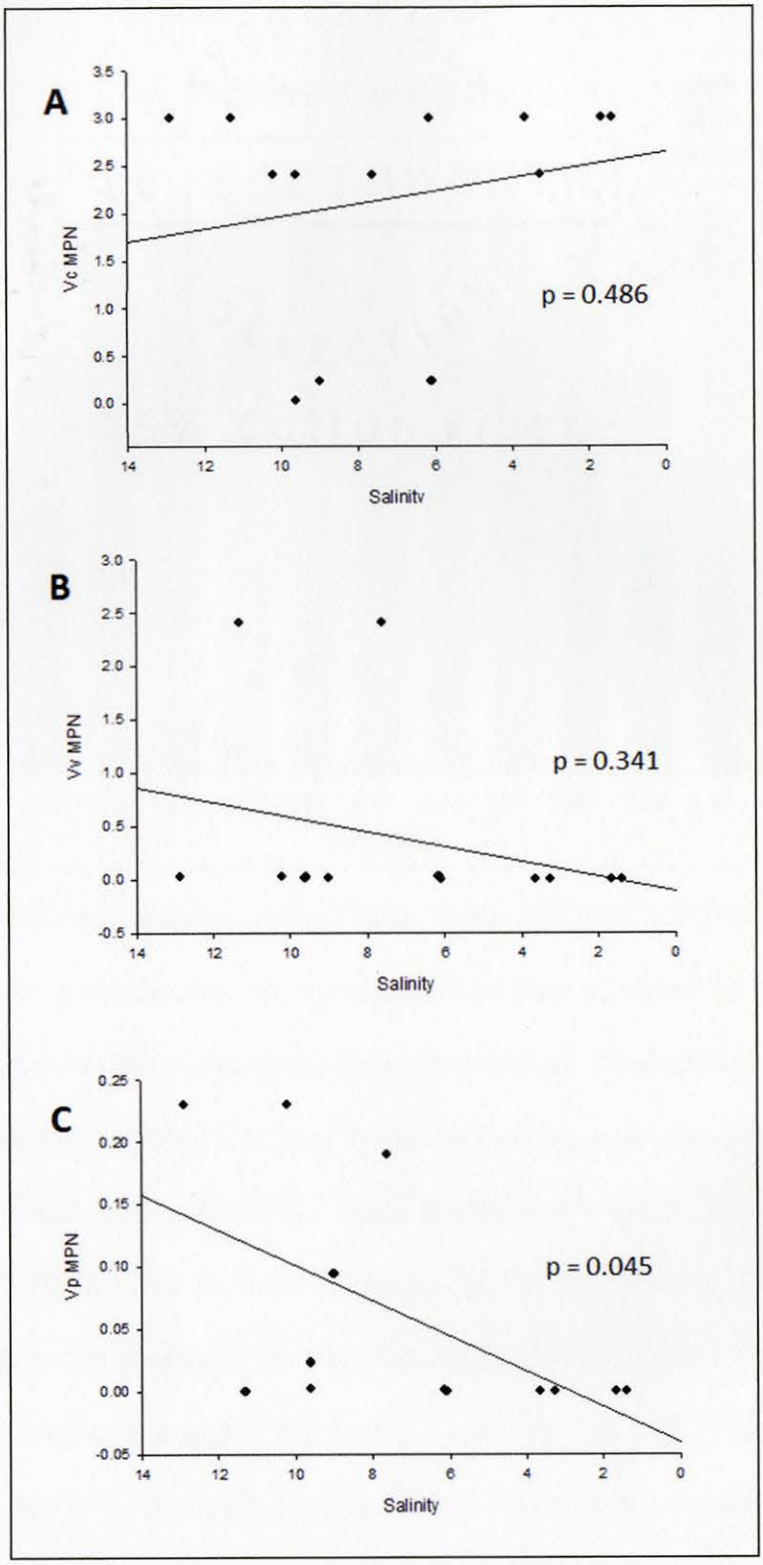

Figure 7. Vibrio Regression Analysis Graphs Including p Values.

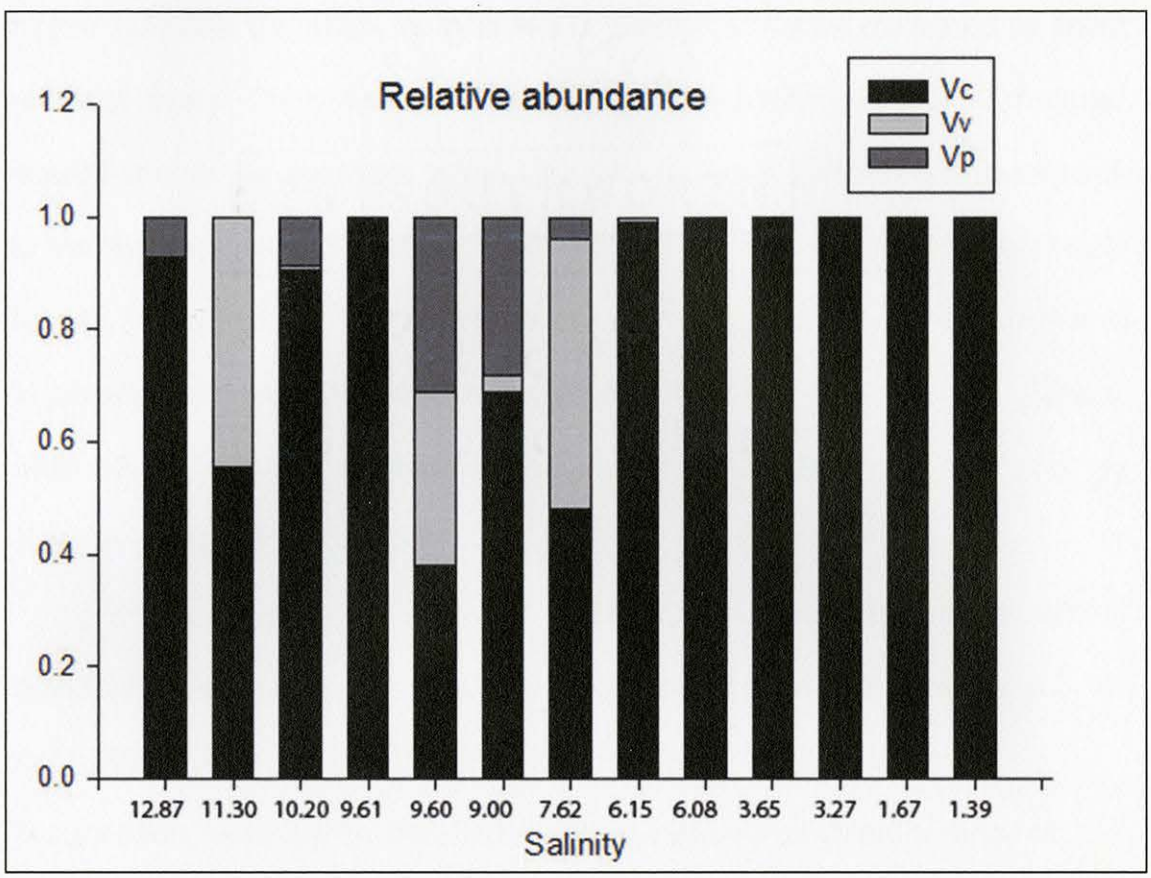

Figure 8. Relative Abundance of the Three *Vibrio* Species in 2011 Samples.

