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Vibrio Parahaemolyticus Interactions With Chitin In Response To Environmental Factors

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VIBRIO PARAHAEMOLYTICUS INTERACTIONS WITH CHITIN IN RESPONSE TO ENVIRONMENTAL FACTORS

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To my friends and family who believed in me and stood behind me every step of the way. And to Mango for always believing that every day can be the best day ever.

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ABSTRACT

Vibrio parahaemolyticus is a naturally occurring chitinoclastic human pathogen that adsorbs to substrates, including chitinous copepods and oysters, as part of its ecology, a phenomenon that is important in its responses to changes in environmental factors such as temperature and salinity. The following *in vitro* studies investigated *V. parahaemolyticus* adsorption and gene expression as a function of substrate, temperature, and salinity as a model for these abrupt changes, using qRT-PCR for absolute and relative quantitation measurements. Results for adsorption studies showed a negative temperature effect on adsorption for temperature shifts from 33 to 10 °C but no substrate or salinity effect, and results indicated that *V. parahaemolyticus* tended toward adsorption to a substrate, regardless of the chemical structure of the substrates tested. Analysis of growth data indicated that *V. parahaemolyticus* did not use chitin as a carbon source in these studies. To more thoroughly understand the response of *V. parahaemolyticus* to varying environmental conditions at a physiological level, additional studies targeted expression of six genes, including two metabolic genes, two virulence factors, two adsorption genes, and one chemotactic gene. Substrate alone had no significant impact on gene expression, and temperature alone had no significant impact on the expression of six of the seven genes investigated, with the exception of VPA1548. However, most genes were expressed at higher levels when salinities were shifted from 10 to 20 grams per liter (gpL) than when shifted from 10 to 35 gpL, with the exception of metabolic gene *thiC*. In addition, chitin caused significant up-regulation of chitobiase and VPA0459 when temperatures were shifted from 33 to 37 °C but for no other temperature shifts. Chitin caused up-regulation and down-regulation

of VP1892 for 33 to 10 °C shifts and for 33 to 25 °C shifts, respectively, and chitin caused up-regulation of VP1892 when shifted from 10 to 35 gpL. This is the first study to examine the response of these genes in *V. parahaemolyticus* in response to changes in three parameters simultaneously and illustrates the potential physiological responses and adaptations of *V. parahaemolyticus* to its changing environment.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Vibrio pathogenesis and the carbon cycle

Vibrios, which are the dominant culturable heterotrophic bacteria in the ocean described to date, are known to attach to and form biofilms on substrates in the environment (Grimes et al., 2009). Several species of vibrio are pathogenic to humans and animals, and *V. parahaemolyticus* is one of 12 that are pathogenic to humans (Grimes et al., 2009). *Vibrio parahaemolyticus* is a major cause of gastroenteritis associated with the consumption of raw and undercooked seafood. It is also associated with wound infections and can cause sepsis in immunocompromised persons (Centers for Disease Control and Prevention, 2013). *V. parahaemolyticus* is halophilic, Gram-negative, and is found free-living and attached to particles, mostly along coastal regions during the warmer months of the year (Joseph et al., 1982; Centers for Disease Control and Prevention, 2013). Temperature is a major determinant of the concentrations of *V. parahaemolyticus* found in the environment (Kaneko and Colwell, 1975). *V. parahaemolyticus* adsorption to solid particles is of concern because adsorption is likely important for the survival and persistence of *V. parahaemolyticus* in the environment, and the availability of adsorption sites may therefore correlate with the probability of human infection.

Vibrios can cause gastroenteritis, spreading necrotic skin lesions, septicemia (Woo et al., 1984). Reported infections include *Vibrio vulnificus* (*V. vulnificus)* open skin lesions, and gastroenteritis (Woo et al., 1984; Strom and Paranjpye, 2000; Butt et al., 2004; Hsueh et al., 2004; Oliver, 2005; Ramamurthy and Nair, 2007; Horseman and

Surani, 2011), *V. parahaemolyticus* ear, eye, blood, gastrointestinal, and wounds infections (Daniels et al., 2000; Butt et al., 2004; Oliver, 2005; Ramamurthy and Nair, 2007), *Vibrio alginolyticus (V. alginolyticus)* ear and wound infections (Oliver, 2005; Ramamurthy and Nair, 2007), and *Vibrio cholerae (V. cholerae)* blood, wound, ear, bile, peritoneal fluid, sputum, and cerebral spinal fluid infections (Oliver, 2005), and *V. mimicus* gastrointestinal and ear infection (Shandera et al., 1983). Vibrio introduction into humans is commonly through ingestion of vibrio carrying raw or undercooked seafood, or open wound exposure to vibrio containing waters.

Responses in the environment may be a direct reflection of how *V. parahaemolyticus* is capable of surviving and causing infection in the human body (Pruzzo et al., 2008). It is hoped that by furthering understanding of *V. parahaemolyticus* responses in the environment, this research may lead to a better understanding of *V. parahaemolyticus* responses as it pertains to human health. In particular, examining vibrio responses to acute and sudden changes in its extracellular milieu can facilitate more informed development of drug delivery systems and antimicrobial drugs and vaccines against *V. parahaemolyticus* and other infectious bacteria (Eko et al., 2003; Anderson et al., 2006; Gao et al., 2007). One potential benefit of this research is the identification of potential targets for vaccine and antimicrobial drug development, such as the cholera vaccine, currently in human trials (Talavera et al., 2006). Characterization of factors involved in *V. parahaemolyticus* environmental stress responses could further our current understanding of *V. parahaemolyticus* ecology. In order to achieve this goal, it is necessary to understand adsorption and gene

regulation related to *V. parahaemolyticus* acclimation and adaptation to changing environmental parameters.

In addition to pathogenicity, vibrios make significant contributions to the environment. The degradation of chitin is a very important factor in the cycling of carbon in the ocean by *V. parahaemolyticus* (Pruzzo et al., 2008; Souza et al., 2011). Chitin, a primary component of the zooplankton exoskeleton, is a known substrate for *V. parahaemolyticus* adsorption. Adsorption is attributable to induction of pili and chitinase production (Grimes et al., 2009). Adsorption in the environment is also of human health importance because the mechanisms of adsorption of *V. parahaemolyticus* to zooplankton and other chitin-containing substances could be indicative of, or may parallel, the mechanisms of adsorption of *V. parahaemolyticus* to human epithelial cells. By use of extracellular enzymes, vibrios break down organic matter into more simple substrates, which can then be taken into the cell for further processing (Arnosti, 2011). Interactions with chitin entail either random collision or chemotaxis, followed by attachment to chitin, degradation of chitin into soluble subunits, and hydrolysis of those subunits into *N*-acetyl glucosamine (Pruzzo et al., 2008). Based on published trends involving interactions between chitin and vibrios, it is reasonable to hypothesize that *V. parahaemolyticus* adsorption in the environment is important for its survival and reproduction.

1.2 Vibrio ecology

It is possible that bacterial adsorption to particles may be advantageous to marine bacteria by enhancing the probability that bacteria will be consumed by particle feeders. Although some particle-bound bacteria are digested during passage through

the guts of particle feeders, more than half appear to survive and multiply in the fecal pellets (Lawrence et al., 1993). Attachment to particles may provide a mechanism for bacteria to gain access to the gut of particles feeders, where they can then adsorb onto nutrient-rich fecal pellets that can be utilized as a source of nutrition.

It has been suggested that if bacteria that have been ingested survive passage through the gut of a particle feeder, they may thrive in fecal pellets, the implication being that attachment to particles may be a survival strategy (Hastings and Nealson, 1977; Lawrence et al., 1993). Likewise, passage through the human intestine allows for bacterial exposure to a nutrient-enriched microenvironment that is advantageous to growth and survival (Worden et al., 2006).

Oysters can also serve as particle attachment sources as the oysters filter feed. It has been reported that oysters can retain small particles (Charles et al., 1992) onto which bacteria may be attached, and can also filter bacteria directly from the filtered water itself (Cabello et al., 2005). Bivalves filter feed by passing water through their gills which capture microorganisms, detritus, and particles via secreted mucus (Cabello et al., 2005). Once these particles pass through the stomach, enzymatic activity digests the particles which are then absorbed (Cabello et al., 2005). Any non-absorbed particles are passed through the oyster and discarded, and some microorganisms can resist the oyster's digestive processes (Cabello et al., 2005).

Several scenarios exist in which vibrios are subjected to sudden environmental shifts. Vibrios often survive these shifts by up-regulating or down-regulating genes that allow them to acclimate in a short period of time. Examples of these scenarios include

sudden changes in temperature or salinity, such as that seen when vibrios are transported via ballast water, when cold raw oysters are consumed by warm-blooded mammals, or when freshly harvested oysters are placed on ice to minimize pathogen growth. In addition, the U.S. Food and Drug Administration recommended in 2012 that oysters should be placed on ice within 5 hours of harvest. Thus, when resident vibrios are subjected to this shock, it is not clear how the cells respond. In a 2005 study, the *gfp* gene that codes for green fluorescent protein was used to identify the fate of *V. parahaemolyticus* when exposed to filtering oysters and found that during postharvest depuration, a small fraction of bacteria remained in the oyster tissue and *V. parahaemolyticus* grew rapidly after storing oysters at room temperature (Cabello et al., 2005).

The key to understanding how *V. parahaemolyticus* interacts in mammalian bodies and aquatic environments is understanding the functions of *V. parahaemolyticus* in its extracellular environment. Thus, understanding the ecology of vibrios and their responses to environmental changes is important.

1.3 Vibrio in the environment

1.3.1 Vibrios in the Gulf of Mexico

Vibrio populations and epidemiology are dominant in the Gulf of Mexico. Clinically, many vibrio infections in the United States have been associated with the Gulf of Mexico and specifically the state of Louisiana, and the state continues to be the dominant source of oysters destined for raw consumption, which is a major risk factor for vibrio infection (DePaola et al., 2003). We have demonstrated previously that total *V. parahaemolyticus* concentrations may be as low as 1 CFU/mL in water and as high as -

83,000 CFU/g in sediment in the northern Gulf of Mexico (Johnson et al., 2012). Thus, research on vibrio populations in the Gulf of Mexico is timely.

1.3.2 Vibrio temperature and salinity

Vibrios are exposed to a variety of conditions in its extracellular environment, including ranges of temperature, salinity, pH, turbidity, and other factors. With an *in vitro* generation time of less than 10 minutes, *V. parahaemolyticus* can have one of the shortest generation times of any bacterium (Martinez-Urtaza et al., 2010). Although *V. parahaemolyticus* can survive and grow at temperatures below 10 °C, growth in the environment is most efficient at 10 to 37 °C; in addition, optimum growth has been reported in salinities ranging from 10 to 34 (Martinez-Urtaza et al., 2010). Within this range of salinities, it has been reported that optimum salinity for *V. parahaemolyticus* in oysters is 23 (Martinez-Urtaza et al., 2010). Johnson et al. (2012) demonstrated previously that temperatures at sampling sites in the northern Gulf of Mexico at which *V. parahaemolyticus* was detected on the Louisiana and Mississippi coasts were 7 to 34 °C, and salinities were 1.3 to 27.1. The temperature and salinities at which the maximum vibrio densities occurred varied with sample type and gene target (Johnson et al., 2012). For example, the maximum densities of *tlh*, *tdh*, *trh*, and *vvh* in water samples occurred at temperatures of 31.9 °C, 12.0 °C, 18.6 °C, and 21.8 °C, respectively, and at salinities of 6.6, 23.7, 24.3, and 15.5, respectively (Table 1). Thus, several studies have examined the relationships between *V. parahaemolyticus* and environmental parameters such as temperature and salinity *in situ*; however, *in vitro* studies remain important in further understanding of *V. parahaemolyticus* responses to changes in its surrounding conditions. Therefore, the salt concentrations and

temperatures used in the current study were chosen based on previous ranges to which vibrios are regularly exposed.

Table 1: Maximum values of target genes

Maximum	Date	CFU/mL, CFU/g	Temperature (°C)	Salinity
tlh wat	8/4/2010	204	31.9	6.6
tdh wat	2/24/2010	66.5	12.0	23.7
trh wat	11/23/2009	23	18.6	24.3
vvh wat	4/7/2010	87.5	21.8	15.5
tlh oys	5/5/2010	21950	28.1	24.5
tdh oys	12/15/2008	241	13.2	26.5
trh oys	12/16/2008	186	12.8	26.6
vvh oys	12/16/2008	19050	12.8	26.6
tlh sed	7/21/2010	116000	30.3	22.3
tdh sed	5/19/2010	409	28.3	7.7
trh sed	5/19/2009	525	20.7	9.2
vvh sed	5/25/2010	35800	29.6	9.7
Mean values			21.7	18.6
CFU = colony-forming units (per milliliter or per gram)				

1.4 Environmental stressors

Although *V. parahaemolyticus* are exposed to long-term natural changes, and several studies have addressed the relationships between the bacteria and their environment via *in situ* studies, little remains known about how *V. parahaemolyticus* respond to abrupt natural and anthropogenic changes. Environmental stressors of particular interest include oxidative stress via exposure to H_2O_2 which can be found both in the aquatic environment and in the human intestine (Yildiz and Schoolnik, 1998; Joelsson et al., 2007), acid shock in the human stomach, detergents, antibiotics, and predatory protozoa (Joelsson et al., 2006) and nutrient deficiency (Yildiz and Schoolnik, 1998). In the natural environment, vibrios encounter environmental stressors that would

cause genetic and physiological changes for acclimation and possible adaptation. Although vibrios are exposed to a range of temperatures, the genus possesses the ability to acclimate to acute changes in both cold and warm temperatures. Genomic analyses of six *Vibrio* spp. have confirmed the ability of these bacteria to thrive in a range of environmental stressors including scenarios in which utilization of readily available ocean substrates and biofilm formation constitute stress responses (Grimes et al., 2009). Cold shock proteins in *V. vulnificus* have been reported to react differently during immediate temperature down-shift (McGovern and Oliver, 1995). Production of 40 proteins were found to increase significantly when *V. vulnificus* was shifted from 23 to 13 °C. Specifically, after 15 minutes, five proteins reached maximum expression, after 30 minutes, 10 proteins reached maximum expression, and after one hour, five proteins reached peak expression (McGovern and Oliver, 1995). In addition, after two hours and four hours, 13 and seven proteins were maximally expressed, respectively (McGovern and Oliver, 1995). These responses to cold shock treatment may represent survival factors that allow for acclimation of bacteria to cold temperatures (McGovern and Oliver, 1995). Another microarray study measuring gene expression for the complete transcriptome of *V. vulnificus* during cold shock conditions reported an overall gene repression when temperature was down-shifted from 35 °C to 10 °C while gene expression was up-regulated below 4 °C (Wood and Arias, 2011). Highest gene expression was observed in cold shock proteins, *cspA* and *cspB* (Wood and Arias, 2011). In addition, ribosomal, protein folding regulators and membrane genes also significantly increased in expression (Wood and Arias, 2011). Highest gene repression

was observed for genes coding for anti-oxidants, sugar uptake, and amino acid scavengers (Wood and Arias, 2011).

Acclimation to warm temperatures is important in the survival of vibrios in the environment. *V. cholerae*, the causative agent of cholera, leads to intestinal infection resulting in diarrhea which is caused by action of the exotoxin, cholera toxin protein (Holmgren, 1981; Spangler, 1992). A 1990 study showed that the cholera toxin gene, *toxR,* was down-regulated at 22 ºC when compared to 37 ºC (Parsot and Mekalanos, 1990). In contrast, expression of the gene encoding heat shock protein HtpG was up-regulated with the same treatment (Parsot and Mekalanos, 1990). The proportional relationship of these two genes is thought to be due to an overlap of the two promoters (Parsot and Mekalanos, 1990) and provides an example of additional factors possessed by vibrios to allow them to acclimate to changing stressors.

It has been suggested that the translocation of pathogenic *V. parahaemolyticus* by means of ballast water discharges was responsible for the spread of *V. parahaemolyticus* infections to Spain and Texas, which resulted in outbreaks (Myers et al., 2003; Martinez-Urtaza et al., 2010). Relocation through ballast water is another example of acute changes in the extracellular environment. Ship ballast water taken aboard in one location when unloading cargo, and released overseas while loading new cargo, introduces the spread of invasive marine species including invading pathogens (Mimura 2005). In a study measuring total concentrations of bacteria in ship ballast water, *V. cholerae* was found in samples from all ships, and strains causing human epidemic cholera were found in 93% of the ships (Ruiz et al., 2000). A previously unreported strain of *V. cholerae* was detected in the Gulf of Mexico after ships with a

last port of call in South America, where there was currently a *V. cholerae* epidemic, were found to have carried the pathogenic strain in their ballast (Drake et al., 2007). As described above, the introduction of the highly pathogenic O3:K6 *V. parahaemolyticus* likely occurred via ballast water transport (Myers et al., 2003; Martinez-Urtaza et al., 2010). While the exact occurrence of this introduction is unknown, it is possible that the pathogen was exposed to a stressor via sudden salinity changes.