While the p values are not significant for either *V. vulnificus* or *V. cholerae*, there does appear to be a change in their abundance. At salinities above 6, all three Vibrios were present; however, once the salinity reached or fell below 6, the only *Vibrio* detected in the water column was *V. cholerae*. The lack of statistically significant results for *V. cholerae and V. vulnificus* is possibly due to a small population size owing to the fact that the small N number of total samples reduced the power of the tests (Vc: 0.101, Vv: 0.153, Vp: 0.391) leading to a decreased ability to detect a significant outcome even in the event one was present. Other factors include the fact that while *V. cholerae* is the only *Vibrio* able to grow without the presence of salt, its growth is not hampered by relatively

higher salinities; therefore, its increase in density cannot be explained by salinity readings alone. The initial hypothesis, supported by this data, that *V. cholerae* would become the dominant *Vibrio*, was based on a reduction in competition due to the decrease in density of the other Vibrios whose growth was retarded by low salinity. The reduction in *V. vulnificus* abundance was not as marked as that of *V. parahaemolyticus*; this can be explained by the knowledge that *V. vulnificus*, while not able to grow without salt, has a higher tolerance to lower salinities than *V. parahaemolyticus* (43, 46).

When analyzing the data from samples collected in 2011, the prevailing determining factor was salinity as the temperature gradient was very small. With the addition of samples collected in 2012, this is no longer the case. Temperature readings for the 2012 samples, still only exhibited a range of approximately 6°C, though the average temperature for 2012 samples is more than 13°C lower than the average for 2011 samples. This factor made it impossible to analyze the pooled data set with a single independent variable. Therefore, multiple linear regression analysis was performed on the pooled data set for each *Vibrio*. Results show that temperature had a highly significant impact ($p=<0.001$) on the *V. cholerae* densities whereas salinity showed no significant effect ($p=0.613$). As has been discussed, this result was not unexpected. Results for *V. vulnificus* (temperature: $p=0.612$, salinity: 0.542) and *V. parahaemolyticus* (temperature: $p=0.677$, salinity: 0.128) showed that neither temperature nor salinity had a significant impact on their densities.

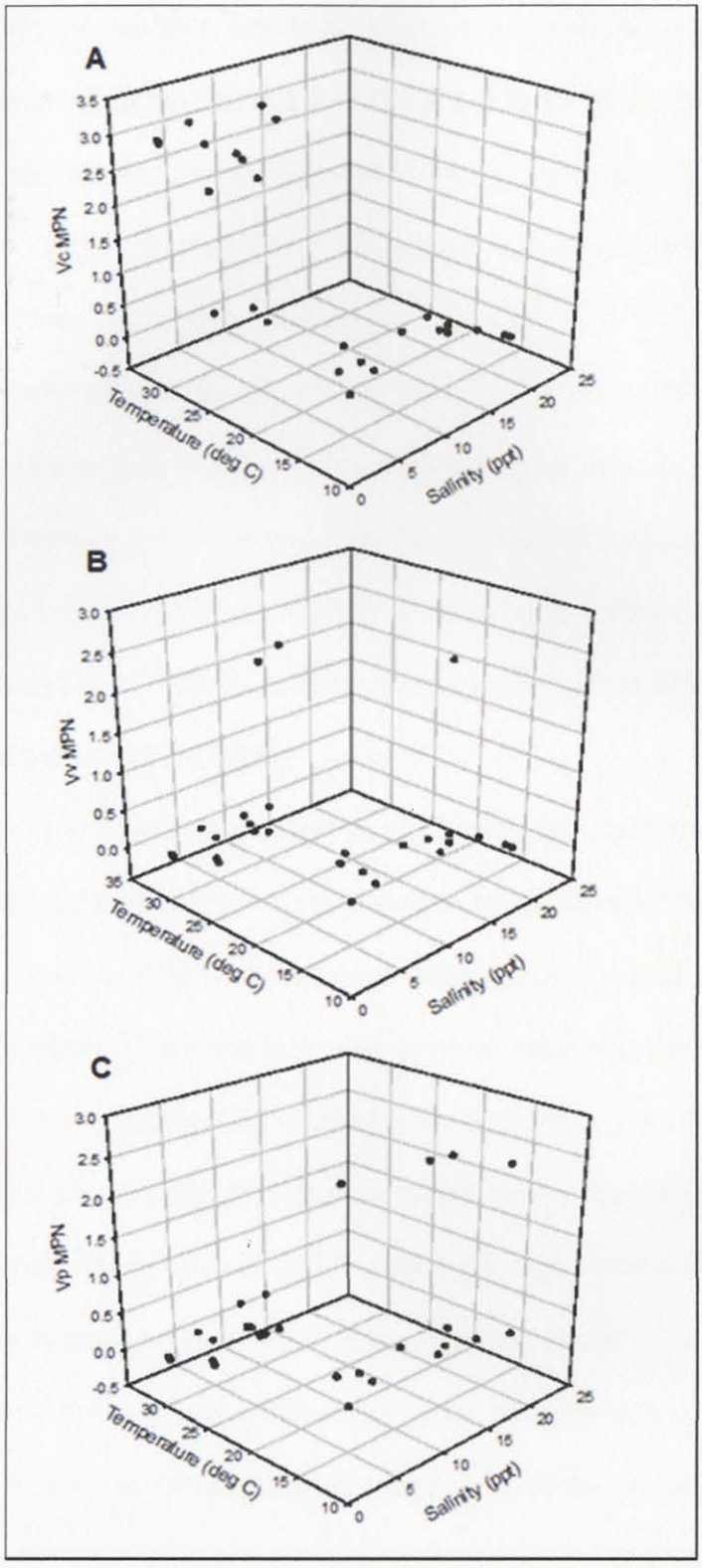

Figure 9. 3D Scatter Plots of (A) *V. cholerae,* (B) *V. vulnificus, and* (C) *V. parahaemolyticus* MPN vs Salinity (ppt) and Temperature (°C).

While the data set as a whole does not show a statistically significant correlation between salinity and the density of the three *Vibrio* species examined, it does show that the salinity decrease did have an effect on relative abundance of these Vibrios. Figure 8 shows that, despite the lower temperature in the 2012 samples, the increase in salinity has shown a rebound in the *V. vulnificus* and especially *V. parahaemolyticus* densities. Were these samples taken when the average water temperature was on par with the time the Bonnet Carre Spillway was open, it is expected that the densities of all three Vibrios would be higher and in a significantly less skewed relative abundance than they were in 2011. A third and final sample collection is planned for the May/June time period of 2012 where this hypothesis will be tested.

A second hypothesis tested in this study was that, with the increase in *V. cholerae* density, there would be an increase in toxigenic strains detected from the Mississippi Sound. This was based on the expectation that, though present in very small numbers, there are toxigenic strains present in the environment and horizontal gene transfer would be facilitated by the higher population density. This hypothesis was not supported by the results of the isolate screening. Out of 130 isolates tested, all were positive for *ompW*, though none tested positive for *ctxA*. There are a few possible explanations for the lack of *ctx*+ isolates, (i) the small sample size prevented the detection of an increase in the toxigenic population; (ii) there was no increase in phage conversion; or (iii) a portion of the population entered the viable but not culturable state preventing its growth and isolation in the lab (38).

Regardless of the lack of toxigenic strain isolation, the increase in *V. cholerae* density was a significant finding in relation not only to the ecology of the Sound, but also as a potential health risk. While the lack of 01 surface antigens and CTXΦ phage in the isolates indicates that outbreaks of cholera are unlikely to occur due to this ecological shift, sporadic infections could occur as diarrheal disease can be caused by *ctx*- strains of *V. cholerae* (49).