Oyster relaying is often used to remove human pathogens from oysters. The relaying process involves the transfer of shellfish from restricted areas to open areas to allow natural decontamination of oysters through biological cleansing (Motes and DePaola, 1996). The movement of oysters from one location to another leads to a possible introduction of abrupt changes in environmental stressors to vibrios within the oysters. A study by Motes and DePaola (1996) described both temperature and salinity differences when oysters were relayed from near-shore harvest sites in coastal Alabama to offshore relay sites. Oyster temperatures at the relay site were lower than temperatures at the harvest site, and average salinities were consistently higher at the relay site than at the harvest site. In a more recent study examining the effects of salinity on oyster relaying as a postharvest processing strategy, *V. parahaemolyticus* and *V. vulnificus* densities decreased by up to 3 orders of magnitude (Audemard et al., 2011). In this study, oysters and their resident vibrios, were shifted from low salinities (14 to 15 psu) and moderate salinities (22 to 25 psu) to high salinities (≥ 30 psu) (Audemard et al., 2011). Thus, results from these and other studies illustrate the responses of vibrios to acute changes in environmental conditions.

Oyster depuration is another method of removing overall bacterial populations from oysters. The depuration process involves purging oysters in clean seawater to remove sand and bacteria (Chae et al., 2009). Previous reports indicated that the depuration process does not successfully reduce vibrio concentrations when conducted at ambient temperature (Colwell and Liston, 1960). However, studies with varying temperature exposures have shown more promising results. In a 1992 study, *V. vulnificus* concentrations increased at temperatures above 23 °C but remained at a constant level at 15 °C (Tamplin and Capers, 1992). In addition, in a 2009 study, an abrupt change in temperature from room temperature to four separate test temperatures (22, 15, 10 and 5 °C), resulted in a higher efficiency of *V. vulnificus* and *V. parahaemolyticus* removal at 15 °C than at 22 °C (Chae et al., 2009). No decrease in vibrio concentrations were seen at 10 and 5 °C which may be due to decreased oyster filter feeding at these temperatures (Chae et al., 2009). Thus, cold and heat shock conditions, oil exposure, ballast water, and oyster relaying and depuration are examples of environmental stressors impacting vibrios.

1.5 Gene expression

Vibrios are chitinoclastic, autochthonous organisms that can be opportunistic pathogens. They possess factors that enable them to acclimate and adapt to a wide range of highly variable extracellular conditions. To adjust to changing conditions gene regulation is one way to enhance survivability in the environment (Mekalanos, 1992; Zhu et al., 2002; Lobo, 2008). Vibrios have been demonstrated to possess a variety of genes that play a role in survival during changing conditions, such as the stressors described above. The time scale of bacterial gene regulation in response to

environmental cues varies from seconds to hours, and this has been shown to hold true for *V. cholerae* (Kanjilal et al., 2010). Specifically, in a 2010 study, regulation of cholera toxin gene expression was compared *in vitro* and *in vivo* under virulence and non-virulence inducing conditions using AKI medium and LB medium, respectively, at various time points of up to 6 hours (Kanjilal et al., 2010). The study demonstrated significant changes in expression of toxin genes such as *tcpA*, *ctxAB*, and *toxT* at time points that included a range of 85 to 210 minutes (Kanjilal et al., 2010). A 1998 study measured temporal expression of *V. cholerae* tricistronic operon *vieSAB* signal transduction genes *in vitro* and during colonization of an infant mouse intestinal tract. Introduction into the 37 °C mouse intestinal tract occurred through injection after incubation at 30 °C, and results showed that transcription of *vieA*, *vieB*, and *vieS* genes differed during *in vitro* and *in vivo* experiments (Lee et al., 1998). Specifically, expression of signal transduction gene *vieB* occurred at 3 hours and 3.5 hours was chosen as an optimal time period in which to measure transcription of *vieB* (Kanjilal et al., 2010).

Gene regulation is crucial when vibrios are exposed to abrupt changes such as interactions with host and aquatic environment. When genes are expressed and how much of the gene is expressed is the basis of gene regulation in an organism. Genes are sections of DNA in a cell that encode for a specific product (e.g., a protein). Genes are coded into mRNA by means of DNA transcription in which a complementary copy of RNA results from the original DNA; all thymines in the original gene code are replaced with uracils (Clancy and Brown, 2008). Reverse transcription, which involves converting an RNA template into cDNA by reverse transcriptase, allows for mRNA to be used as a

measurement of the amount of targeted genes present. During varying stresses and environmental conditions, increasing or decreasing the expression of particular genes for the formation of specific enzymes and proteins allows the organism to acclimate (Lobo, 2008; Shaw, 2008). In bacteria, genes are regularly expressed, but can be turned off by repressor proteins that regulate expression by binding to the operator DNA sequence near a cluster of co-regulated genes (Shaw, 2008). Binding of repressor proteins prevents RNA polymerase from binding and causes the repression of gene expression, which is sensitive to a ligand that binds to the repressor and signals the environmental conditions (Shaw, 2008). An example of this type of gene expression is the regulation of metabolism in conjunction with nutrient levels in the environment (Shaw, 2008). The inability to control specific gene expression could be detrimental to the survival of bacteria in adverse environments. Because gene expression can be measured by the amount of mRNA in a cell, the amount of mRNA present in cells at specific intervals allows for quantitation of up-regulation and down-regulation of specific genes. Measurement of these genes can be carried out by use of quantitative real-time PCR (qRT-PCR), which enables quantitation of targeted genes in real-time. The result is the generation of a threshold cycle (Ct) value that allows for the estimation of the absolute and relative gene concentrations present for each individual template. Absolute quantitation is used to determine the amount of targeted genes by comparing Ct values of targeted genes to a standard curve of known concentrations. Relative quantitation measures the amount of gene expression of a sample relative to another sample, such as a control. This method of measuring gene expression can also be carried out with the use of a reference gene, usually a stable housekeeping gene within

the organism of interest, to which one compares other targeted gene expressions in order to measure the amount or fold change[1] occurring within each reaction.

Acclimation and adaptation of *V. parahaemolyticus* to changing environmental factors can also be attributed to the expression of, and acquisition of, genes through horizontal gene transfer that provide the bacteria with a cache of variable fitness factors (Johnson, 2013). The pathogenicity island on chromosome 2 of *V. parahaemolyticus* carries genes encoding for the acclimation of the bacterium to various environmental parameters encountered in marine environments, including pathogenicity factors, antibiotic resistance, and effector proteins (Makino et al., 2003). Because of its existence in variable environments, including marine and host systems, the ecology of *V. parahaemolyticus* is dependent on several factors for survival, including nutrient acquisition, immune evasion factors, biofilm formation, acclimation to environmental stressors (including temperature and salinity variations), and attachment and colonization (Johnson, 2013).

Expression of stress and toxicity genes have been measured in vibrios in association with changing salinity. A 2008 study showed that when comparing *in vitro* and *in situ* stress and virulence gene expression in *V. vulnificus*, genes required for *in vitro* survival were not necessarily required for *in situ* survival (Jones et al., 2008). Kao et al. (2009) reported that there was increased survival of *V. anguillarum* with an up-shift of salinity from 8.5 gpL to 15 gpL, than 8.5 gpL to 35 gpL. In a 2009 study of *V. anguillarum*, Omp26La, a porin-like outer membrane protein (OMP) with a high

[1] Fold change is a metric of how much something has changed from an initial to final value. For example, if something changes from an initial value of 5 to a final value of 10, there has been a twofold change.

sequence identity to *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *Photobacterium damselae* porin-related proteins, exhibited high expression levels at 8.5 gpL, indicating its use as a salt response adaptation to lower salinities (Kao et al., 2009). Also, expression of maltoporin, a protein responsible for permeation of saccharides across the bacterial outer membrane, was reduced at higher salinity (Kao et al., 2009). In contrast, OmpW and OmpU were up-regulated at 35 gpL (Kao et al., 2009). OmpW has been shown to be regulated in response to environmental conditions such as nutrients, temperature, and osmoregularity, in other vibrios including *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*; OmpU is associated with bile resistance and biofilm formation (Kao et al., 2009). These reports show that salinity significantly impacts gene expression, and they illustrate the importance of gene expression profiles for acclimation to a range of factors.

It has also been reported that bacterial adsorption can increase with increasing temperature, possibly due to changes in bacterial morphology and/or possible changes in thermodynamics involved in adsorption (Fletcher and Marshall, 1982; Mai and Conner, 2007). Laboratory models are important in the study of bacterial trends and responses in the environment, and many of these models incorporate temperature as a variable to assess the effects associated with physiological and genetic modifications in response to temperature changes (Membre et al., 2005; Gross, 2006). Information collected from these models aids in the prevention of human infection by harmful bacteria associated with food contamination and potentially pathogenic bacteria in the environment.

In addition to sudden changes in salinity, sudden changes in temperature have also been demonstrated to play a role in vibrio gene expression. For example, a 2006 study investigated *in vitro* (cold shock from 22 to 4 ºC) and *in situ* (<15 ºC estuarine waters) genes associated with the viable but non-culturable state (VNBC) of *V. vulnificus* (Smith and Oliver, 2006). This study reported detection of *vvhA* hemolysin gene expression during *in situ* investigations only up to one hour after treatment (Smith and Oliver, 2006). It was suggested that *vvhA* plays a role in osmoprotection and/or cold shock response (Smith and Oliver, 2006).

This dissertation examines *V. parahaemolyticus* interactions with chitin in response to environmental factors. The studies described in chapter 2 were conducted to determine the effects of temperature and salinity on the adsorption of *V. parahaemolyticus* to chitin and plastic substrates and what role bacterial growth and cell density play in *V. parahaemolyticus* adsorption. Chapter 3 describes studies that were conducted to determine the effect of temperature, salinity, and chitin on the expression of *thiC*, *tdh*, chitobiase, VPA1598, VP1892, VPA0459 and VPA1548. Chapter 4 includes a research summary and implications of the effects of environmental parameters on changes in *V. parahaemolyticus* adsorption and gene expression.

CHAPTER 2: EFFECTS OF TEMPERATURE AND SALINITY ON *VIBRIO PARAHAEMOLYTICUS* ADSORPTION *IN VITRO*

2.1 Abstract

Vibrio parahaemolyticus is a naturally occurring chitinoclastic human pathogen that adsorbs to chitinous organisms as part of its ecology, a phenomenon that is important in its responses to environmental factors such as temperature and salinity. The current study aimed to investigate *V. parahaemolyticus* adsorption *in vitro* as a function of substrate type, and shifting temperature and salinity, in the absence of confounding environmental factors. The microcosm studies included parameter combinations of substrate (chitin and plastic), salt concentration (20 and 35 gpL) of artificial seawater (ASW), and temperature (10, 21, 25, 30, and 37 °C). Following shifts from growth parameters (33 °C, 10 gpL) to test parameters, microcosms were incubated for 3.5-hour periods, and quantitative real-time PCR was used to enumerate copy numbers following exposures. Interestingly, there was an effect of abrupt temperature change on adsorption but no effect of substrate or change of salinity, and results indicated that *V. parahaemolyticus* preferred adsorption to a substrate based on the tested substrates. The lack of a substrate effect may have been due to the absence of a chemical gradient from the degradation of chitin, which would have led the bacteria to the chitin. Indeed, growth similarities between chitin and plastic indicated *V. parahaemolyticus* did not use chitin as a carbon source in these studies. This research illustrates the relationship between abrupt changes in temperature and salinity on adsorption, and the methodology is a complementary approach to studies investigating responses of vibrios in their native marine and estuarine environment.

2.2 Introduction

Several environmental factors have been linked to the abundance and distribution of *V. parahaemolyticus*, including temperature, salinity, and turbidity (Colwell, 1996; Johnson et al., 2010; Johnson et al., 2012). Temperature is a major factor that influences the concentrations of *V. parahaemolyticus* in the environment (Kaneko and Colwell, 1975). Because increasing temperature and increasing concentrations of *V. parahaemolyticus* are positively correlated, natural and anthropogenic influences on temperature could lead to short-term and long-term increases in the abundance of *V. parahaemolyticus* in the environment and could therefore lead to an increase in the number of human infections (Martinez-Urtaza et al., 2008). Natural and anthropogenic influences have been reported to likely alter the risk of vibrio-related disease in the northern hemisphere (Eiler et al., 2007). In a study to determine the effect of low tide exposure on *V. parahaemolyticus* levels, *V. parahaemolyticus* densities were four to eight times greater after maximum exposure than during initial exposure in oysters exposed to ambient air during low-tide events (Nordstrom et al., 2004).

Environmental factors have also been demonstrated to play a role in adsorption of vibrios. A 2006 study investigating the culturable and non-culturable form of *V. cholerae* in an estuary reported that although *V. cholerae* are found attached to copepods in the varying salinities encountered in the study, bacteria at high salinity, 30 to 35 psu, were detected more prevalently in the non-culturable state (Thomas et al., 2006). This implies that *V. cholerae* may attach to copepods during adverse conditions, and may indicative a survival strategy of the bacteria (Thomas et al., 2006). Studies in

Bangladesh have demonstrated the importance of vibrio adsorption to copepods. Specifically, *V. cholerae* populations in water, and by association risk of cholera illnesses, were significantly decreased via filtration with the commonly used sari cloth (Huq et al., 2010). In a 1984 study examining attachment of *V. cholerae* to live copepods at 5, 10, and 15 gpL, highest attachment was demonstrated at 15 gpL when bacteria were added to microcosms at 22 ºC after initial growth at 30 ºC (Huq et al., 1984). In this same study, *V. cholerae* attachment had a highest attachment at 30 ºC at a salinity at 1 gpL when compared to 5 and 15 gpL (Huq et al., 1984). In another study, *V. cholerae* and *V. parahaemolyticus* were demonstrated to adsorb to live copepods at 0.2, 15 and 22 gpL at ambient temperature (23 to 26 ºC) (Huq et al., 1983). *V. parahaemolyticus* has similarly been demonstrated to adsorb to live copepods in 5 gpL natural seawater, a 5 gpL NaCl solution, and a 15 gpL NaCl solution at 20 ºC (Kaneko and Colwell, 1975). Thus, changes in environmental conditions may not only lead to an increase in *V. parahaemolyticus* concentrations, but may also effect *V. parahaemolyticus* adsorption.

Vibrios are chitinoclastic and adsorb to chitin in aquatic environments (Kaneko and Colwell, 1975; Pruzzo et al., 2008). Chitin, the most abundant renewable polymer in the ocean and the second most abundant polymer on Earth, is a polymer of ß-1,4-linked *N*-acetyl glucosamine (GlcNAc) found throughout the environment in organisms such as zooplankton (Rinaudo, 2006; Souza et al., 2011; Blokesch, 2012). Chitin is an important source of carbon and nitrogen in the ocean and is utilized by free-living and adsorbed chitinolytic bacteria, which play a large role in the cycling of nutrients in the ocean (Souza et al., 2011).

Vibrio adsorption to chitin has been attributed to production of chitinases, which break down the major components of chitin (Gooday, 1990; Pruzzo et al., 2008) and enable vibrios to utilize chitin as a source of carbon. *V. parahaemolyticus* chemotaxis has been demonstrated to guide vibrio toward chitin monomer GlcNAc gradients that are produced by the breakdown of chitin that has come into contact with chitinase secreted by stressed vibrio cells (Pruzzo et al., 2008). Substrate adsorption is an important phase of the life cycle of environmental bacteria, and the interactions between bacteria and their adsorption sites are influenced by both the bacteria and the surfaces to which they attach. The influential factors include salt concentration, temperature, pH, charge, hydrophobicity, bacterial growth phase, and other factors (Zeraik and Nitschke, 2012). Monomers of GlcNAc are also found as sugar residues on the surface of human epithelial cells. The tendency of vibrios to respond to a GlcNAc gradient could therefore have implications for human infections (Jude et al., 2009). *V. parahaemolyticus* adsorption to particles and solid substrates is of concern because adsorption is important for the survival and persistence of *V. parahaemolyticus* in the environment and consequently increases the probability of human infection. Interactions between bacteria, ocean cycling of carbon and nitrogen, and animal/human contact and infection could be better understood by elucidating bacterial adsorption to particles and solid substrates.