Many factors beyond salinity are responsible for determining the composition of the diverse microbial community in the Mississippi Sound, and this study did not seek to lessen their significance. The purpose of looking at the effect of a singular environmental factor on *Vibrio* densities was to assess the impact of the population due to this significant, unanticipated anthropogenic event. The findings here show that despite the short window to collect affected water resulting in a relatively small sample size with which to analyze, there was a measurable effect on *Vibrio* ecology in the Mississippi Sound during the time the spillway was open. Intermediate follow-up samples collected in early 2012 indicate that even with low sea surface temperatures reducing the overall densities of Vibrios in the Mississippi Sound, a long-term shift in relative abundance was not apparent. Water samples taken when sea surface temperatures reach peak *Vibrio* growth conditions, and tissue samples taken as the oyster reefs recover from the salinity-induced die-back, will further elucidate potential long term changes to the risk posed by *V. cholerae* in the Mississippi Sound.

CHAPTER IV

ENUMERATION OF VIBRIO PARAHAEMOLYTICUS IN THE VIABLE BUT NONCULTURABLE STATE USING DIRECT PLATE COUNTS AND RECOGNITION OF INDIVIDUAL GENE FLUORESCENCE IN SITU HYBRIDIZATION

Abstract

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium indigenous to marine and estuarine environments and it is capable of causing food and water-borne illness in humans. It can also cause disease in marine animals, including aquacultured species. Currently, culture-based techniques are used for quantification of *V. parahaemolyticus* in environmental samples; however, these can be misleading as they fail to detect *V. parahaemolyticus* in the viable but nonculturable (VBNC) state which leads to an underestimation of the population density. In this study, we used a novel fluorescence visualization technique, called recognition of individual gene fluorescence *in situ* hybridization (RING-FISH), which targets low copy, chromosomal DNA for enumeration. A polynucleotide probe labeled with Cyanine 3 (Cy3) was created corresponding to the ubiquitous *V. parahaemolyticus* gene that codes for thermolabile hemolysin (*tlh*) as well as pathogenicity factors thermostable direct hemolysin (*tdh*), and tdh-related hemolysin (*trh*). When coupled with the Kogure method to distinguish viable from dead cells, RING-FISH probes reliably enumerated total, viable *V. parahaemolyticus*. The probes were tested for sensitivity and specificity against a pure culture of *V. parahaemolyticus*, *V. vulnificus*, *V. harveyi,* and *V. fischeri,*

and a mixed environmental sample. A limited field study was also conducted to show the applicability to environmental samples. This research will provide additional tools for a better understanding of the risk these environmental organisms pose to human health.

Introduction

Vibrio parahaemolyticus is a Gram-negative bacterium indigenous to marine and estuarine environments worldwide. A known etiologic agent of food and water-borne illness in humans, clinical manifestations of *V. parahaemolyticus* illness include wound infection, septicemia, and gastroenteritis (22, 23, 44, 70). It is also known to cause of disease in marine animals (3). The most common human illness is gastroenteritis, resulting from the consumption of raw, undercooked, or mishandled seafood (20, 26). From 2003 through 2006, *V. parahaemolyticus* was the most common clinical *Vibrio* isolate reported to the Center for Disease Control (CDC) (5, 8-10). *V. parahaemolyticus* is one of more than 60 species of bacteria that have been described to enter a dormant growth state known as viable but nonculturable (VBNC) (56, 66). In this state, cells remain viable and metabolically active but are not culturable on routine culture media in the laboratory (56, 77). This dormant phase allows for non-spore-forming bacteria to survive adverse environmental conditions such as low temperature, salinity extremes, or low nutrient levels; they accomplish this through metabolic, physiologic and structural changes, including reduced cellular respiration, reduction in cell size and membrane morphology changes (34, 40, 56, 62). Many studies have shown that pathogenic strains retain their virulence

while in the VBNC state and many strains will return to a culturable state when introduced into a more favorable environment (1, 14, 15, 58). This lack of culturability poses a detection problem as standard methods for *V. parahaemolyticus* rely on the ability to grow the cells *in vitro* followed by molecular probing for typing and enumeration. This implies that cells in the VBNC state are missed with standard techniques, leading to a potentially serious underestimation of total *V. parahaemolyticus* densities in environmental samples. There are methods that simultaneously identify cells of interest while determining viability such as the indirect fluorescent antibody technique for *V. cholerae*. However, this method is not applicable to *V. parahaemolyticus*, as there are more than 70 known *V. parahaemolyticus* serotypes and many as yet untypeable environmental strains (13, 26).

Fluorescence *in situ* hybridization (FISH) is an ideal way to enumerate VBNC cells when coupled with the Kogure method for distinguishing viable cells microscopically (45). The Kogure method involves treating cells with a low dose of yeast extract (0.025%) and nalidixic acid (0.002%), a DNA gyrase inhibitor. The treatment prevents the cells from dividing while allowing them to continue to metabolize, resulting in cells that are fattened and/or elongated, making them easily distinguished microscopically from those that are no longer metabolically active. FISH can be problematic for identifying different species of Vibros due to closely related species having very similar 16s rRNA sequences which are often the target of choice for this method (66). We therefore employed a novel type of FISH known as Recognition of Individual Gene Fluorescence *In Situ*

Hybridization (RING-FISH) which targets low copy chromosomal DNA (79). This technique allowed us to combine traditional colony hybridization with microscopic RING-FISH counts using the same sequence probe that targets the ubiquitous thermolabile hemolysin gene (*tlh*) of *V. parahaemolyticus.* By targeting *tlh* we were able detect the whole population of *V. parahaemolyticus*, both pathogenic and non-pathogenic strains. Once lab studies showed the method to be a reliable means of detecting total *V. parahaemolyticus*, it was tested in field studies. This short field study was conducted in February and March during times when water detection rates for *V. parahaemolyticus* are low and decreased sea surface temperatures are expected to increase VBNC numbers. The method was then extended to encompass direct screening for known *V. parahaemolyticus* pathogenicity factors by creating probes to detect thermostable direct hemolysin (*tdh*) and thermostable direct-related hemolysin (*trh*) genes.

Materials and Methods

Bacterial Cultures

V. parahaemolyticus FIHES, a tlh^+, tdh^-, and trh^- strain, was the reference culture selected for the *tlh* portion of this study (FDA A. DePaola). *V. parahaemolyticus* F11 was used both for *tdh* and *trh. Vibrio vulnificus* 99-645 was used as a negative control, and *V. harveyi* 35084, *V. alginolyticus* AQHL0007, and *V. fischeri* VSL2 strains were used for specificity testing. Stock cultures were maintained on T1N3 slants (10 g Tryptone, 30 g NaCl, 10 g agar per 1000 mL diH_2O, pH 7.2) or on Difco Marine agar 2216 (37.4 g Marine broth, 10 g agar per 1000 mL diH_2O) slants at ambient temperature. Bacteria from

stock cultures were grown overnight on T1N3 plates at 33°C. One colony of each culture was inoculated into 10 mL of 10X alkaline peptone water (APW; 100 g Bacto-Peptone, 10 g NaCl per 1000 mL diH_2O, pH 8.5) and grown overnight for VBNC experiments.

Induction into the VBNC State

A 100-µL aliquot of overnight *V. parahaemolyticus* FIHES growth in APW was centrifuged at 5000g for 5 minutes and washed twice with sterile 25 artificial sea water (ASW) to remove all nutrients from the cells. The cell pellet was resuspended in 100 µL ASW, and the entire volume was added to 100 mL ASW in a 250-mL Erlenmeyer flask. An aliquot was then removed for initial enumeration and the culture was incubated at 4°C. The removal of all available nutrients and incubation at low temperature promoted entry of the cells into the VBNC state.