When oysters such as *Crassostrea virginica* filter feed, particles are ingested, and often these particles are colonized with vibrios. It has been reported that vibrio attachment to particles may be a response mechanism during unfavorable environmental conditions (Cottingham et al., 2003; Alam et al., 2006; Belkin and

Colwell, 2006; Lutz et al., 2013). Switching between free floating and surface-attachment allows vibrios to enhance their microenvironments (Cottingham et al., 2003). Attachment of vibrios to particles allows for retrieval of excess nutrients found on the surfaces (e.g. chitin) of these particles and may be a survival mechanism in nutrient-limited environments (Lutz et al., 2013). Reports have also shown varying temperature influences on attachment to particles. Although in one study, attachment of *V. cholerae* to zooplankton increased with increasing temperature above 15 °C, another study reported more prevalent concentrations in the water column than attached to phytoplankton at 19 °C (Lutz et al., 2013).

Conflicting results indicate several factors may influence attachment (Lutz et al., 2013). Several approaches to reducing the levels of vibrios in oysters meant for human consumption have been used, including temperature regulation of postharvest processes. Historically, depuration methods have been attempted with limited success. Depuration is the process of purging shellfish of sand and bacteria by filter feeding oysters in ASW (Chae et al., 2009). This process is used in an attempt to reduce the amounts of resident microbial contaminants in shellfish before refrigeration and consumption (Su and Liu, 2007). Although depuration can reduce the amounts of some bacteria, studies have shown that the reduction of vibrios was not effective, and reduction of concentrations to less than 10^4 cells per gram of oyster is rarely reported (Su and Liu, 2007). In a 2002 study to measure the effect of depuration on the concentrations of *V. cholerae* and *V. parahaemolyticus* in molluscs, it was reported that *V. cholerae* only declined one log in concentration after 24 hours and remained at that concentration for the 44-hour experiment duration (Croci et al., 2002). *V.*

parahaemolyticus was reduced even less and remained at a concentration of 10^3 MPN g^{-1} after 44 hours, with most reduction occurring within the first five hours of treatment (Croci et al., 2002). Comparison of concentrations of both vibrio species to *E. coli*, whose concentrations continuously declined approximately 3 log throughout the 44-hour exposure, indicates the necessity for further studies investigating attachment of vibrios (Croci et al., 2002). Further investigation of vibrio attachment may contribute understanding to trends and responses of vibrios in oysters.

A better understanding of the dynamics of vibrio adsorption to copepods and chitin could lead to a better understanding of vibrio abundance and distribution in its natural environment. However, little remains known about the precise nature of the environmental impacts on vibrio adsorption to animate and inanimate objects in its surrounding, particularly under stressor conditions. Thus, there is a need for further evaluation of environmental changes in association with *V. parahaemolyticus* adsorption.

2.3 Materials and methods

2.3.1 Sampling site

Sampling for collection of bacterial isolates was conducted off the coast at Port Fourchon, LA (Figure 1) as described previously (Johnson et al., 2012). Specifically, oysters were in the northern Gulf of Mexico in Louisiana. At all sampling stations, 20 – 25 oysters were collected. All samples were transported to the laboratory in coolers containing ice or ice packs. Oyster were scrubbed, shucked, and homogenized as described previously (Johnson et al., 2010).

V. parahaemolyticus was enumerated by spreading 0.1 g and 0.01 g oyster on T_1N_3 agar plates (1% tryptone, 3% NaCl, pH 7.2). Detection of *tdh* + and *trh*+ *V. parahaemolyticus* was accomplished using 1 L, 100 mL, and 10 mL water enrichments and 10 g. All samples were incubated at 33 ºC for 16 – 18 hours.

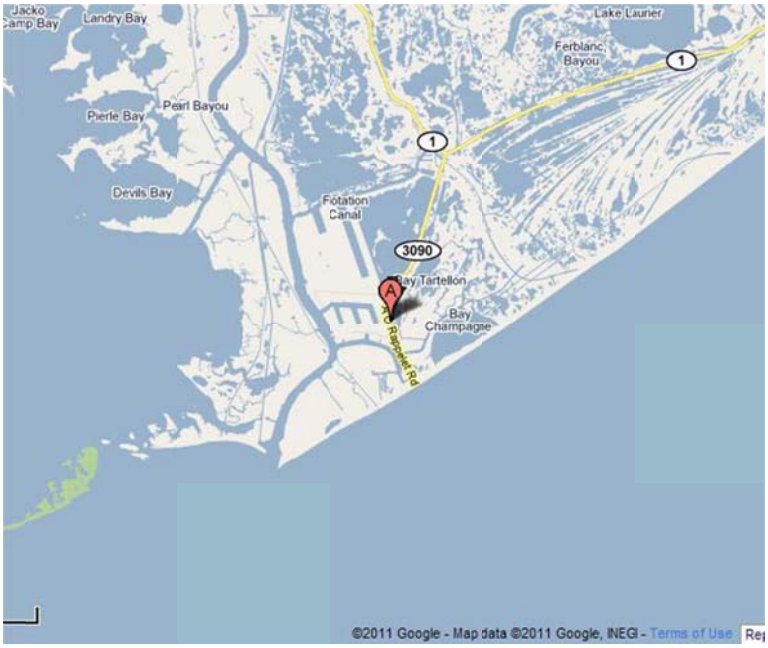

Figure 1: Sampling site, Port Fourchon. Louisiana, for Vp342 *V. parahaemolyticus* strain (courtesy of Google Maps).

2.3.2 Adsorption of *V. parahaemolyticus* to substrates

After trial and error, the following method was used for substrate adsorption studies. Adsorption to beads was carried out by exposing a *V. parahaemolyticus* strain to organic (chitin) and inert (plastic) beads *in vitro* and then quantifying adsorption using quantitative real-time PCR (qRT-PCR). Bead concentrations were quantified with a Z1 Coulter Particle Counter (Beckman Coulter, Brea, CA). Magnetic chitin beads (50–70 µm) (New England BioLabs, Ipswich, MA), and magnetic polyethylene beads (53–63 µm) (Cospheric, Santa Barbara, CA) were placed into 2-ml siliconized centrifuge tubes

with 1 ml Instant Ocean artificial seawater (ASW, United Pet Group, Inc., Cincinnati, OH) at salt concentrations of either 20 or 35 gpL. The total surface area for each type of bead was calculated from the bead concentration and assumed diameters of 60 μm and 58 μm for the chitin and polyethylene beads, respectively (Appendix B.2). Equal surface areas of beads, 14 mm^2, were added to each sample tube.

A *tdh+ V. parahaemolyticus* strain, *V. parahaemolyticus* 342, which was isolated from oysters in 2010 off the coast of Louisiana, was cultured in 10X alkaline peptone water[2] (APW, 10% peptone, 10 gpL of NaCl, pH 8.5) at 33 °C. The culture was centrifuged at 14,000 × g and washed with ASW. Tubes were prepared containing an equal surface area of either chitin or plastic beads in 1 mL of either 20 or 35 gpL ASW. The washed cells grown at 33 °C were added to these tubes at an initial concentration of 3.8×10^6 or 7.9×10^6 copies per mL. Differing initial bacterial concentrations were a result of growth in media for 2 hours. Mixtures were incubated at 10, 21, 25, 30, and 37 °C for 3.5 hours. Thus, cells were shifted from a salt concentration of 10 gpL to 20 and 35 gpL and from a temperature of 33 °C to temperatures of 10, 21, 25, 30, and 37 °C. The duration of the incubations was 3.5 hours, which previous reports have shown to be sufficient to establish equilibrium between adsorption and detachment of bacteria (Belas and Colwell, 1982; Kogure et al., 1998). Samples were shaken at 125 rotations per minute to simulate environmental conditions.

[2] Peptone water is a growth medium that is used for the growth of *Vibrio* species. It contains peptone as a source of carbon, nitrogen, vitamins, and minerals and NaCl for osmotic balance. Peptone is a combination of various protein derivatives that are formed by the partial hydrolysis of proteins.

2.3.3 Quantitation of *V. parahaemolyticus* adsorption and cell density

After exposure to tested salinities, temperatures, and substrates, beads were separated from the mixtures by placing a neodymium magnet directly below each tube until the beads separated out of suspension. The top 800 µl (the aqueous portion) and the remaining 200 µl (the portion containing the beads) were removed and placed in fresh microcentrifuge tubes. DNA was extracted by boiling for 10 minutes. Samples were immediately placed on ice and stored at –20 °C until analysis by real-time qPCR (qRT-PCR).

The qRT-PCR was carried out as described previously (Nordstrom et al., 2007), but only *tdh* and an internal amplification control were targeted. PCR reactions included final concentrations of 0.2 µM each of the forward (5'-TCCCTTTTCCTGCCCCC-3') and reverse (5'- CGCTGCCATTGTATAGTCTTTATC-3') primers, 5 mM $MgCl_2$, 0.2 mM dNTPs, 1X PCR buffer, 0.45 units/µL Platinum *Taq* polymerase (Life Technologies, Grand Island, NY), 0.075 µM probe (5'-FAM-TGACATCCTACATGACTGTG-MGBNFQ-3', Applied Biosystems, Grand Island, NY), and 5 µL template. PCR cycling conditions included an initial 95 °C denaturation for 60 sec, followed by 45 cycles of amplification at 95 °C for 5 sec and a combined primer annealing/extension step at 59 °C for 45 sec (Nordstrom et al., 2007). The qRT-PCR was carried out on a Cepheid SmartCycler II system (Sunnyvale, CA) using the software's default settings, except that fluorescence units were changed to 15 for greater sensitivity against background fluorescence, as described previously (Nordstrom et al., 2007). The number of replicates measured ranged from a minimum of 4 to a maximum of 8.

The qPCR standard curves were generated by preparing ten-fold serial dilutions of DNA extracted from a fresh culture grown for 6 hours in 10X APW at 33 °C, boiled for 10 min, and immediately placed on ice (Appendix A.3.3). DNA concentrations were quantified using a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE). Dilutions were analyzed by qPCR as described above, and threshold cycle values were regressed against corresponding DNA concentrations. The relationship between DNA concentrations and copy numbers was determined from the total genome size of *V. parahaemolyticus* strain RIMD 2210633 strain (5,165,770 bp), the mass of DNA per individual base pair ($1.026 * 10^{-21}$ g / bp), and the fact that most *V. parahaemolyticus* carry two copies of *tdh* (Iida et al., 1998), as described previously (Biosystems, 2003).

2.3.4 Data analysis

The data were analyzed on the assumption that at equilibrium the relationship between the concentrations of attached (Y) and unattached (X) bacteria were related by the Langmuir adsorption isotherm (Langmuir, 1916):

$$Y = \frac{AX}{B + X} \qquad \text{Eq. (1)}$$

We used a Hanes-Woolf (Haldane and Stern, 1932, p. 119) transformation to linearize Eq. 1 by using Eq. 2 below:

$$\frac{X}{Y} = \frac{B}{A} + \frac{X}{A} \qquad \text{Eq. (2)}$$

Although the initial concentrations of unattached bacteria were similar in all experiments, differences in growth and adsorption during the subsequent 3.5-h incubations resulted in final unattached *V. parahaemolyticus* concentrations of 1.7–166

$\times 10^6$ cells ml^{-1}. This range of concentrations was more than adequate to identify the relationship between X and the ratio of unattached to attached bacteria (X/Y).

2.4 Results

2.4.1 Effect of temperature, salinity, and substrate type on adsorption

When the bacteria were transferred from growth conditions at 33 °C to experimental temperatures 21 to 37 °C (Figure 2) and a salinity from 10 gpL NaCl to 20 and 35 gpL (Figure 3), there was no apparent difference between adsorption to chitin or plastic.

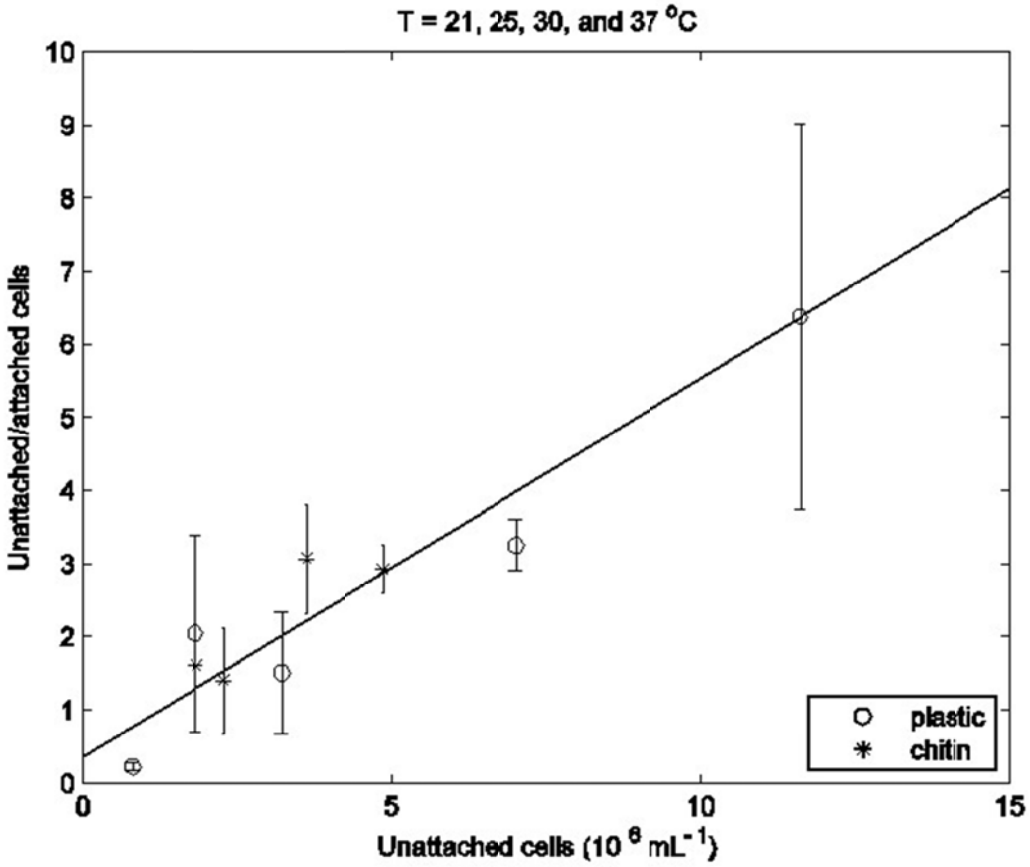

Figure 2: Comparison of adsorption of *V. parahaemolyticus* to chitin and plastic beads at different temperatures.The data are median concentrations of attached bacteria following temperature shifts from 33 °C to the tested range of 21 to 37 °C. Error bars are median absolute deviations.

We therefore pooled the chitin and plastic results in the subsequent analyses. After pooling the chitin and plastic results, we found no effect of salinity on the Langmuir adsorption isotherm. However, the slope of the Hanes-Woolf plot (Haldane and Stern, 1932) when the temperature was changed from 33 °C to 10 °C was 4 to 5 times the slope of the analogous plot when the temperature was changed from 33 °C to 21 to 37 °C (Figure 3). An analysis of covariance revealed that the difference of the slopes was significant at p = 0.005.

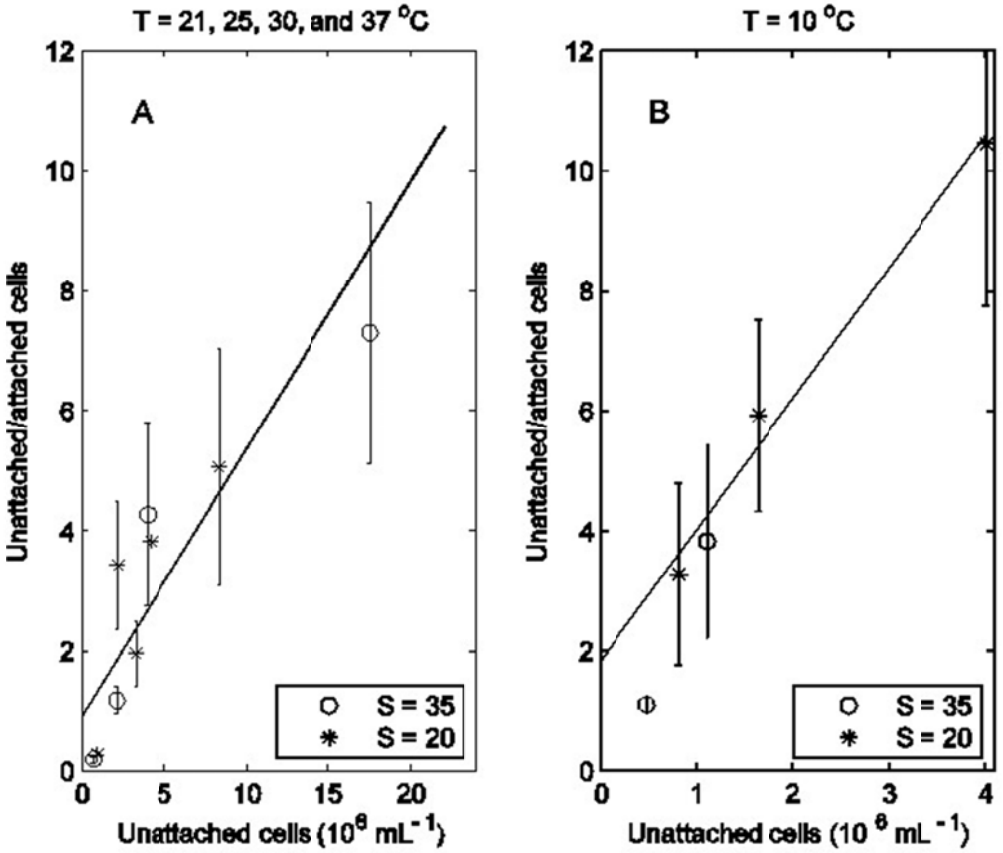

Figure 3: Comparison of adsorption of *V. parahaemolyticus* to chitin and plastic beads at different temperatures and salt concentrations. In the left panel, the data are median concentrations of attached bacteria following temperature shifts from 33 °C to the tested range of 21 to 37 °C. In the right panel, the data are median concentrations of attached bacteria following a shift from 33 to 10 °C. Error bars are standard deviations about the means of the number of attached bacteria in each bin. The regression lines are least squares fits of Eq. (1) to the experimental data.