Probe Generation

The polynucleotide probes used in this study target the *tlh, tdh,* and *trh* genes and were generated as previously described with the following changes (79). Initially, the probe template was produced via standard PCR to amplify a 208-bp region, 232-bp region, and 272-bp region respectively *Vibrio parahaemolyticus* control strains. Template DNA was acquired using a standard phenol:chloroform extraction method following overnight growth of pure FIHES culture (41). The primer sequences used were (T3 promoter sequence underlined), fwd-FISH-tlh5'-

<u>ATAGGTATTAACCACTAAAGGG</u>ACTCAACACAAGAAGAGATCGACAA-3', and

rev-FISH-tlh 5'-GATGAGCGGTTGATGTCCAAA-3'; fwd-FISH-tdh5'-ATAGGTATTAACCACTAAAGGGTCCCTTTTCCTGCCCCC-3' and rev-FISH-tdh5'-CGCTGCCATTGTATAGTCTTTATC-3'; fwd-FISH-trh5'-ATAGGTATTAACCACTAAAGGGTTGCTTTCAGTTTGCTATTGGCT-3', and rev-FISH-trh5'-TGTTTACCGTCATATAGGCGCTT-3'. The PCR parameters were as follows: 100 ng template DNA, 50 pmol of each primer, 80 nmol dNTP, 10X Mg free PCR buffer (New England Biolab, Ipswich, MA), 3 mM $MgCl_2$, and 3 U Taq-polymerase (New England Biolab, Ipswich, MA) in a volume of 100 µL. For *tlh* an initial denaturation of 3 minutes at 94°C was followed by 30 cycles of a 60-second denaturation at 94°C, a 60-second annealing at 62°C, and a 60-second elongation at 72°C, ending with a final elongation of 72°C for 5 minutes. For *tdh* and *trh* the annealing temperature was lowered to 54°C. The resulting *tlh* and *trh* products were purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA), *tdh* produced multiple bands so the one corresponding with ~230-bp was extracted from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). All purified products were *in vitro* transcribed incorporating Cyanine 3 (Cy3) dUTP (GE Healthcare, Buckinghamshire, UK) to label the *tlh* and *trh* probes, and Fluorescein dUTP (Fermental Life Sciences, Glen Burnie, MD) to label the *tdh* probe. The transcription reaction, run for 4 hours at 37°C, included 1-2 µg purified PCR product, 6 µL 5X transcription buffer (Fisher Scientific, Fair Lawn, NJ), 3 µL T3 polymerase (Fisher Scientific), 3 µL dithiothreitol (Fisher Scientific), 1.5 µL RNAse Out (Invitrogen, Carlsbad, CA), and 200 nmol NTP in a 1:1:1:0.35:0.65 ratio (ATP, CTP, GTP, unlabeled UTP,

labeled UTP) in a 34-µL volume. Any remaining DNA was removed with 3 µL RQ1 RNAse free DNAse (Fisher Scientific) and 4 µL reaction buffer (Fisher Scientific) for 15 minutes at 37°C. The DNAse was inactivated by the addition of DNAse stop solution (Fisher Scientific) and incubation at 65°C for 10 minutes. The labeled probes were precipitated with 4.4 µL 3 M sodium acetate and 110 µL 100% ethanol at -80°C overnight. After centrifugation (14000 g x 15 min) and rinsing with 70% ethanol, the probes were suspended in 10 µL glass distilled water (Teknova, Hollister, CA). The concentration of the probes were measured using a NanoDrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE) and the sequences were verified via cloning into *E. coli* followed by sequencing.

Transformation, Cloning, and Sequencing

In order to verify that the probe was the correct size and sequence to target *tlh*, the *in vitro* transcription template (PCR product) was transformed into *E. coli* cells using the TOPO TA Cloning Kit with One Shot TOP10´ Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA). Transformed cells were plated on LB agar plates containing 50 µg/mL ampicillin with X-Gal /IPTG overlay and grown overnight at 37°C. Six white colonies were picked and inoculated into tubes containing 5 mL LB broth and grown overnight at 37°C. A 1.5-mL aliquot was taken from each of the turbid cultures and purified via standard plasmid mini prep. Three of the six samples were sequenced forwards and backwards. The sequencing reaction was run with the GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit following the manufacturer's protocol (Beckman

Coulter, Fullerton, CA). Samples were sequenced on the Beckman Coulter CEQ8000following the manufacturer's protocol.

Cell Treatment and Fixation

In order to verify the viability of hybridized cells microscopically, prior to fixation, cells were treated according to the Kogure method (Figure 10) (45). The Kogure method prevents cells from dividing causing them to grow large and fat thereby making them easily distinguishable from non-viable cells. Throughout the 4°C incubation of the culture to induce the cells to enter the VBNC state, 1-mL aliquots were periodically removed, cells were harvested via centrifugation at 5000g for 5 minutes and treated with 0.025% yeast extract and 0.002% nalidixic acid for 5 hours (45). Treated cells were harvested and fixed as previously described by Zwirglemaier et al. (79). Briefly, cells were washed with phosphate buffered saline (PBS) (0.58 g Sodium dihydrate phosphate (Fisher Scientific, Fair Lawn, NJ), 2.5 g sodium monohydrate phosphate (Fisher Scientific, Fair Lawn, NJ), 8.5 g sodium chloride (Fisher Scientific, Fair Lawn, NJ), in 1 L DI water) and fixed in 1 part PBS and 3 parts 4% paraformaldehyde for 5 hours at 4°C. Cells were harvested, washed with PBS, preserved in PBS and 100% ethanol in a 1:1 ratio and stored at -20°C until hybridized.

Direct Plate Counting

In order to determine what portion of the total viable microscopic count was still culturable, simultaneous to the microscopic aliquot, a second aliquot was removed for direct plate counts. Specifically, 200 µL was removed from the culture; 100 µL was spread plated onto a T1N3 plate and 100 µL was serially

diluted followed by spread plating on T1N3 plates. The plates were incubated overnight at room temperature (23°C). Colonies were counted on the least diluted sample containing individual, countable colonies and back calculated (counted CFU x dilution factor x 10) to give a colony forming unit (CFU) per milliliter density.

Hybridization of VBNC Cells Using V. parahaemolyticus tlh-Specific RING-FISH Probe

The preserved cell suspension was mixed and 10 µL was added to Teflon lined 5-mm well slides. Slides were dried for 5 minutes at 65°C, followed by dehydration for 2 minutes each in 50%, 80% and 100% ethanol. Twelve microliters of hybridization buffer (75-mM NaCl, 20-mM Tris-HCL pH 8.0, 0.01% SDS, 5% formamide) was added to the well with 3-4 µg probe. The slide was placed in a humid chamber containing a buffer soaked towel, denatured for 20 min at 80°C, and hybridized at 35°C for 30 hours. After hybridization, the slide was washed in deionized water, stained with 50 µL of 4',6-diamidino-2-phenylindole (DAPI) and air dried prior to reading. The slide was viewed with a Zeiss Axiostar Plus microscope (Carl Zeiss, Germany) using the appropriate filter sets. All fattened and/or elongated cells exhibiting Cy3 fluorescence were counted as viable *V. parahaemolyticus* (Figure 10).