2.4.2 Effects of substrate type, changes of salt concentration and temperature, and adsorption surface presence on growth

Growth was defined to be the final concentration of intact bacterial cells at the end of each 3.5-hour experiment expressed as a percentage of the initial concentration of intact cells.

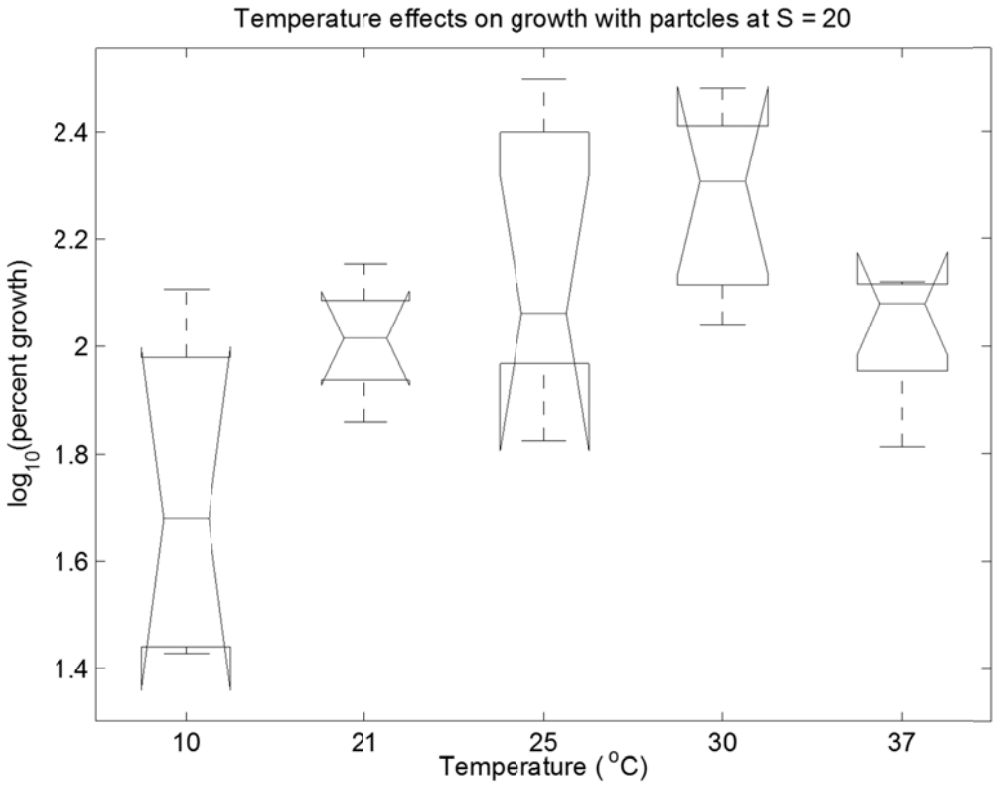

Figure 4: Temperature effects on growth in presence of particles at S = 20. Temperature effects are significant at p = 0.0175 based on a Kruskalwallis test.

With respect to substrate type, the median growth in the presence of plastic substrates was 496% (n = 28, 95% confidence interval, 130 to 582%), whereas that in the presence of chitin was 276% (n = 29, 95% confidence interval, 109 to 283%); there was no significant difference between the growth in the presence of chitin versus growth in

the presence of plastic (p = 0.29). Temperature significantly affected growth at 20 gpL (p = 0.0175) and at 35 gpL (p = 0.0037), as illustrated in Figures 4 and 5. In addition, salinity shifts affected growth at p = 0.0017. Substrate presence affected growth at p = 0.0303 (Figure 6).

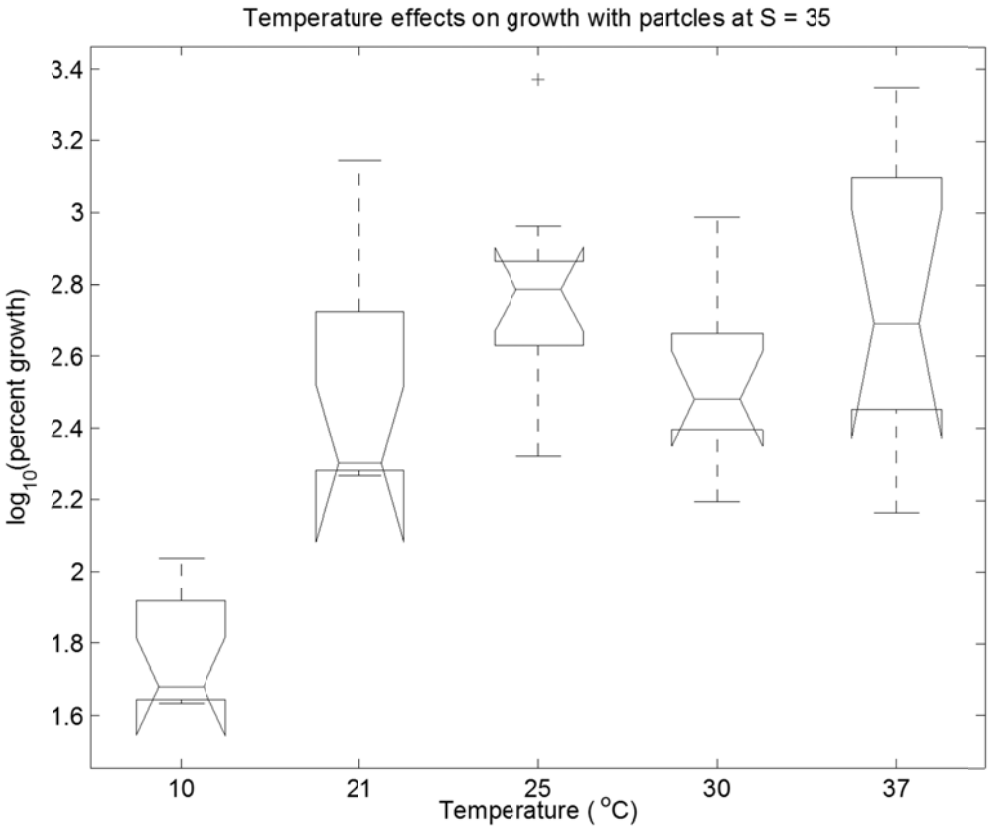

Figure 5: Temperature effects on growth in presence of particles at S = 35. Temperature effects are significant at p = 0.0037 based on a Kruskalwallis test.

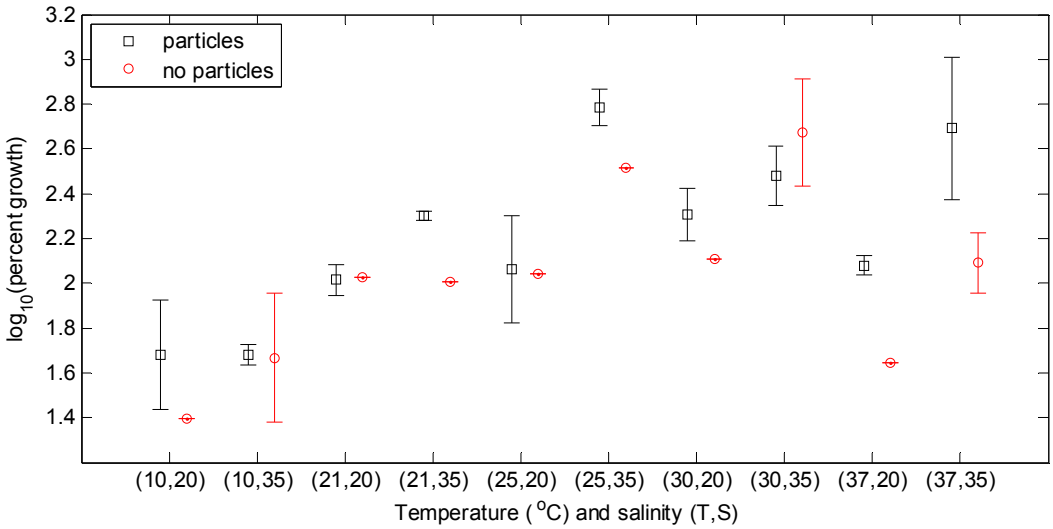

Figure 6: Paired t-tests comparing growth in the absence (no particles, red circles) or presence (particles, black squares) of an adsorption substrate indicated there was a significant substrate effect (p = 0.0303).

2.5 Discussion

Results from this study revealed that, whereas substrate type had no significant impact on adsorption or growth, temperature had a significant impact on both, and both temperature and salt concentration had an impact on growth. Specifically, there was significantly less adsorption and significantly less growth following the 10 °C shift than at any of the other four temperatures tested, likely due to bacterial shock and death. There was increased growth at warmer temperatures, as expected. Temperature significantly impacted growth regardless of the salinity tested, and regardless of the presence of substrates as potential adsorption sites. Salinity impacted growth at the warmer temperatures tested, and the impact of salinity on growth was only significant in the presence of substrates. The presence of substrate impacted growth only at 37 °C and only at 35 gpL.

This project yielded several surprising results. A major surprise of this study was the lack of a difference between adsorption to chitin and adsorption to plastic, an ostensibly inert substrate. There was also no significant impact of substrate on growth. Chitin is a primary component of the zooplankton exoskeleton and a common *V. parahaemolyticus* adsorption and degradation target in the ocean via pili and chitinase production (Grimes et al., 2009). Such adsorption is also clinically relevant because the mechanisms and molecules involved often parallel the mechanisms of adsorption of *V. parahaemolyticus* to human epithelial cells, e.g., N-acetyl glucosamine binding protein. It was hypothesized that *V. parahaemolyticus* would use chitin as an adsorption and carbon source, as described previously (Huq et al., 1983; Huq, 1984; Jude et al., 2009; Johnson, 2013). However, in the current study, the impact of chitin was no different than the impact of plastic. The chitobiase-containing *V. parahaemolyticus* strain used in the current study did not use chitin as a carbon source in the timeframe tested, although such use has been described in other studies (Arutchelvi et al., 2008; Johnson, 2013). Forming a bacterial community on the surface of a substrate may allow for quorum sensing between individual bacterial cells, which would allow for protection in unfavorable environments, and controlled metabolism and reproduction (Davey and O'Toole G, 2000; Callow and Callow, 2006; Nadell et al., 2008).

A very interesting result from this research was the finding that growth of *V. parahaemolyticus* was significantly higher in the presence of either adsorption substrate than when adsorption sites were absent, and this was the case at most combinations of temperature and salt concentrations. This indicates that *V. parahaemolyticus* fared better in the presence of a substrate regardless of the chemical nature of the substrates

tested. It also supports the above implication that although the strain tested in this study carried the chitobiase gene, it did not appear to use chitin as a carbon source, even under starvation conditions.

The impact of temperature on growth in the current study was not surprising, but the impact of temperature on adsorption was unexpected. It was originally hypothesized that lower temperatures would be associated with increased adsorption to chitin particles when exposed to the acute stressors examined in this study: abrupt changes in nutrients, temperature, and salt concentrations. The rationale was that in its natural marine and estuarine environment, *V. parahaemolyticus* would display increased adsorption to chitin as a response to acute stressors, including protection from unfavorable temperatures, and biofilm formation as a response to abrupt starvation conditions to provide bacteria with a carbon source (Venkateswaran et al., 1990; Marshall, 1996; Hall-Stoodley et al., 2004; Kokare et al., 2009; Lutz et al., 2013). In the current study, however, the opposite was true, even when accounting for the impact of *in vitro* growth.

Another surprise was the increased growth of the *V. parahaemolyticus* strain tested even despite removal of nutrients during incubations. This indicates that physical removal of nutrients and exposure to nutrient-free conditions do not impact bacterial replication *in vitro* over a timeframe of 3.5 hours. Indeed, a 1982 study of vibrio starvation did demonstrate that vibrios grown in nutrient-free liquid conditions were capable of large increases in cell numbers (Kjelleberg et al., 1982). As described by Wai et al. (1999), mechanisms used by *V. cholerae* to cope with starvation deprivation include morphological changes, changes in cellular composition, extracellular

polysaccharides, rugosity, and biofilm formation. The role of *in vitro* growth in the current study was a factor in the interpretation of the data. It was unexpected that the *V. parahaemolyticus* strain would increase in copy number during the 3.5-hour incubation in ASW in the absence of nutrients.

Overall, this study has several implications for vibrio ecology. The absence of a substrate effect on either adsorption or cell density indicated that *V. parahaemolyticus* likely adsorbed to particles with no regard to the chemical nature of the substrate. This is a pattern also seen with biofouling (Dang and Lovell, 2000; Callow and Callow, 2006; Shikuma and Hadfield, 2010; Bixler and Bhushan, 2012; Dobretsov et al., 2013). The question has been previously posed whether bacteria attach due to the presence of specific chemicals on substrates, or if adsorption is based on mere collision with substrates. A study conducted by Yu et al. (1993), suggests that bacterial adsorption is initiated by attraction of bacteria to exudates released by sick or dying organisms. Then there is a release of extracellular chitinases, which causes a chitin catabolic cascade that attracts additional bacteria to adsorb. This postulation supports the results from our study using inanimate chitin beads. Without having living chitinous organisms as the source of adsorption, it is possible that the bacteria did not have a chemical exudate to trigger initial adsorption. Hence, there would not be a cascade effect to attract additional bacteria. Because of this, bacterial response in the presence of chitin beads would mirror that of bacterial responses in the presence of plastic beads, as was observed in the current study.

With respect to the impact of temperature on adsorption, this has important implications for sudden temperature changes, such as postharvest icing of oysters and

previously unsuccessful attempts at depuration of pathogens from oysters destined for raw consumption.

One method for decreasing vibrio concentrations used in the oyster industry is depuration. In a 2009 study, depuration of oysters in ASW resulted in the highest decrease of *V. parahaemolyticus* and *V. vulnificus* populations at 22 and 15 °C, while there was no decrease in populations at 10 and 5 °C (Chae et al., 2009). In another study, harvested oysters were stored at temperatures of 10, 13, and 18 °C and ambient outside air temperature of 23 to 34 °C (Cook, 1994). Results from this study concluded that there was no multiplication of *V. vulnificus* at 13 °C and below, and an increase of *V. vulnificus* in oysters held at 18 °C and under ambient conditions when compared to initial harvest levels (Cook, 1994). This result indicates changes in bacterial concentrations in oysters due to up-shift and down-shift of temperatures. In addition, a 1984 *V. cholerae* study demonstrated significantly higher growth and adsorption at 30 °C than at lower temperatures (Huq et al., 1984). In a 2003 study, an abrupt change in temperature may have contributed to decreased adsorption of *V. parahaemolyticus* to particles when compared to other temperatures tested. The study reported induction of cold-shock proteins (Csps), which play a role in cold acclimation, when *V. cholerae* was exposed to 15 °C over the course of 30 to 300 minutes (Datta and Bhadra, 2003). Thus, if there is a significant decrease in vibrio attachment following icing of oysters, or an abrupt change from ambient temperature to iced conditions, then this may warrant revisiting the idea of oyster depuration.