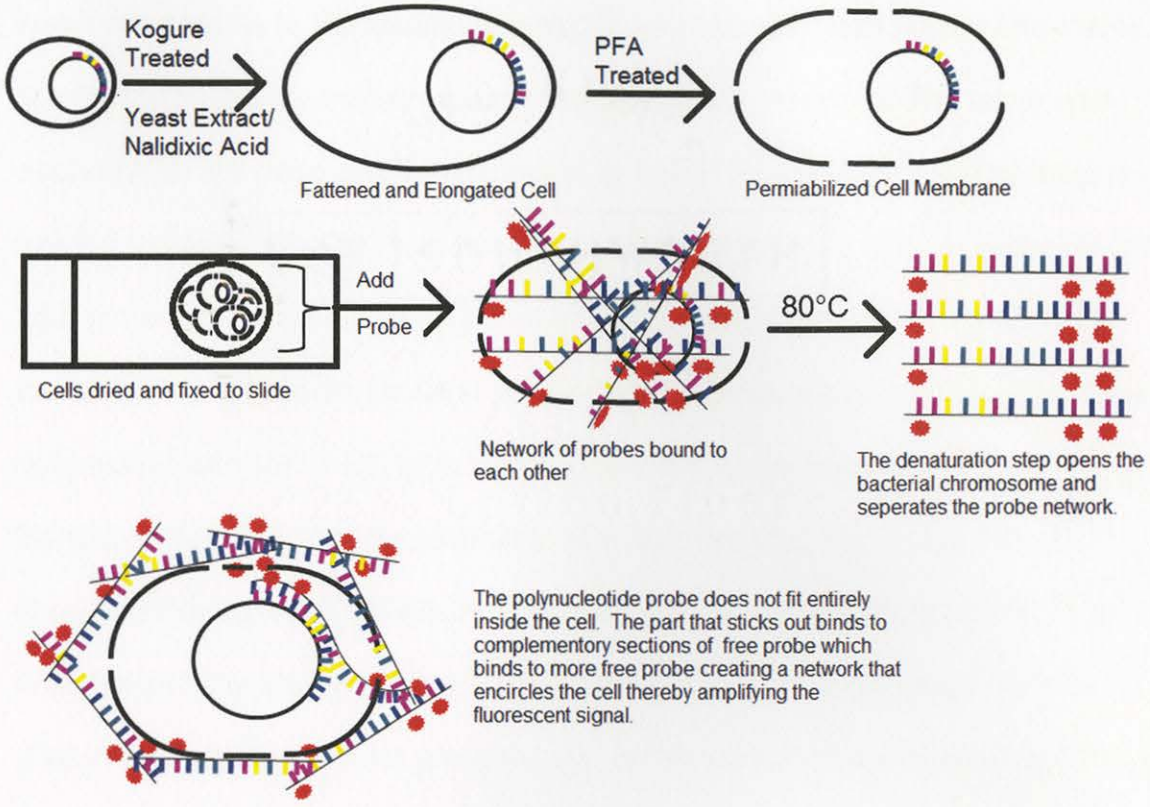

Figure 10. Artistic Rendition of the RING-FISH Method for Detection of Viable *V. parahaemolyticus* Cells.

Resuscitation of VBNC cells to culturable state

Once an undiluted aliquot of the VBNC culture yielded no colonies on T1N3 agar, it was removed from 4°C, 10 mL of sterile 10X APW was added, and it was incubated overnight at 33°C. A 100-µL aliquot of the resulting turbid culture was spread plated on CHROMagar Vibrio (CHROMagar, Paris France) and T1N3 agar plates and incubated overnight at room temperature.

Enumeration of VBNC V. parahaemolyticus in Environmental Samples

Water samples were collected from the Davis Bayou area of The Mississippi Sound in January and February in sterile 1-L bottles. Samples were transported back to the lab where they were immediately processed. In the lab,

water samples were vigorously agitated; 25-mL aliquots were placed into sterile 50-mL conical tubes and centrifuged at 5000g for 10 minutes. The water was decanted off the pellet and resuspended in 1mL PBS. One aliquot was Kogure treated and fixed as described above. A second aliquot was plated onto a T1N3 agar plate and grown for 16-18 hours at 33°C. After incubation the cells were transferred to Whatman 541 filter paper discs (GE Lifesciences, Piscataway, NJ), cells were lysed with 1 mL lysis buffer (0.5 M sodium hydroxide (Fisher Scientific, Fair Lawn, NJ), 1.5 M sodium chloride (Fisher Scientific, Fair Lawn, NJ), the filters were dried, washed with 2 M ammonium acetate (Fisher Scientific), and rinsed with 1X standard saline citrate solution (SSC) (8.77 g sodium chloride (Fisher Scientific), and 4.41 g sodium citrate dehydrate (Fisher Scientific). Filters were then treated with proteinase K, hybridized with alkaline phosphatase labeled oligonucleotide probe (DNA Technologies, Risskov, Denmark) and visualized with nitro blue tetrazolium chloride 5-Bromo-4chloro-3-indoyl phosphate, toluidine salt (NBT/BCIP) substrate (Roche Diagnostics, Indianapolis, IN) (18).

Colonies were enumerated by counting purple spots indicative of the enzymatic breakdown of the NBT-BCIP substrate by the bound probe. Fixed cells were hybridized as described above and visualized under florescent microscopy. Fattened and/or elongated cells exhibiting fluorescence under the appropriate wavelength filter were counted as viable. The calculated difference between microscopic counts and filter counts was determined for enumeration of the VBNC fraction of the population.

Results and Discussion

The work we present provides a potential method for addressing a persistent problem in bacterial surveillance - enumerating the viable but nonculturable portion of the *V. parahaemolyticus* bacterial population in the environment. It has been shown that many bacteria, including many human pathogens, adapt to unfavorable conditions by entering the VBNC state; however, there is no widely accepted method for determining the prevalence of VBNC cells (40, 56). By utilizing RING-FISH, the same gene used in the widely accepted colony hybridization detection method for Vp may be targeted, allowing for direct comparison of the two counts with the VBNC population being calculated as the difference between the total and plateable estimates. This method can be extended for use with other species, including non-marine human pathogens, by coupling with appropriate methods for total enumeration.

The RING-FISH probes were queried with BLAST which verified the sequences as specific to *V. parahaemolyticus* (http://blast.ncbi.nlm.nih.gov). To verify the primers would produce the expected probe sequence, the *tlh in vitro* template was cloned into *E. coli* cells and then sequenced. Results showed the probe to be the correct sizes and sequence. The primers used for *tlh*, *tdh*, and *trh* are not novel to this project. Therefore, the proof of concept shown with cloning and sequencing the *tlh* template was taken as verification that the other two primer sets would produce the expected templates and *tdh* and *trh* sequencing was deemed unnecessary. To measure both sensitivity and specificity, the probes were tested against actively growing cultures of *V.*

vulnificus, *V. harveyi, V. fischeri*, *V. alginolyticus*, and *V. parahaemolyticus*. *V. vulnificus* was chosen to test the specificity of the probes to their respective genes because they are not associated with *V. vulnificus* and it is commonly found in conjunction with *V. parahaemolyticus* in environmental samples (Johnson et al., unpublished results). *V. harveyi*, *V. alginolyticus*, and *V. fischeri* were chosen because they contain either *tlh* or a related hemolysin gene (39, 65, 71). None of the non-*V. parahaemolyticus* slides displayed probe specific fluorescence indicating a lack of nonspecific binding of the probe (data not shown). The *V. parahaemolyticus* culture demonstrated the high sensitivity of the RING-FISH probes; all DAPI stained cells displayed Cy3 fluorescence (Figures 11, 12, and 13).