This group (Johnson et al., 2012) determined previously that temperature accounted for 11%, 18%, and 34% of the variability of *V. parahaemolyticus* abundance

in water, oyster, and sediment samples, respectively. That study also determined that suspended particulate matter concentrations accounted for 16%, 6%, and 7% of the variability of *V. parahaemolyticus* abundance in water, oyster, and sediment samples, respectively. The relationship with turbidity described in that study was consistent with the tendency of vibrios to attach in the environment (Johnson et al., 2012). In the absence of characterizing the exact nature of turbidity during that study (i.e., quantifying the proportion of the suspended particles that were predominantly chitin particles, sediment, or copepods), the current study is an intriguing complement to the previous study and others. Specifically, the relationship between changes in temperature and adsorption of *V. parahaemolyticus* was examined with respect to adsorption as a model for suspended chitin particles or live zooplankton under conditions of constant turbidity, i.e., constant particle concentrations and surface area. Thus, the current research investigated in further detail the relationship between *V. parahaemolyticus*, changes in temperature, and adsorption of *V. parahaemolyticus* to suspended particles. The results indicated that *V. parahaemolyticus* adsorption is significantly lower when the temperature is abruptly changed from 33 °C to 10 °C than when the temperature is changed abruptly from 33 °C to 21–37 °C, regardless of the substrate types tested.

With respect to the impact of temperature on growth, the results were as expected. Low-temperature growth results from this study coincide with results from other studies in association with postharvest bacterial growth, such as Cook (1994), who reported no multiplication of *V. vulnificus* at 13 °C and below, and an increase of *V. vulnificus* in oysters held at 18 °C and ambient temperatures (23 to 34 °C) when compared to initial harvest levels. A 1993 study also demonstrated that *V. vulnificus*

grew more that 100-fold at 30 °C but were reduced by 10- and 100-fold at temperatures of 2 to 4 °C and 0 °C, respectively (Kaspar and Tamplin, 1993). In a 2003 study, growth occurred when exposed to 10 °C after preincubation at 15 °C, while no growth occurred at 37 °C (Datta and Bhadra, 2003). Growth at 15 °C prior to exposure at low temperature may have primed the induction of cold shock proteins (Datta and Bhadra, 2003). Lower growth at 10 °C than at 21 to 37 °C in the current study may have not only been a result of 10 °C being the threshold below which *V. parahaemolyticus* will not grow (Kaneko and Colwell, 1973), but may also have been due to an abrupt change in temperature from bacterial growth at 33 °C to 10 °C resulting in cell death. Cook and Ruple (1989) reported that vibrios in oysters increased at 22 °C and 30 °C and did not grow at 10 °C, after refrigeration from ambient outside temperatures. Findings from the current study showing that an abrupt change in temperature impacts the growth of *V. parahaemolyticus* and even causes death provides additional information for the fate of *V. parahaemolyticus* in oysters, and in return could lead to better informed decisions in oyster processing and consumption.

Although most studies support the importance of temperature *in situ*, *in vitro*, and *in vivo* (Huq et al., 1983; Huq et al., 1984; Carli et al., 1993; DePaola et al., 2003; Stauder et al., 2010; Johnson et al., 2012), one contradictory 2007 study determined that temperature alone did not affect vibrio growth, but temperature in conjunction with dissolved organic matter was significant (Eiler et al., 2007). A 2012 study (Zeraik and Nitschke, 2012) demonstrated media-specific temperature effects on *in vitro* adsorption of *Listeria monocytogenes* and other bacterial species to polystyrene plates. Adsorption of all five species was greater at 35 °C than at 25 °C when the cells were grown in

TSYEA medium. However, when the cells were grown in lactose agar or peptone agar, the correlation between temperature and adsorption was dependent on the species. The effects of changes in temperature on the synthesis of heat and cold shock proteins by *Listeria monocytogenes* have been studied by Phan-Thanh and Gormon (1995), and Meylheuc et al. (2001). They found that changes in temperature were sufficient to alter the surface characteristics of *L. monocytogenes* and hence potentially its tendency to adsorb to surfaces. These studies implicate the importance of testing combined environmental stressors.

The impact of salinity on growth is of importance in this study. A 1994 study reported the detection of *V. cholerae* in 5 of 19 ships surveyed with salinities of 12, 13, 14, 20, and 32 gpL, indicating a range of salinity acclimation by these bacteria (McCarthy and Khambaty, 1994). Another study investigating the ability of *V. cholerae* to acclimate to ballast water exchange at sea described an overall decrease in bacterial concentrations over 30 days at salinities of 8, 16, and 32 gpL, whereas the decrease was minimal in sterile seawater (McCarthy, 1996). This may indicate salinity acclimation, and acclimation of vibrios may differ in combination with varying environmental stressors. In the current study, growth was significantly higher when the salt concentration was 35 gpL versus 20 gpL. The 20 and 35 gpL salt concentrations used in this study were chosen to simulate estuarine and marine environments, respectively. Thus, these results indicated *V. parahaemolyticus* populations with characteristics similar to the strain used in this study may exhibit more robust adsorption in an open ocean environment than in an estuary. Although only two salt concentrations were tested in the current study, these results contrast with previous studies that have

identified 23 gpL as the optimal salinity for *V. parahaemolyticus* (Zimmerman et al., 2007; Martinez-Urtaza et al., 2008). Thus, the results indicate that the salinity in an estuarine or marine setting would affect the abundance and growth of *V. parahaemolyticus* but not its adsorption tendencies.

A key 1975 study demonstrated that adsorption of *V. parahaemolyticus* to chitin particles and copepods, was greater at lower salt concentration and pH, and adsorption to copepods was lower than adsorption to chitin (Kaneko and Colwell, 1975). The current study attempted to build on this research question but used inanimate chitin particles, a culture-independent enumeration method, and a *V. parahaemolyticus* strain carrying the *tdh* gene. Recent studies have also demonstrated that temperature is the main factor influencing gene expression in *V. cholerae* and that biofilm formation and binding to chitin from crab shells increase with temperature (Stauder et al., 2010; Stauder et al., 2012).

Although the current study identified no significant impact of abrupt changes in salt concentration on *V. parahaemolyticus* adsorption, previous studies have reported different results. In a previous study, *V. parahaemolyticus* adsorption to copepods after 6 hours was more efficient in filtered estuarine water sampled from lower salinities in the salinity range 1.6 to 15.8 gpL (Kaneko and Colwell, 1975). There also appeared to be a higher adsorption in artificial saltwater than in natural saltwater, which after filtration would have contained many of the original components from field samples (Kaneko and Colwell, 1975). *V. parahaemolyticus* cells were grown in a 3 gpL NaCl medium and abruptly washed with corresponding experimental salinities (Kaneko and Colwell, 1975).

A 1982 study reported strong adsorption at 10, 15 and 20 gpL and low adsorption at 35 gpL after 3.5 hours (Belas and Colwell, 1982).

Gordon and Millero (1984), in contrast, examined adsorption of *V. alginolyticus* to hydroxyapatite particles and did find little difference between percent adsorption at salinities of 20 and 35 after 18 hours, which is in agreement with the findings of the current study. That study reported that at high bacterial concentrations, there may be a salting out effect in which cell-to-cell adhesion leads to clusters of bacteria, or flocs, which sink rapidly and do not appear as suspended particles. This effect may have caused an overestimation of adsorption when assuming that cells that were not in suspension were attached. This is because at high bacteria-to-adsorption site ratios (i.e., on the asymptote of the adsorption curve) there are too few adsorption sites, and perceived changes in adsorption could be a function of this fact and not an indication of bacterial tendency to attach. The bacterial concentrations used in this study fell in the 10^6 to 10^7 cells per mL range, which would likely fall in the linear range of the adsorption isotherm described in the Gordon and Millero (1984) study; therefore, cell concentrations outside the isotherm do not explain the lack of a salinity effect on adsorption. We have demonstrated previously that *V. parahaemolyticus* concentrations may be as low as 1 CFU/mL in water and as high as 83,000 CFU/g in sediment in the northern Gulf of Mexico (Johnson et al., 2012). The current study used *V. parahaemolyticus* at a concentration of 10^6 cells / mL, because these concentrations were detectable using molecular methods with the most reproducible results; future studies will provide more sophisticated enumeration methods that may permit use of lower concentrations of bacteria in lab studies.

In its natural environment, where *V. parahaemolyticus* falls on the adsorption isotherm is unclear. However, adsorption of bacteria to surfaces is a complex process that can potentially be affected by a variety of biotic and abiotic factors, including, *inter alia*, hydrophobicity (Zita and Hermansson, 1997), cellular surface charge (Ukuku and Fett, 2002), outer membrane proteins (Danese et al., 2000; Torres and Kaper, 2003), the ionic strength of buffers (Sheng et al., 2008), the presence of fimbriae and flagella (Goulter et al., 2009), the production of exopolysaccharides (Donlan, 2002), the composition of growth media, the growth of the bacteria, and temperature (Zeraik and Nitschke, 2012). Simplistically, bacterial adsorption is envisioned as involving a two-step process. The first step, an initial reversible adsorption, is followed by an irreversible adsorption in the second step. The forces involved in reversible adsorption are relatively weak and include van der Waals forces, electrostatic forces, and hydrophobic interactions between the bacterial cell and the adsorption surface (Vanloosdrecht et al., 1987). Irreversible adsorption involves stronger forces that may include covalent bonds, hydrogen bonds, and strong hydrophobic interactions (Vanoss et al., 1988) and may also involve exopolysaccharides and surface structures such as flagella and fimbriae (Jones and Isaacson, 1983; Hancock). Considering the long list of factors that can potentially influence adsorption, it is not surprising that investigations of the effect of a single factor without rigorous control over all others have sometimes led to rather disparate results (Goulter et al., 2009; Zeraik and Nitschke, 2012).

This project experienced several shortcomings that will be addressed in future studies. It must also be noted that the high noise levels within the individual microcosms may have masked treatment effects; variance within replicates was 65%, and variance

between treatments was 35%. As is apparent from the scatter of the adsorption data in the current study, our efforts to minimize the noise in our experimental results by control of growth conditions met with limited success. Given the factor of 10 to 30 scatter in the data at a given temperature, it is likely that in the absence of a large dataset the effect of temperature on adsorption would have been statistically insignificant.

The reason for higher adsorption at tested temperatures ranging from 21 to 37 °C when compared to 10 °C is likely because of cell death. With the change in temperature from initial growth at 33 °C to microcosm temperature at 10 °C, it is possible that, due to the sudden down-shift of temperature, changes in bacterial cellular physiology and subsequent death occurred (Phadtare, 2004). Cold shock response is attributed to a decrease in membrane fluidity, reduced efficiency of mRNA translation and transcription, inefficient folding of some proteins, and hindered ribosome function (Phadtare, 2004). These factors could contribute to decreased concentrations of attached *V. parahaemolyticus* to particles.

Although the goal of the microcosm approach was to isolate combinations of specific environmental parameters without the background noise of uncontrollable factors such as weather, rainfall, or oil spills, the interactions between *V. parahaemolyticus* and the environment remain very complex. It will be necessary to examine a wider range of salinities and bacterial concentrations, exposure times, animate and inanimate substrates, acute decreases in salt concentration associated with rainfall and runoff, various nutrients, and strains of diverse phylogenetic lineages, as well as other scenarios, to more thoroughly understand these autochthonous organisms. It will be necessary to systematically vary the bacterial concentration as

previously described (Gordon and Millero, 1984) so as to determine the characteristics of the adsorption isotherm at each salinity and temperature. With the increasing availability of genomics approaches, it will become more cost-effective to examine gene expression changes en masse following exposures to these environmental parameters. Future studies will use preincubation temperature closer to each tested temperature to minimize introduction of physiological effects due to preincubation temperatures and abrupt temperature changes. Similarly, more appropriate preincubation salt concentrations will be used for the same reason. Also, a wider range of salt concentrations will be used so that more finely spaced impacts may be assessed.

This study was unique in several aspects. A major strength of this study in comparison to previous similar research is the use of inanimate substrates in controlled microcosms with defined temperatures and salinities. This group and others have investigated the relationships between environmental parameters and concentrations of *V. parahaemolyticus* in environmental settings (DePaola et al., 2003; Zimmerman et al., 2007; Parveen et al., 2008; Johnson et al., 2010; Johnson et al., 2012), and others have used live or dead copepods (Huq et al., 1983; Huq et al., 1984). Our approach was to assess the adsorption characteristics of *V. parahaemolyticus* in the absence of zooplankton immunological effects (Kurtz and Franz, 2003; Kurtz, 2007) and other factors that are difficult to control.

Another strength is the use of a qPCR approach to increase detection sensitivity over enumeration methods associated with culture-based and spectrophotometric methods. The role of aggregates when using culture-based methods makes it difficult to accurately enumerate bacterial cells as colony forming units without disrupting

adsorption. The use of magnetic beads in this research allowed for quick and accurate separation of adsorbed bacteria versus free floating bacteria in the experimental supernatant.

A third strength is the wide range of temperatures studied, which included a range of temperatures designed to simulate conditions encountered by *V. parahaemolyticus* seasonally, and in the human body. This range of temperatures allows for a more thorough representation of conditions to which *V. parahaemolyticus* may be exposed due to shifting of temperatures during preharvest to postharvest, and consumer ingestion conditions. Using a large temperature range is important to understand the trends and fluctuations associated with *V. parahaemolyticus* exposure to these environmental settings.

Thus, this study, investigating *V. parahaemolyticus* attachment and growth as a result of a combination of environmental stressors, provides additional insight to factors encountered throughout its life cycle. Information from this study contributes knowledge of *V. parahaemolyticus* ecology, trends, and responses, in association with environmental and mammalian interactions.

CHAPTER 3: EFFECTS OF TEMPERATURE AND THE PRESENCE OF CHITIN ON *VIBRIO PARAHAEMOLYTICUS* GENE EXPRESSION

3.1 Abstract

Vibrio parahaemolyticus is a naturally occurring chitinoclastic human pathogen that adsorbs to chitinous copepods as part of its ecology, a phenomenon that is important in its responses to environmental factors such as temperature and salinity. This study aimed to investigate *V. parahaemolyticus* gene expression as a function of the presence of chitin and abrupt changes of temperature and salinity, in the absence of confounding environmental factors. Microcosms included parameter combinations of substrate (chitin and no chitin), shifting salt concentrations (20 gpL and 35 gpL), and shifting temperatures (10, 21, 25, 30, and 37˚C). Real-time qPCR was used to target *thiC*, *tdh*, chitobiase, VPA0459, VPA1548, VP1892, and VPA1598, genes that encode thiamine biosynthesis, thermostable direct hemolysin, chitin breakdown, collagenase, lateral flagellin, methyl-accepting chemoreceptor, and GlcNAc-binding protein A, respectively. Substrate impacted the expression of *tdh*, VPA0459, and VPA1598. There was a significant temperature correlation at 20 gpL with expression of VPA1548 and VPA1598, and salinity had a significant impact on *thiC* expression. In addition, chitin caused significant up-regulation of chitobiase and VPA0459 when the temperature was shifted from 33 to 37 °C but for no other temperature shifts. Chitin caused up-regulation of VP1892 when temperatures were shifted from 33 to 10 °C, and up-regulation of VP1892 when salt concentrations were shifted from 10 to 35 gpL. Increased expression ratios were observed for VPA1598 with increasing temperature and for VPA1548 when cells were shifted from 33 to 20 gpL. This is the first study to examine the response of

these genes in *V. parahaemolyticus* in response to three parameter changes simultaneously and sheds light on the potential physiological responses of *V. parahaemolyticus* to its changing environment.

3.2 Introduction

In the environment, several species of bacteria are normally found attached to the external surfaces, egg sacs, and guts of copepods. Attachment is thought to provide the bacteria with nutrients (Montgomery and Kirchman, 1993). Of the bacteria found on copepods, one of the most commonly found is the genus *Vibrio* (Carli et al., 1993). *V. cholerae* adsorption to chitin-containing substrates is facilitated by chitin-binding proteins produced by the bacteria that break down the copepod chitin exoskeleton.

The mechanisms of adsorption of *V. parahaemolyticus* to zooplankton and other chitin-containing substances may be similar to mechanisms when exposed to human epithelial cells. Pathogenic bacterial adsorption mechanisms involve hydrophobic and ionic bonds and lectin membrane proteins that recognize target sequences of glycoprotein sugars present on host cells (Montgomery and Kirchman, 1993; Tarsi and Pruzzo, 1999). *N*-acetyl glucosamine (GlcNAc) is a monosaccharide derivative of glucose. GlcNAc-specific proteins and chitin-binding proteins have been described in some species of vibrios (Tarsi and Pruzzo, 1999). Specific factors aiding in vibrio colonization in human hosts include type 4 pili in *V. cholerae,* which play roles in intestinal cell adsorption and environmental biofilm formation (Hacker and Kaper, 2000), type 3 secretion system complex factors and effector proteins that are used in host cell adsorption in vibrios (Grimes et al., 2009), and membrane proteins that are thought to be involved in immune evasion or adsorption to host cells (Makino et al., 2003). It is

thought that the capacity to attach to substrates in seawater has evolved into the ability to attach to substrates in human hosts and has occurred to provide an advantage to pathogenic bacteria that can use the same types of adsorption factors in both environments (Zampini et al., 2005). Understanding adsorption properties in the extracellular environment can contribute to the understanding of adsorption capabilities, mechanisms, and responses of *V. parahaemolyticus* in human hosts; thus, it is important to examine the changes in adsorption and gene expression under varying conditions representative of stressor conditions.

Vibrios display various stress responses to environmental stressors. Specifically, gene expression can allow for vibrios to acclimate to abrupt environmental changes and fluctuations. Some studies exposing *V. parahaemolyticus* to environmental stressors include gene expression assays following shock treatments in which temperature is up-shifted and down-shifted, sometimes including temperatures representative of the ocean and human host environment (Datta and Bhadra, 2003; Chiu et al., 2008; Iordache et al., 2008; Yang et al., 2009), varying salt concentrations (Kaneko and Colwell, 1975; Iordache et al., 2008; Whitaker et al., 2010), ethanol exposure (Chiang and Chou, 2008), and varying pH (Kaneko and Colwell, 1975; Whitaker et al., 2010). Shock treatments can be of great importance not only in acclimation in the environment but also when related to oyster harvesting and consumption. Cold shock proteins were suggested to be responsible for tolerance and survival of *V. vulnificus* in oysters during shellstock temperature control studies (Limthammahisorn et al., 2009) and the same has been reported for *V. parahaemolyticus (Yang et al., 2009).* Like many bacteria, *V. parahaemolyticus* acclimates to adverse and changing environments by means of

selective gene expression to ensure survival, including *tdh* and the stress response sigma factor gene *rpoS* (Mahoney et al., 2010; Whitaker et al., 2010).

Vibrio adsorption to particles may give vibrios a competitive advantage relative to unattached bacteria to the extent that particles are a source of organic substrates. There is no question that bacteria can utilize surface-bound substrates as carbon sources (Cooksey and Wigglesworth-Cooksey, 1995). Adsorption to particles may be a mechanism to gain access to the gut of particle feeders and subsequent incorporation into the nutrient-rich environment of fecal pellets, and may also allow bacteria to utilize surface-bound substrates. Because of this, it is reasonable to hypothesize that bacterial adsorption to particles may be negatively correlated with the availability of dissolved organic substrates in the water and positively correlated with bacterial demand for organic substrates, i.e., with the metabolic rate of the bacteria. Vibrio metabolic rates are known to be influenced by temperature (Boyer, 1994). Examples of this include metabolism-related genes down-regulated with a temperature shift from 37 °C to 10 °C (Yang et al., 2009), a decrease in superoxide dismutase and catalase activity with a temperature shift from 4 °C to 47 °C (Chiang and Chou, 2008), and a 25-fold increase of urease activity with a temperature increase from 30 °C to 37 °C (Park et al., 2009). Because it has been shown that temperature affects metabolic activities, and therefore, gene expression of metabolic system genes, there are compelling reasons to study the effects of temperature on metabolic gene expression in *V. parahaemolyticus*. The changes in the expression of metabolic genes resulting from exposure to abrupt temperature shifts could help to describe responses of *V. parahaemolyticus* in varying

environmental conditions, including temperatures commonly found in the environment and within hosts.

Changes in gene expression under different temperature conditions have been described. As reported in Wong et al. (2002), several unique proteins are up-regulated when *V. parahaemolyticus* is exposed to heat shock treatment from 4 ºC for eight hours to 44 ºC for 30 minutes (Wong et al., 2002). On the opposite end of the spectrum, cold-shocked *V. parahaemolyticus* up-regulated genes associated with adsorption and pathogenicity (Yang et al., 2009). As described by Boroujerdi et al. (2009), several vibrio species exhibit temperature-dependent pathogenicity, which they demonstrated with experiments that resulted in changes in up-regulation and down-regulation of metabolites of *Vibrio coralliilyticus*. Increases of gene expression in association with exposure to stressors could lead to increased understanding of fitness factors and other factors involved in cell density and physiology. Genes expressed under various conditions causing production of factors associated with adsorption and pathogenicity in human hosts may resemble factors associated with adsorption to substrates and pathogenicity in the environment (Pruzzo et al., 2008). T6SS1 and T6SS2 are type VI secretion systems used in protein transfer into a host and are associated with virulence in *V. parahaemolyticus.* The importance of gene expression of T6SS1 and T6SS2 in pathogenic vibrios was demonstrated by Salomon et al. (2013), who reported that T6SS1 was active at warm temperatures and high salinity, whereas T6SS2 was active only at low salinity, the implication being that T6SS1 is expressed under conditions found in the environment, whereas T6SS2 was expressed in marine organisms. Interestingly, neither T6SS1 nor T6SS2 was expressed at 37 °C, the indication being

that these protein secretion systems are not involved in human host infection and are therefore likely of relevance to survival in the marine environment (Salomon et al., 2013).

The studies described below highlight the importance of understanding gene regulation in relation to environmental parameters, including temperature, salinity, and substrate. Gene regulation is beneficial to the fitness of organisms by allowing them to quickly sense and respond to their environment (Kalisky et al., 2007). Gene regulation in the environment is implemented in order to utilize available resources and to survive stressful conditions (Kalisky et al., 2007). The current research examined the expression of seven genes, including two metabolic genes, two virulence factors, two adsorption genes, and one chemotactic gene. The following genes were targeted in this study as representatives of each of these functional categories.

3.2.1 *thiC* (thiamine biosynthesis metabolic possesses)

The gene involved in thiamine biosynthesis, *thiC*, is found *in V. parahaemolyticus* and is used in metabolic processes. *V. cholerae* has been reported to increase levels of gene expression of *thiC* when exposed to increased concentrations of 3',5'-cyclic diguanylic acid (c-di-GMP levels) (Beyhan et al., 2006). As an important regulator of biofilm formation, c-di-GMP acts as a messenger for integrating environmental signals that affect cellular physiology (Beyhan et al., 2006). *ThiC* was reported to be up-regulated in two pathogenic *V. parahaemolyticus* strains and was highly up-regulated in a *tdh-/trh+/T3SS2*β+ strain belonging to the pandemic O3:K6 serovar when exposed to oyster hemocytes (Noriea III, 2012). Acclimation of *V. parahaemolyticus* to various environments allows for greater adjustment to changing environmental parameters,

including sudden changes in temperature such as those encountered during shellfish harvesting and marine and terrestrial mammal transmission, as described above. Examination of metabolic responses to various environmental conditions is important in understanding the ecology and ability of this species to acclimate in its environment, whether that environment is the skin of a bottlenose dolphin, the mantle fluid of an eastern oyster, or the intestinal walls of a human.

3.2.2 *tdh* (virulence factor)

Pathogenic gene expression is a key component of the persistence and survival of vibrios in the environment and in eukaryotic hosts. Indeed, pathogenicity factors can serve as fitness factors that may render *V. parahaemolyticus* better acclimated or more fit with respect to fluctuations in environmental conditions. Bacterial strains positive for this virulence factor exhibit a phenomenon known as the Kanagawa phenomenon, in which clear halo rings of lysed red blood cells are produced around colonies of the *V. parahaemolyticus* grown on Wagatsuma blood agar (Miyamoto et al., 1969). The *tdh* gene causes hemolysis of erythrocytes, toxicity, and increased vascular permeability in rabbit skin, and alters ion fluctuations in intestinal cells that lead to a diarrheal response (Nishibuchi and Kaper, 1995). Thus, it is important to understand how *tdh* expression responds under various environmental conditions, as this information can yield clues about the ecology of *V. parahaemolyticus*.

3.2.3 chitobiase (chitin catabolism)

Vibrio chitin catabolism is associated with substrate adsorption and chitin degradation, which enables the vibrio to use chitin as a source of carbon (Figure 7). The breakdown of chitin into its constituent derivatives is important in ocean carbon cycling.

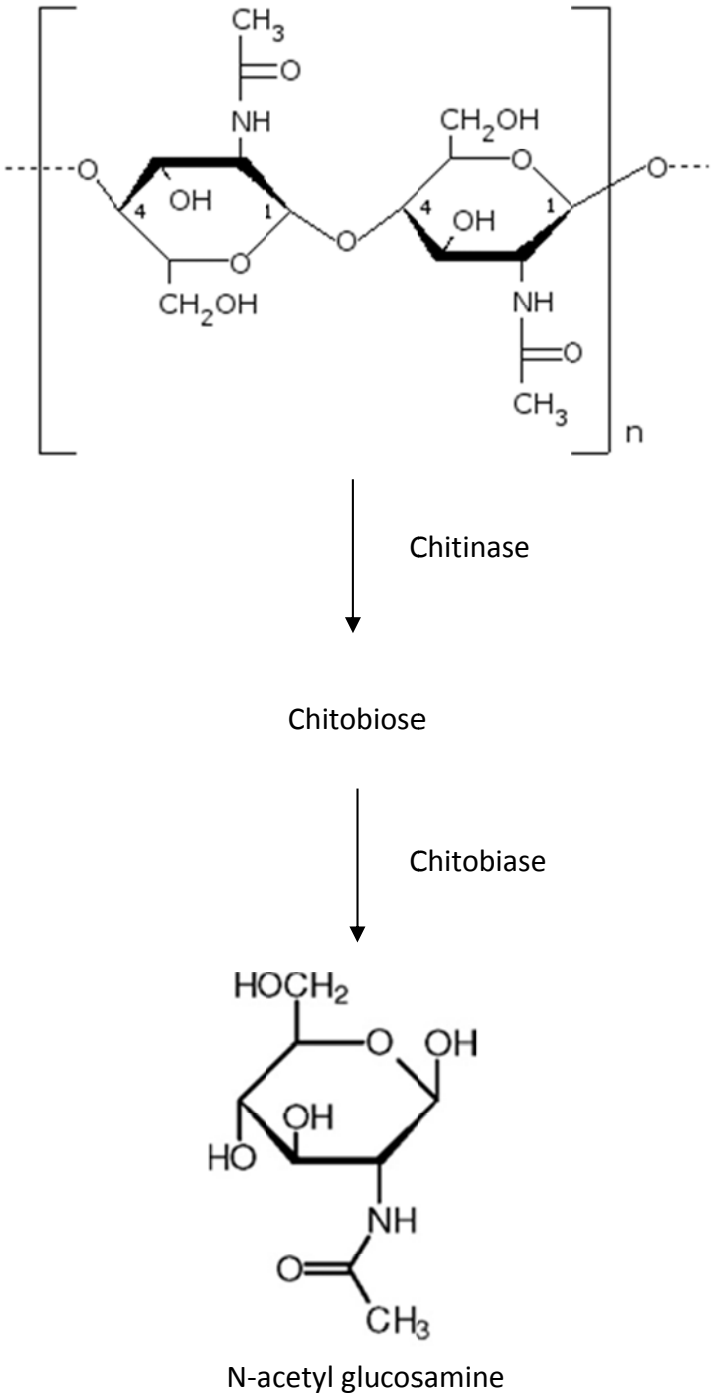

Figure 7: Schematic of role of chitobiase in chitin breakdown. Modified from (Sylvia et al., 2005)

In the marine environment, GlcNAc is also found in marine mammal respiratory mucosa and could play an important role in *V. parahaemolyticus*-marine mammal infections. Vibrios catabolize chitin by means of several genes, one of which is chitobiase (Keyhani and Roseman, 1996; Keyhani et al., 2000). Chitobiase activity has been demonstrated to occur in the periplasm and cytoplasm and is used as part of a system used to break down chitin (Zhu et al., 1992).

The components of chitin breakdown are also important for their uses in pharmaceuticals such as anti-tumor and anti-aging agents, and for the stimulation of immune system response to infection (Suginta et al., 2010). GlcNAc is found on the surface of human epithelial cells and could also play a role in vibrio survival.

3.2.4 VPA0459 (collagenase virulence factor)

Collagenase, in bacteria, cause tissue damage by degradation of collagen and are used to disintegrate connective tissue, cleave gene products, tenderize meat, and remove necrotic tissue from burns, ulcers, and bed sores (Yu, 1999). Vibrio collagenase is a zinc proteinase that cleaves the collagen sequence -Pro-X↓-Gly-Pro- where X can be any amino acid (Takeuchi et al., 1992; Yu, 1999). Gode-Potratz previously reported that the collagenase gene VPA0459 was expressed only during surface colonization (Gode-Potratz et al., 2010), and expression of this gene increased by >125-fold when *V. parahaemolyticus* were grown on a solid surface with a NaCl concentration of 15 gpL (Gode-Potratz et al., 2011). Collagenase in vibrios is an additional virulence (Belas and Colwell, 1982) factor that contributes to the pathogenicity of the microbe with respect to human health. It is thus important to understand how its expression levels change under various combinations of parameters potentially encountered by *V. parahaemolyticus*.

3.2.5 VPA1548 (lateral flagellin)

Vibrios possess two types of flagella, a polar flagellum, which is present and used in liquid and solid environments, and a lateral flagellum, which is formed in solid environments only (Shinoda and Okamoto, 1977). *V. parahaemolyticus* has two sets of flagellar gene sets, each containing 35 structural and regulatory genes (Stewart and McCarter, 2003). When *V. parahaemolyticus* is in a liquid environment, the first set of genes allows for polar flagellar propulsion of the bacterium through the environment. When cells come in contact with and attach to a surface, a second set of genes, which code for the formation of lateral flagellae, allows the vibrio to use both polar and lateral flagella, which are located randomly over the entire cell (Shinoda and Okamoto, 1977; Stewart and McCarter, 2003). During this time, the bacterial cell elongates, and the previously sheathed polar flagella no longer are sheathed (McCarter and Silverman, 1990). VPA1548 is the open reading frame that encodes for the lateral flagellin gene, *lafA*, which encodes for the lateral flagellum in *V. parahaemolyticus*. Expression of *lafA* was demonstrated to be up-regulated 641-fold when *V. parahaemolyticus* was grown on a solid surface compared to growth in a liquid medium (Gode-Potratz et al., 2011). Up-regulation under certain conditions can cause an increase of specific genes that are involved in marine and human infection.

3.2.6 VP1892 (methyl accepting chemoreceptor used in chemotaxis)

Methyl accepting chemoreceptor (MCP) genes are part of a chemotaxis system that can be found in Bacteria and Archaea (Gestwicki et al., 2000). Methyl-accepting chemoreceptors take part in a signal cascade that results in the formation of a phosphorylated chemotaxis protein that causes the bacterial flagellum to switch rotation

from counterclockwise to clockwise, thereby allowing the bacteria to sense chemical gradients and allowing movement toward or away from the sensed gradient (Baker et al., 2006). This formation occurs upon binding a chemoattractant or a repellant, a process that requires methylation of MCP's (Chaparro et al., 2010). Expression of VP1892, a methyl-accepting chemoreceptor gene in *V. parahaemolyticus*, decreased 10-fold when *V. parahaemolyticus* was grown on a surface compared to liquid (Gode-Potratz et al., 2011). Understanding VP1892 gene expression could allow for further understanding of environmental cues that would allow *for V. parahaemolyticus* movement toward and away from favorable and unfavorable environments, respectively.

3.2.7 VPA1598 (attachment to GlcNAc-containing substrates)

VPA1598, also known as GbpA, is a secreted protein that aids in vibrio adsorption to GlcNAc-containing substrates, including chitinous zooplankton and human epithelial cells (Kirn et al., 2005; Zampini et al., 2005; Matson et al., 2007; Stauder et al., 2012; Wong et al., 2012). Initial adsorption of vibrios to GlcNAc-containing intestinal cells, by means of GbpA, allows for up-regulation of mucin genes in the intestinal cells. The up-regulation of these genes in turn attracts more vibrios to form a biofilm on the surface of the infected cells (Stauder et al., 2012). VPA1598 (GbpA), as reported by Gode-Potratz, increased in gene expression >110-fold during *V. parahaemolyticus* growth on a surface compared to growth in a liquid (Gode-Potratz et al., 2011). Vibrio adsorption in aquatic environments may have evolved to allow for vibrio to persist in other environments, such as human hosts (Zampini et al., 2005). The ability of vibrios to use GbpA as an adsorption factor in the aquatic environment and in human-bacterial

interactions allows for bacterial persistence in environments that would otherwise be unfavorable for their survival. Further understanding of the characteristics of VPA1598 gene expression and its response to differing environmental parameters will provide insight concerning *V. parahaemolyticus* endurance and survival in the environment through adsorption to chitin substrates.

3.2.8 *pvsA* (stable housekeeping gene required for vibrioferrin biosynthesis)

The *pvsA* gene, as found in *V. parahaemolyticus*, is a stable housekeeping gene that is required for biosynthesis and transport of the siderophore vibrioferrin and has been used in qRT-PCR reactions as a reference gene for normalizing expression of other genes (Coutard et al., 2007; Sylvander et al., 2013). Because of this, *pvsA* was chosen as the reference gene for this study, and the expression of other targeted genes was compared to the expression of *pvsA* as a reference.