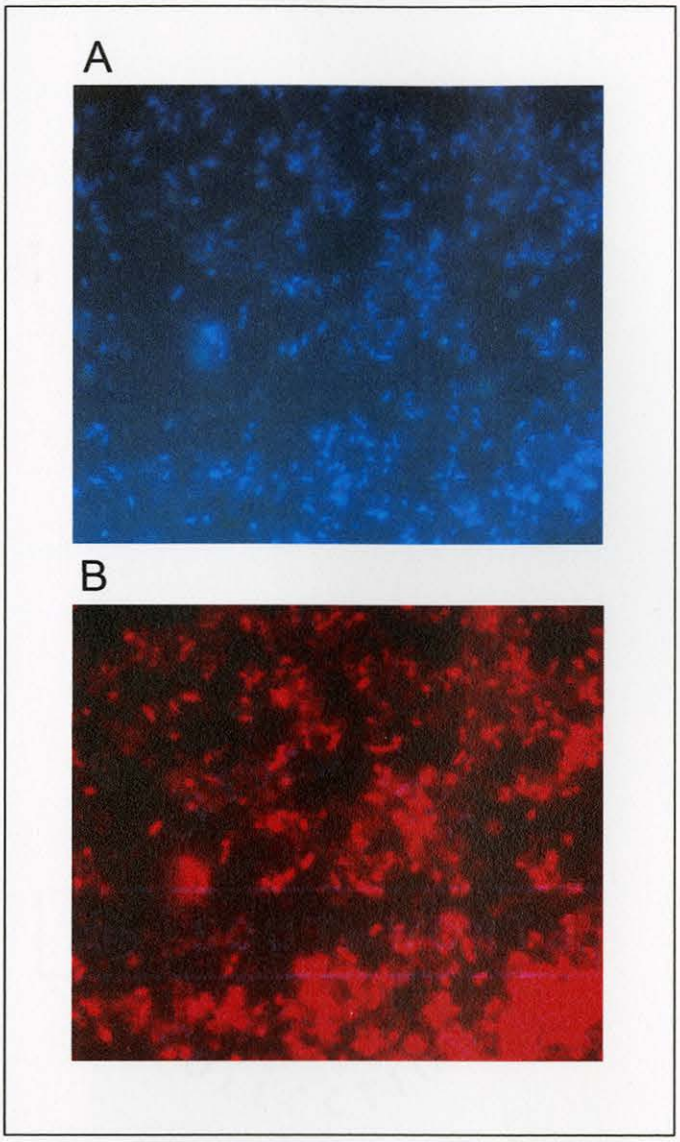

Figure 11. RING-FISH Displays a High Sensitivity for *V. parahaemolyticus* in Monoculture. A. *V. parahaemolyticus* culture stained with DAPI. B. V. parahaemolyticus hybridized with Cy3 labeled RING-FISH probe. (32)

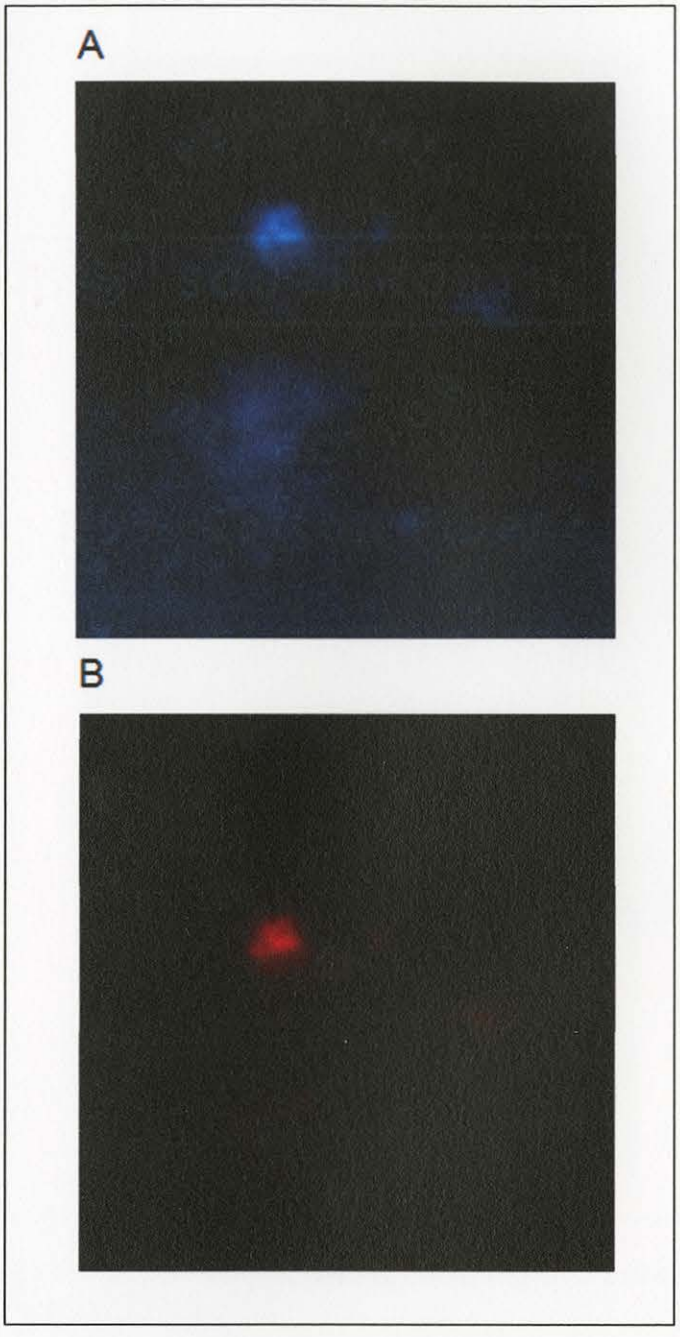

Figure 12. V. parahaemolyticus Cells Stained with (A) DAPI and (B) Hybridized with *trh* Specific RING-FISH Probe. (32)

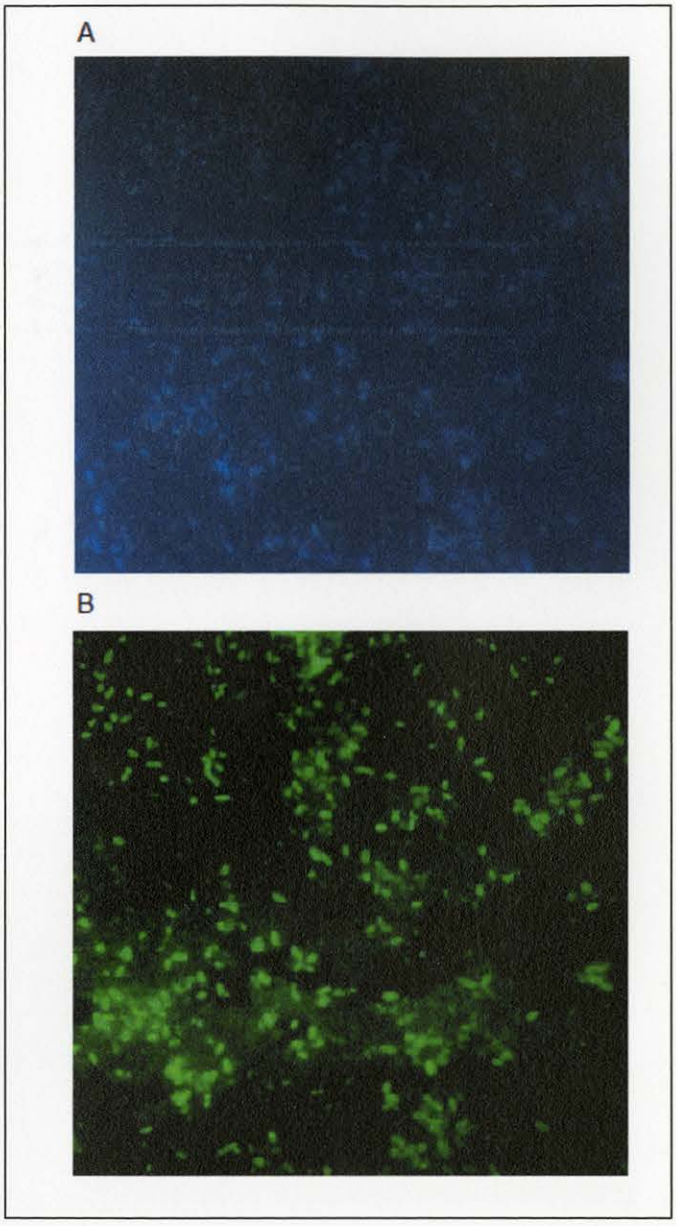

Figure 13. V. parahaemolyticus Cells Stained with (A) DAPI and (B) Hybridized with *tdh* Specific RING-FISH Probe.