Understanding *V. parahaemolyticus* responses can help in discovering adsorption and how these microbes interact with cells. Mechanisms of adsorption and injection by *V. parahaemolyticus* can lead to further discoveries, such as the development of anti-fouling agents and antimicrobial drugs and vaccines (Knight and Bouckaert, 2009; Axner et al., 2011; Walden et al., 2012). The response of *V. parahaemolyticus* to particles is not well described, and it is of concern because adsorption to substrates is important for the survival and persistence of *V. parahaemolyticus* and other vibrios in the environment. For this reason it is essential that the relationship between vibrios and copepods be further examined to expand the understanding of possible routes of human infection by pathogenic vibrios, and connection of environmental parameters to vibrio-copepod interactions.

3.3 Materials and methods

3.3.1 Sampling site

Sampling for collection of bacterial isolates was conducted off the coast at Port Fourchon, LA (Figure 8) as described previously (Johnson et al., 2012). Specifically, oysters were in the northern Gulf of Mexico in Louisiana. At all sampling stations, 20 – 25 oysters were collected. All samples were transported to the laboratory in coolers containing ice or ice packs. Oyster were scrubbed, shucked, and homogenized as described previously (Johnson et al., 2010).

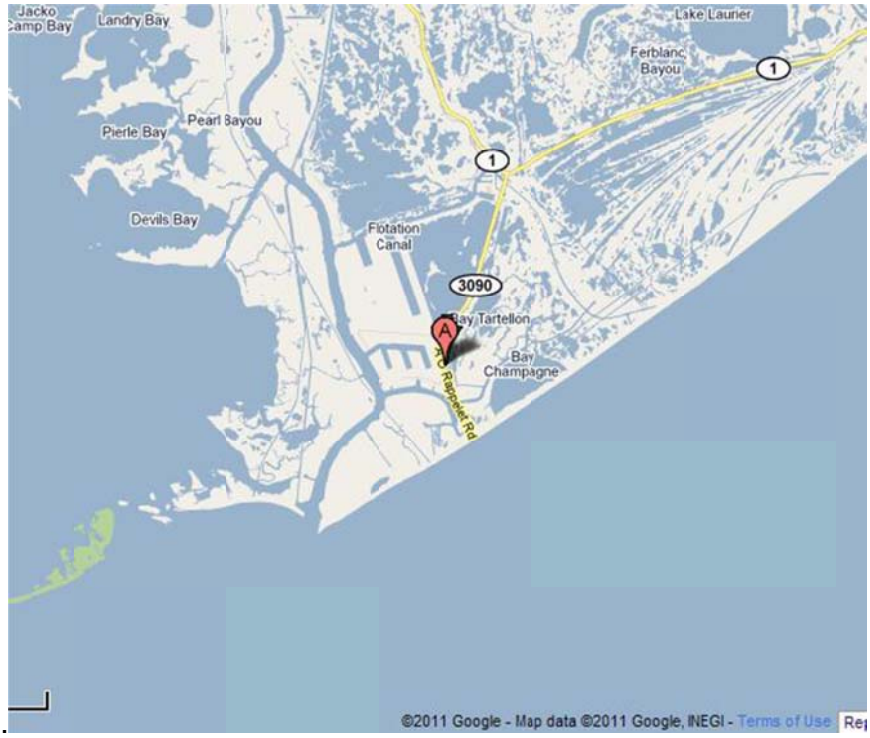

Figure 8: Sampling site, Port Fourchon, Louisiana, for Vp357 *V. parahaemolyticus* strain (courtesy of Google Maps).

V. parahaemolyticus was enumerated by spreading 0.1 g and 0.01 g oyster on T_1N_3 agar plates (1% tryptone, 3% NaCl, pH 7.2). Detection of *tdh* + and *trh*+ *V.*

parahaemolyticus was accomplished using 1 L, 100 mL, and 10 mL water enrichments and 10 g. All samples were incubated at 33 ºC for 16 – 18 hours.

3.3.2 Microcosms

To determine changes in gene expression in the presence of chitin, microcosms were used to expose a *V. parahaemolyticus* strain to chitin beads *in vitro*, and relative gene regulation was measured using quantitative real-time PCR (qRT-PCR). The concentration of beads (number per ml) was measured with a Z1 Coulter Particle Counter (Beckman Coulter, Brea, CA). Magnetic chitin beads (50–70 μm diameter) (New England BioLabs, Ipswich, MA) were placed into 2-ml siliconized centrifuge tubes with 0.5 mL Instant Ocean artificial seawater (ASW, United Pet Group, Inc., Cincinnati, OH) at concentrations of either 20 gpL or 35 gpL.

A *tdh*+ *V. parahaemolyticus* strain, VP357, which was isolated from oysters in 2010 off the coast of Louisiana was cultured in 10X alkaline peptone water (APW, 10% peptone, 1% NaCl, pH 8.5) at 33 °C. The culture was centrifuged at 14,000 × g and washed with ASW. Tubes were prepared containing an equal surface area of chitin beads in 1 mL of either 20 or 35 gpL ASW. The washed cells grown at 33 °C were added to these tubes at an initial concentration of 3.8×10^6 or 7.9×10^6 copies per mL. Differing initial bacterial concentrations were a result of growth in media for 2 hours. Mixtures were incubated at 10, 21, 25, 30, and 37 °C for 3.5 hours. Thus, cells were shifted from a salt concentration of 10 gpL to 20 and 35 gpL and from a temperature of 33 °C to temperatures of 10, 21, 25, 30, and 37 °C. The incubation time of 3.5 h was chosen based on previous reports of the time required for equilibrium between adsorbing and detaching bacteria (Belas and Colwell, 1982; Kogure et al., 1998).

Microcosms were shaken at 125 rotations per minute to simulate environmental conditions. At the end of 3.5 hours, 1 ml of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) was added to each reaction tube to immediately harvest all RNA present at the 3.5-hour mark. Measurements consisted of 4 replicates for each tested condition.

3.3.3 Relative quantitation of *V. parahaemolyticus*

After addition of RNAprotect, tubes were incubated at 25 °C on a shaker for 5 minutes and centrifuged at 5,000 * g for 10 minutes. RNA was then extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) as described by the manufacturer. Reverse transcription was carried out by use of a High Capacity cDNA Reverse Transcription Kit (Invitrogen, Grand Island, NY). Reactions included 2 μl 10X RT Buffer, 0.8 μl 25X dNTP mix, 2 μl 10X RT Random Primers, 1 μl MultiScribe Reverse Transcriptase, and 10 μl template, respectively, per each 20 μl reaction. Reverse transcription conditions included an initial step at 25 °C for 10 minutes, a second step at 37 °C for 120 minutes, and a final step at 85 °C for 5 minutes. Applied Biosystems Veriti 96 Well Thermal Cycler was used for all reverse transcription reactions.

qRT-PCR reactions using AgPath-ID™ One-Step RT-PCR Reagents (Life Technologies, Grand Island, NY), included final concentrations of 0.4 μM each of the forward and reverse primers (Table 2), 1X buffer, 1X enzyme mix, and 0.12 μM probe and 1 μL template in 10 μL reactions. PCR cycling conditions included an initial reverse transcription deactivation step at 95 °C for 15 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, followed by annealing at 60 °C for 1 minute. Applied Biosystems 7900 fast real-time PCR system (Grand Island, NY) was used for all qPCR reactions.

Table 2: Gene target primers and probes

Gene	Product size	Primer/probe sequences 5' to 3'
*pvsA*F	112	CTCCTTCATCCAACACGAT
pvsAR		CGAAATTCGGGCGAGATAA
pvsA probe		56-FAM/AGCCAATTT/ZEN/CAACACGCTCAAGGC/3IABkFQ
Vp1892F	103	AGCGAAGACATCAAGAGCTTAG
Vp1892R		TCACACCGAGTATTGAGGAAAC
Vp1892 probe		56-FAM/CAGCGACAT/ZEN/TGAACAAGTGCGTGA/3IABkFQ
chitobiaseF	102	GAAGTACAGCGAAGGTGAGAAG
chitobiaseR		GACCACTCGTACACTGATGAAG
chitobiase probe		56-FAM/ACACACGCG/ZEN/TTAACTTCTGGGATGT/3IABkFQ
*thiC*F	103	GTGTCTAGCACATCACCAAGAA
*thiC*R		CAAGCCATCACCAAGAGATAGAG
thiC probe		56-FAM/ACCCATTTCCGCGAGATCTGTGAA/3IABkFQ
VPA1598F	101	CGAACCCAATTTACCTGCAATC
VPA1598R		ACCATCAACCGTGAGTGAATAG
VPA1598 probe		56-FAM/AGCCAATTT/ZEN/CAACACGCTCAAGGC/3IABkFQ
VPA0459F	101	CACGATAGCAACCGCATTTAC
VPA0459R		GAACGAGACAACGCCAATAAG
VPA0459 probe		56-FAM/CCTGGCAGT/ZEN/TCGCTTCATGCTAGA/3IABkFQ
VPA1548F	122	GGCGCAGTTACTTTCCAAATC
VPA1548R		TCACCGATACCGTCAGTGATA
VPA1548 probe		56-FAM/AGACGTAGA/ZEN/CGCATCTGCAAGCAT/3IABkFQ
tdh F	232	TCCCTTTTCCTGCCCCC
tdh R		CGCTGCCATTGTATAGTCTTTATC
tdh probe		56-FAM/TGACATCCTACATGACTGTG/3IABkFQ

3.3.4 Data analysis

For analysis of the impact of abrupt changes of temperature on gene expression, cycle threshold values for each gene of interest (GOI) were normalized to the housekeeping reference gene *pvsA* to generate expression ratios (ER). Specifically, real-time PCR amplification efficiencies were calculated for each GOI and for *pvsA* by creating standard curves of serial dilutions and using the slopes of the resulting Ct (threshold cycle) values in the equation $E = (10^{-1/slope}-1) * 100$, where E = efficiency, as described previously (Pfaffl; Pfaffl, 2001; Pfaffl et al., 2002) (Appendix C). To determine the expression ratios for use with temperature data, in the absence of a negative control for temperature, as is needed for the more conventional -ΔΔCt model, the equation ER = E-ΔCt was used, where ER is the expression ratio, or the ratio of the GOI to the reference gene *pvsA*, E = PCR amplification efficiency, and $-\Delta Ct = Ct_{pvsA} - Ct_{GOI}$. Thus, ER represented the amount of GOI RNA normalized to the reference gene *pvsA*. Statistical analysis of temperature data was carried out using N-way analysis of variance (N-way ANOVA), *ad hoc* linear regression, and Tukey's statistical tests, and Matlab 2012a software. Salinity data were treated similarly, and statistical analysis was carried out with student's t-tests and Matlab 2012a.

The substrate data were analyzed using fold change (FC) values. These were calculated by comparing the ΔCt values for the genes of interest ($Ct_{GOI,control} - Ct_{GOI,sample}$) to the ΔCt values for the reference gene *pvsA* ($Ct_{ref,control} - Ct_{ref,sample}$). Specifically, the FC was calculated as $FC = \frac{\left[(E_{GOI})^{\Delta Ct_{GOI,control-sample}}\right]}{\left[(E_{ref})^{\Delta Ct_{ref,control-sample}}\right]}$, where E is PCR efficiency, GOI is the gene of interest, control is the negative control containing no

chitin substrate, sample is sample containing chitin substrate, and ref is the reference gene *pvsA*. Thus, fold change represented the amount of change in gene expression levels that resulted from the presence of chitin. Statistical analysis of FC values was performed with the Relative Expression Software Tool (REST) developed by Pfaffl, et al. (Pfaffl et al., 2002) using bootstrapping techniques with 2000 resamplings.

3.4 Results

3.4.1 *thiC* (thiamine biosynthesis metabolic possesses)

The impacts of temperature and substrate presence on *thiC* expression were not significant (Figure 9). However, when controlling for temperature and salinity, a paired t test revealed that expression of *thiC* was affected by salinity, and expression was higher following the shift to 35 gpL ASW ($p = 3.0 \times 10^{-4}$).

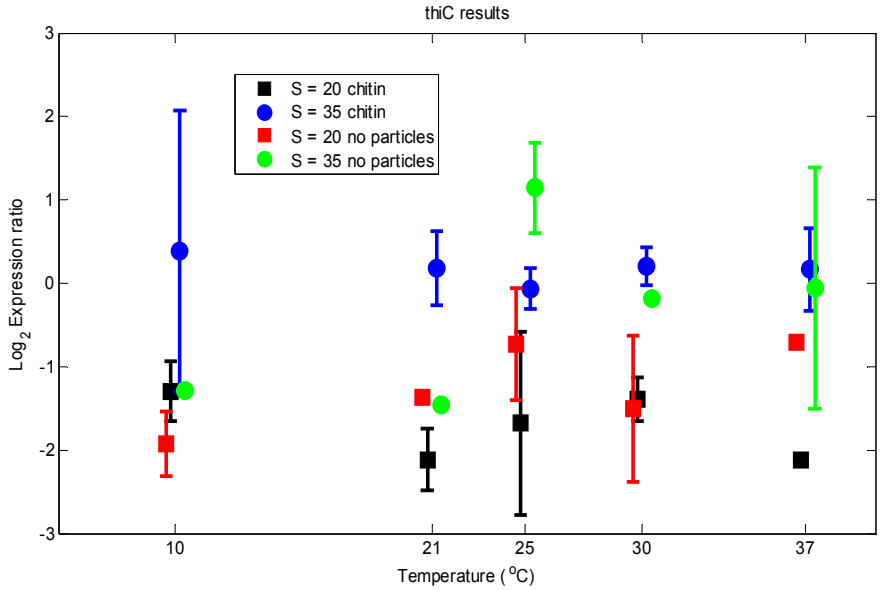

Figure 9: When controlling for temperature and salinity, expression of thiC was impacted by salinity (P = 3.0259 * 10-4). Data are median values, and error bars are median absolute deviations.

3.4.2 *tdh* (virulence factor)

No reliable results were obtained at T = 10 °C, S = 35 in the absence of chitin. To make it possible to carry out a complete paired t test, it was assumed that the expression ratio under these conditions was the same as the expression ratio at S = 20. This assumption was reasonable because there appeared to be no salinity effect on the expression of *tdh*. A paired t test on the results revealed that there was a substrate effect at $p = 0.055$ (Figure 10). In 8 of 10 comparisons, the *tdh* expression ratio was higher in the presence of chitin. There was also a marginally significant temperature effect at S = 35; the correlation coefficient between temperature and the *tdh* expression ratio (–0.36) was significant at $p = 0.064$. These results are subject to the caveat that the expression ratios at T = 10 °C in the absence of chitin were assumed to be the same at S = 20 and S = 35. This issue will be addressed in subsequent work because of the importance of the *tdh* gene.

3.4.3 chitobiase (chitin catabolism)

The effects of the environmental parameters examined in these studies on chitobiase expression ratios were not significant. Bootstrapping analysis indicated chitin caused a significant up-regulation of chitobiase expression following the 37 °C shift ($p = 0.057$) but not at any other tested temperatures examined. It is apparent from Figure 11 that this conclusion is based very much on the results at S = 35 in the absence of particles, because there was only one data point at S = 20 in the absence of particles at T = 37 °C.

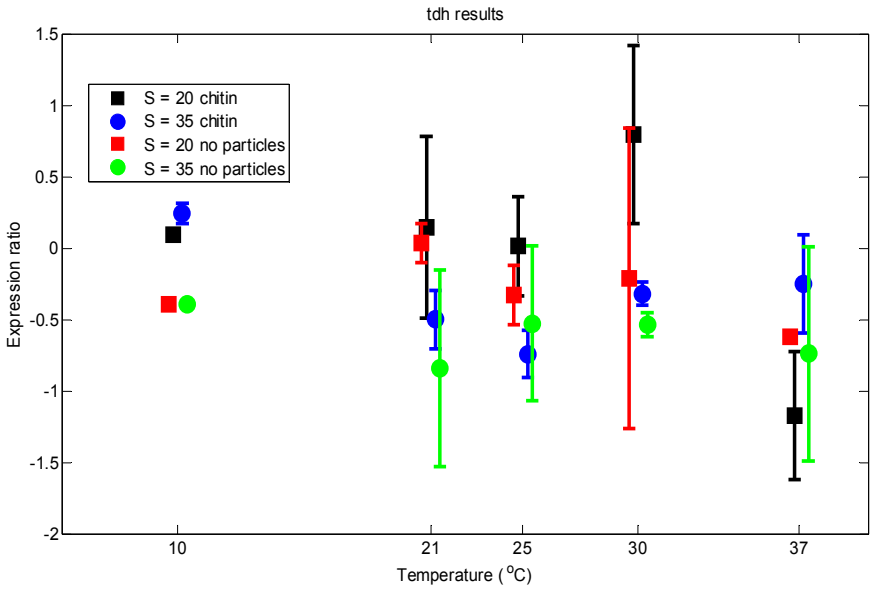

Figure 10: Expression ratios of *tdh* as a function of temperature, salinity, and presence or absence of chitin. Data are median values, and error bars are median absolute deviations. The data point at S = 35 and T = 10 °C in the absence of chitin was equated to the expression ratio at S = 20, T = 10 °C in the absence of chitin.