The probe is specific to *V. parahaemolyticus* in mixed culture as well. Figure 7 shows an environmental water sample with probe bound to only two of the many DAPI stained cells.

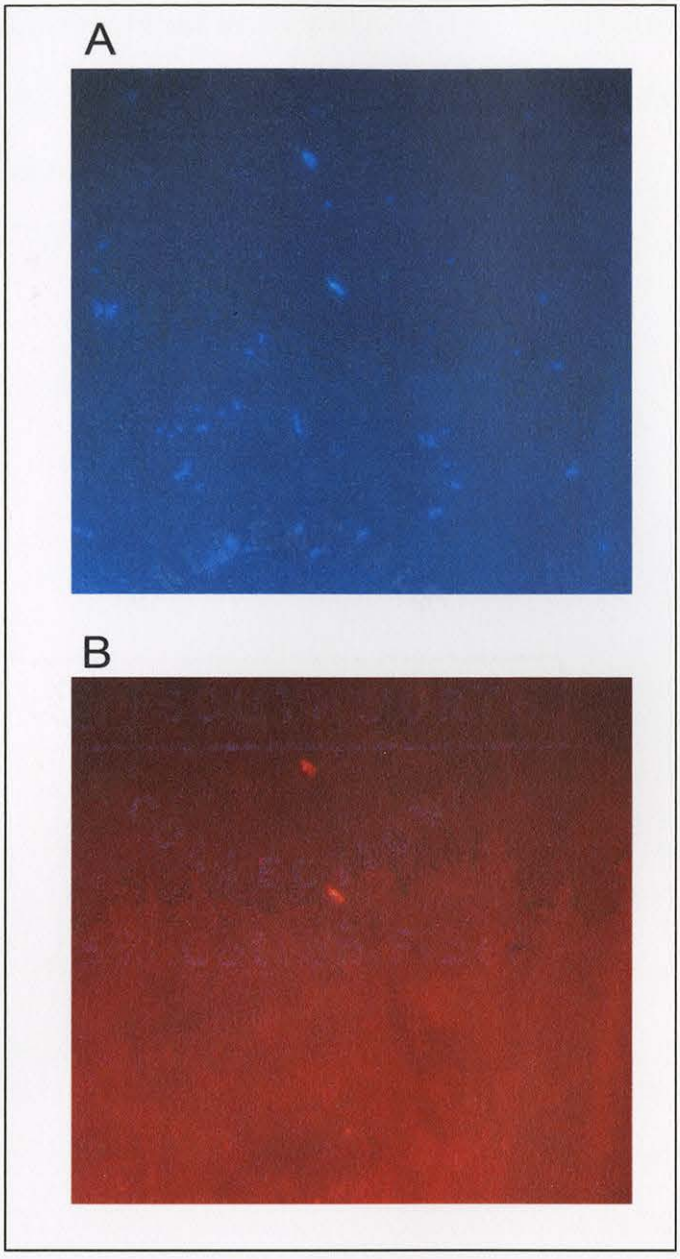

Figure 14. RING-FISH Displays a High Specificity for *V. parahaemolyticus* in Environmental Samples. A. Environmental sample stained with DAPI showing total bacteria. B. Environmental sample hybridized with Cy 3 labeled RING-FISH probe showing only V. parahaemolyticus. (32)

To test the efficacy of using RING-FISH to enumerate VBNC cells, a pure culture of *V. parahaemolyticus* was induced to enter the VBNC state and this progression was followed by hybridization with the *tlh* probe. Culturability of the

VBNC culture was determined by spread plating on T1N3 agar. The starting CFU/mL was over 18 million declining to 57,600 by day 10, 17,900 by day 20, 1560 by day 32, and finally reaching 0 by day 53 (Figure 15).

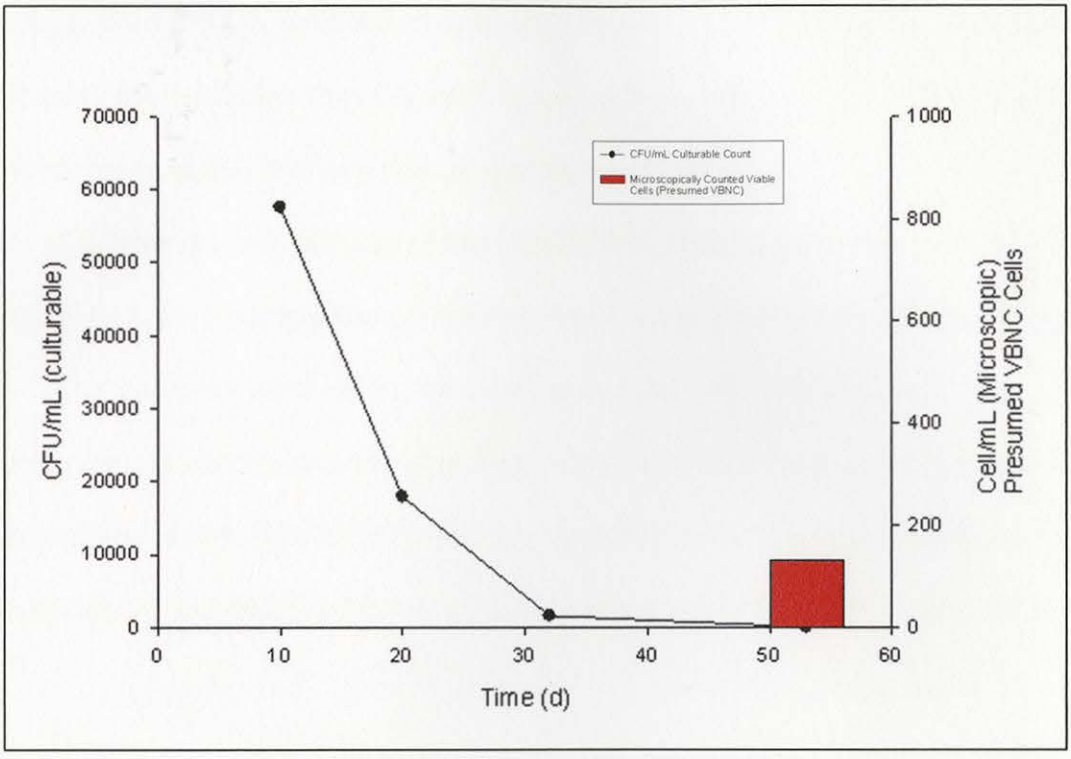

Figure 15. The Decline in Culturabiltiy of *V. parahaemolyticus* Over Time with the Presence of Viable Microscopic Cells in the Ending Time Point. (32)

The treated and fixed cells corresponding to the day 53 culture were hybridized for a microscopic total viable count. There were a total of 13 viable cells on the slide which corresponded to a density of 130 VBNC cells per milliliter in the total culture.

Restoring culturability to VBNC cultures is not always successful. Some cultures have been restored with temperature upshift alone, while others have used both addition of nutrients combined with temperature upshift (63, 74). In our study, culturability was restored by addition of APW and incubation at 33°C

overnight. The resulting turbid culture produced a lawn of growth on T1N3 agar and a lawn of purple growth on CHROMagar Vibrio plates which is indicative of *V. parahaemolyticus* (33). This growth from a culture that previously produced no plateable colonies, and was therefore considered undetectable by standard methods, demonstrates that the cells visualized microscopically by the Kogure method were viable and capable of resuscitation (45).

To truly test the efficacy of the RING-FISH method on environmental enumeration, four samples were collected and tested in the method presented here. The samples were collected in winter months when VBNC cells are expected to be more prevalent due to decreased sea surface temperatures. The results showed that RING-FISH did have an increased detection over the colony hybridization method indicating a detection of some VBNC cells (Figure 16).

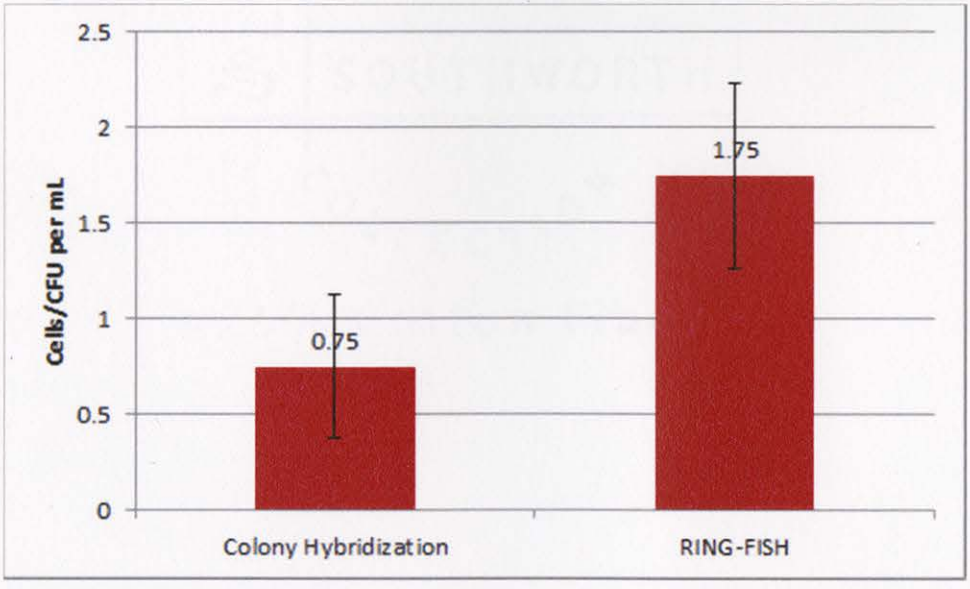

Figure 16. The Mean Values for Detection of Total *V. parahaemolyticus* Using Colony Hybridization and RING-FISH.

The *tlh* gene targeted for the full VBNC study is ubiquitous in *V. parahaemolyticus* and is therefore the one widely used for enumeration of population density in risk assessment models (69). The work presented here with the virulence factors *tdh* and *trh* and in related work with the Type III Secretion System is the first step in attempting to enumerate pathogenic strains of *V. parahaemolyticus (55).* These strains are not prevalent in large numbers in the water column even in summer months when the water temperature is elevated for sustained periods of time. They are often encountered in a greater frequency in sediment and oyster samples (42). Building on the research presented here to explore hybridization methods for these and other possible sample types could prove to be an even greater boon to the assessment of risk posed to human health by these bacteria.

CHAPTER V

CONCLUSIONS

The *Vibrio* genus is currently comprised of 90 distinct species of bacteria. Of these, nine have been shown to cause human illness. Three of these pathogens, *Vibrio vulnificus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*, are the focus of this body of work. All three are endemic to coastal habitats and, as such, the detection and accurate enumeration of these pathogens is of significant importance to human health protection.

Chapter II focuses on a novel agar (VVX) developed for the detection and direct enumeration of *Vibrio vulnificus* from oyster tissue. *V. vulnificus* is a significant human pathogen, which has been reported to be the number one cause of death from seafood related illnesses in the US (57). As such, the accurate and rapid enumeration of this bacterium from oyster tissue, commonly consumed raw, is very important to mitigate infection risk. As has been discussed, there are many methods for enumeration of *V. vulnificus* from environmental samples, though VVX agar has here been shown to be the only one capable of direct enumeration without requiring stringent growth conditions. Limited studies completed on water samples also showed promising results. With more extensive evaluation of this sample matrix in warmer months when *V. vulnificus* densities are at detectable levels, it is expected that VVX recovery from water will be on par with oyster tissue recovery. The validation results of this new agar formulation with its high sensitivity (87.5% recovery), and specificity

(98% positivity rate) have proven it will be a valuable tool in density determination assessment to mitigate the infection risk of *V. vulnificus*.

Chapter III presents a unique study of the densities of *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* in response to a major ecological shift. The 2011opening of the Bonnet Carre Spillway to prevent the Mississippi River flooding New Orleans quickly diverted a large amount of river water into the Mississippi Sound. This diluted the brackish water of the Sound significantly, and dropped the salinity in the western-most MS County to just above 1. The river water effect on the Sound was not as profound in the central and eastern counties which set up a salinity gradient presenting a unique sampling opportunity. Two hypotheses were tested; one, that *V. cholerae* would become the dominant *Vibrio* in the Sound as it is freshwater tolerant, and two, that this increase in *V. cholerae* density would facilitate gene transfer from toxigenic to non-toxigenic strains increasing the density of toxigenic *V. cholerae*. The results of the sampling efforts showed that the first hypothesis was supported. Linear regression analysis did not find a significant relationship between salinity and *V. vulnificus* or *V. cholerae* but there was a significant relationship with *V. parahaemolyticus.* This was not unexpected as *V. parahaemolyticus* has the highest salinity range of the three and would therefore be the first and hardest impacted by a reduction in salinity. Analysis of the relative abundance of the three Vibrios however revealed that once the salinity reached 6 *V. cholerae* was the only one of the three detected in the water column. The second hypothesis however was not supported by the data. None of the isolates collected

throughout the sampling period showed presence of the *ctx* gene responsible for production of the cholera toxin. Possible reasons for this non-detection of *ctx*+ isolates include a small sample size, no increase in toxigenic strains occurred, or the bacteria had entered the VBNC state preventing their cultivation. Follow-up samples were taken in January – February 2012 to determine if the relative abundance had returned to normal after the effects of the Spillway had dissipated. This evaluation was complicated however by the temperature difference between the two sample time periods being too great to allow for a single variable analysis. A second issue was the density for all three Vibrios was low owing to the reduced water temperature. A more accurate follow-up analysis is planned to be completed when the water temperature is within the same range as 2011 before this work is submitted for publication.

Chapter IV focuses on another detection method for *Vibrio* bacteria. This method is for the detection of *V. parahaemolyticus* in the Viable but Nonculturable State (VBNC). Cells enter the VBNC state in order to withstand harsh environmental conditions such as temperature or salinity changes or a lack of nutrient availability. Cells in this state have a reduced metabolic function and are not culturable on routine artificial media, but they do retain virulence and are able to cause disease upon gaining entry into a host. Current detection methods for *V. parahaemolyticus* rely heavily on the culturability of the cells. This methodology presents a potential underrepresentation of the true cell density as it does not take into account the VBNC population. The method presented in chapter four, recognition of individual gene - fluorescent *in situ* hybridization

(RING-FISH), when used alone provides a tool for total enumeration of all viable *V. parahaemolyticus* cells. When used in conjunction with established colony hybridization enumeration, this new method is able to enumerate just the VBNC portion of the population. The method was initially validated with the *V. parahaemolyticus* gene, thermolabile hemolysin (*tlh*), present in all cells for total enumeration. It was then extended to target known pathogenicity factors, thermostable direct hemolysin (*tdh*) and tdh-related hemolysin (*trh*). Employment of this new method either alone, or in conjunction with currently established methods can greatly improve upon risk assessment models now in place for *V. parahaemolyticus*.

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