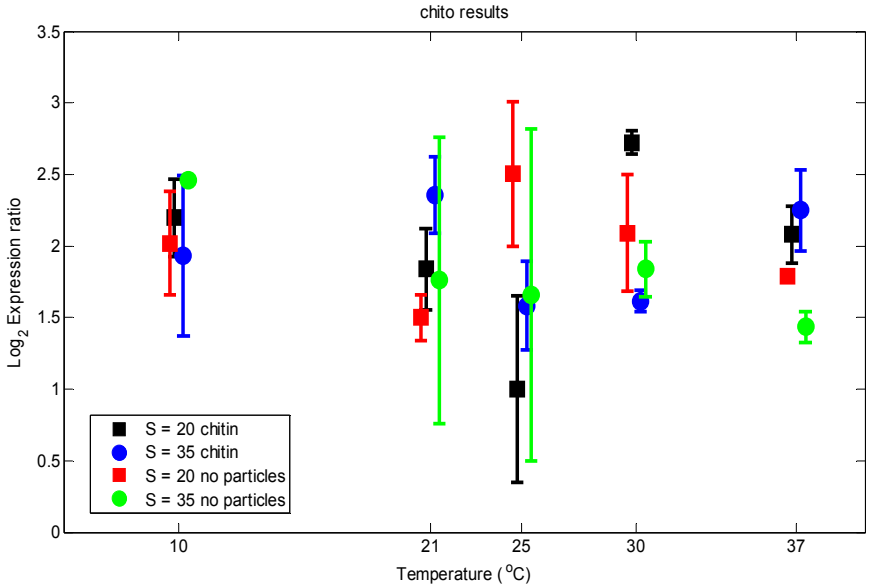

Figure 11: Log_2 of the expression ratios of the chitobiase gene as a function of temperature, salinity, and presence or absence of chitin. Data are median values, and error bars are median absolute deviations.

3.4.4 VPA0459 (collagenase virulence factor)

The effects of the temperature and salinity on VPA0459 expression ratios were not significant (Figure 12). When controlling for temperature and salinity, there was a significant chitin effect (p = 0.0224). Bootstrapping analysis indicated chitin caused an up-regulation of VPA0459 expression following the 37 °C shift (p = 0.037).

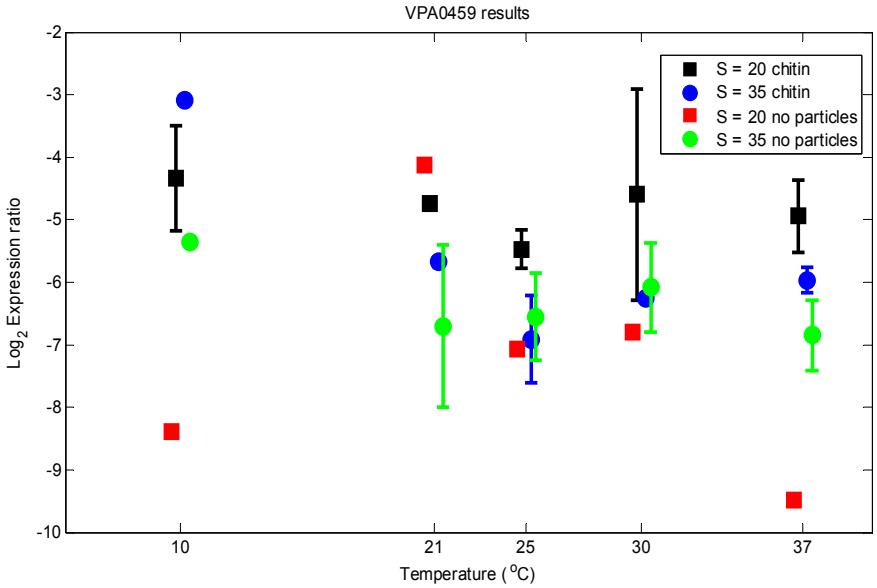

Figure 12: Log_2 of the expression ratios of the VPA0459 gene as a function of temperature, salinity, and presence or absence of chitin. Data are median values, and error bars are median absolute deviations.

3.4.5 VPA1548 (lateral flagellin)

In the absence of chitin, there was a significant temperature correlation for VPA1548 expression at 20 gpL (red squares) but not at 35 gpL (p = 0.0227); i.e., there was a general increase in VPA1548 expression (Figure 13). In the presence of chitin, there was a significant (p < 0.05) nonlinear temperature effect. VPA1548 expression was lowest when shifted from 33 to 21 degrees. Expression of VPA1548 in the

presence (blue circles) and absence (green circles) of chitin was similar, indicating chitin had no effect on its expression.

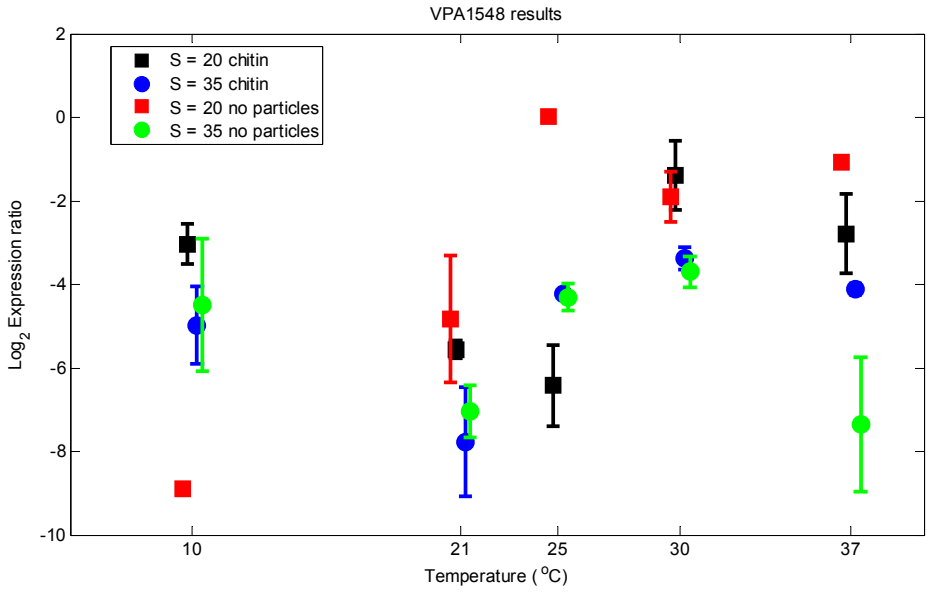

Figure 13: Log_2 of the expression ratios of the VPA1548 gene as a function of temperature, salinity, and presence or absence of chitin. Data are median values, and error bars are median absolute deviations.

3.4.6 VP1892 (methyl-accepting chemoreceptor used in chemotaxis)

There was a significant ($p < 0.05$) nonlinear relationship between temperature and expression ratios in the absence of particles (Figure 14). Peak activity occurred at 25 °C. In the presence of particles, there was a significant ($p = 0.0262$) positive correlation with temperature at 35 gpL. Bootstrapping analysis indicated chitin caused up-regulation of VP1892 at 10 °C ($p = 0.001$) but did not significantly impact its expression at other temperatures examined; it also caused VP1892 up-regulation at 35 gpL ($p = 0.01$).

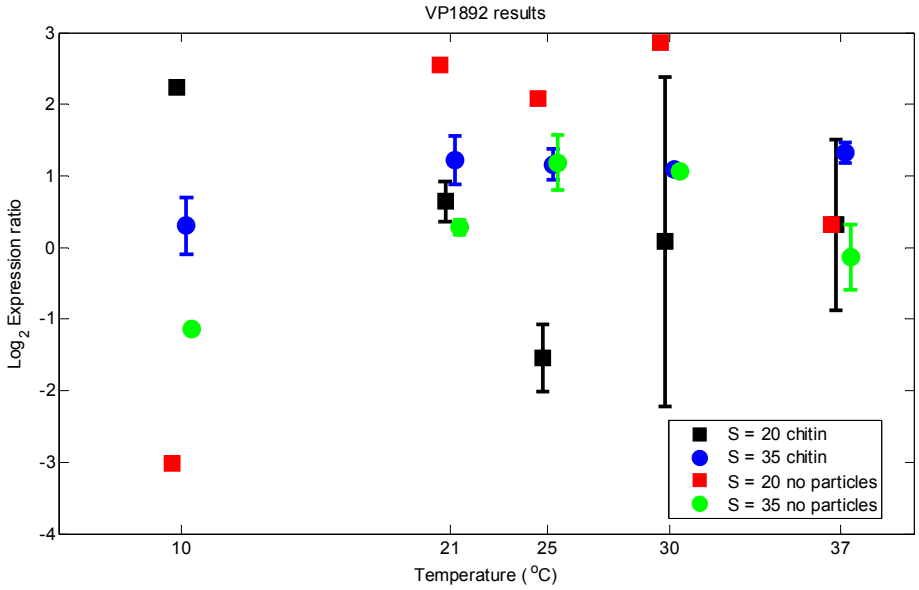

Figure 14: Log_2 of the expression ratios of the VPA1892 gene as a function of temperature, salinity, and presence or absence of chitin. Data are median values, and error bars are median absolute deviations.

3.4.7 VPA1598 (attachment to GlcNAc containing substrates)

There was a chitin effect when controlling for salinity and temperature (p = 0.0430), and that effect is dependent on temperature; i.e., there is a big chitin effect at low temperatures, and the chitin effect is less obvious at higher temperatures (Figure 15). In the absence of chitin, there was a significant correlation between temperature and VPA1598 expression at 20 and 35 gpL (p = 0.0294).

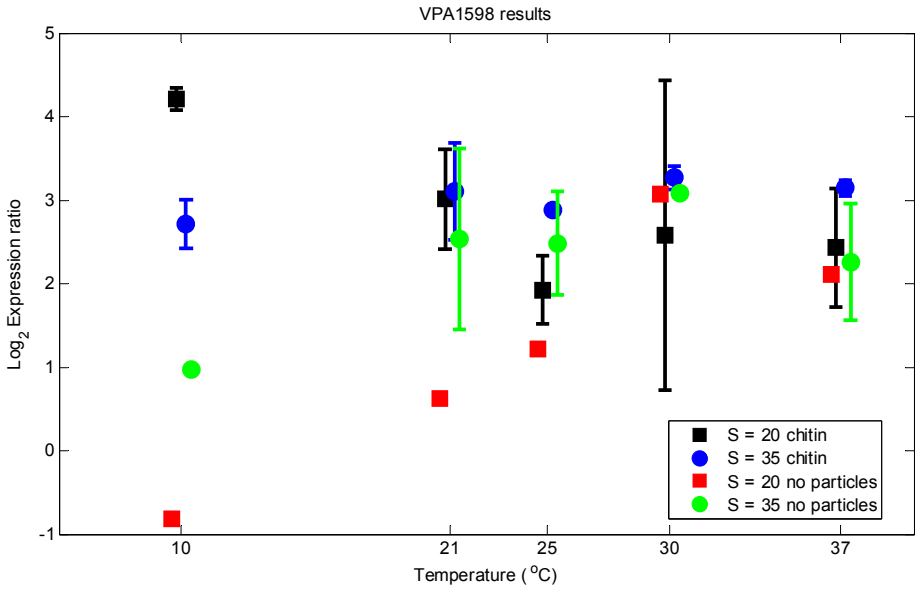

Figure 15: Log_2 of the expression ratios of the VPA1598 gene as a function of temperature, salinity, and presence or absence of chitin. Data are median values, and error bars are median absolute deviations.

3.4.8 Summary of results

When controlling for salinity, the impact of temperature on expression ratios was minimal. Only expression ratios for chitobiase at 20 gpL and VPA0459 at 35 gpL were significantly impacted. When controlling for temperature, the impact of salinity on expression ratios was more interesting. In general, expression ratios for *thiC* and chitobiase were impacted by salinity at warmer temperatures, and expression ratios for VPA0459, VPA1598, and VP1892 were impacted by salinity following shifts to 10 gpL. A summary of the genes targeted and their respective responses is provided in Table 3.

Table 3: Summary of impact of conditions on gene expression ratios.

Gene Expression Ratio *p*-values				
thiC				
Effect of salinity at 10°C	Effect of salinity at 21°C	Effect of salinity at 25°C	Effect of salinity at 30°C	Effect of salinity at 37°C
0.2823	0.1523	0.1034	**0.0034**	**0.018**
Effect of temperature at 20 gpL	Effect of temperature at 35 gpL			
0.6329	0.4877			
Chitobiase				
Effect of salinity at 10°C	Effect of salinity at 21°C	Effect of salinity at 25°C	Effect of salinity at 30°C	Effect of salinity at 37°C
0.8378	0.5847	0.5154	**0.0036**	0.3877
Effect of temperature at 20 gpL	Effect of temperature at 35 gpL			
0.0301	0.2833			
VPA0459				
Effect of salinity at 10°C	Effect of salinity at 21°C	Effect of salinity at 25°C	Effect of salinity at 30°C	Effect of salinity at 37°C
0.0841	0.3365	0.3009	0.1966	0.1424
Effect of temperature at 20 gpL	Effect of temperature at 35 gpL			
0.9031	**<0.001**			
VPA1598				
Effect of salinity at 10°C	Effect of salinity at 21°C	Effect of salinity at 25°C	Effect of salinity at 30°C	Effect of salinity at 37°C
0.0035	0.4782	0.4665	0.1632	0.2446
Effect of temperature at 20 gpL	Effect of temperature at 35 gpL			
0.4178	0.4362			
VPA1548				
Effect of salinity at 10°C	Effect of salinity at 21°C	Effect of salinity at 25°C	Effect of salinity at 30°C	Effect of salinity at 37°C
0.2832	0.3065	0.5805	0.2641	0.3141
Effect of temperature at 20 gpL	Effect of temperature at 35 gpL			
0.6032	0.126			

(Table 3 continued)

Gene Expression Ratio *p*-values				
VP1892				
Effect of salinity at 10°C	Effect of salinity at 21°C	Effect of salinity at 25°C	Effect of salinity at 30°C	Effect of salinity at 37°C
0.0047	0.7171	0.6849	0.1805	0.3423
Effect of temperature at 20 gpL	Effect of temperature at 35 gpL			
0.5684	0.2726			

3.5 Discussion

3.5.1 *thiC* (thiamine biosynthesis metabolic possesses)

As part of thiamine biosynthesis, *thiC* is used for metabolic processes. Thiamine has been found in organisms, including bacteria, fungi, animals, and plants. It plays a role in acclimation to stressors such as fungal, bacterial, and viral infection; salinity; heat; hypoxia; and disease (Tunc-Ozdemir et al., 2009). This may explain the up-regulation of *thiC* when salinity shifted from 10 to 35 gpL and the increased expression ratio of *thiC* at 35 gpL when compared to 20 gpL. In the aquatic environment, a salinity of 35 is less than optimal for *V. parahaemolyticus*, but in the current studies there was a significantly higher growth at a salt concentration of 35 gpL than at 20 gpL. While it is possible that the *thiC* up-regulation resulted from the stress of a salinity shift, it is not possible to rule out the fact that increased metabolism may also have been due to the increased growth. Nevertheless, environmental stressors in aquatic environments, such as increased salinity associated with relaying and depuration, may trigger the production of thiamine, which in turn alleviates the effects of environmental stresses.

Indeed, other metabolic changes in response to environmental stressors have been described. With respect to changes in metabolism, it has been previously reported that metabolism increases with increasing salinity. Leahy and Colwell (1990) reported that microbial-hydrocarbon degradation was positively correlated with salinity in estuarine sediments. Earlier studies have also reported observations of increased naphthalene mineralization with increasing salinities in a comparison of estuarine and less saline sites (Leahy and Colwell, 1990). Bacterial metabolism of naphthalene results in cis-1,2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol, salicylic acid, and catechol metabolites (Heitkamp et al., 1987). The results from the aforementioned studies are in contrast with the results of the current study, in which salinity had no effect on *thiC* expression. This is the first study to address the effect of temperature, substrate, and chitin on expression of *thiC* in *V. parahaemolyticus*, and it provides insight on how metabolic genes may respond to changes in environmental parameters.

3.5.2 chitobiase (chitin catabolism)

Chitobiase, which is involved in the breakdown of GlcNAc, was up-regulated in the presence of chitin following the 37 °C shift but not at any of the other temperatures examined in this study. GlcNAc is a monomeric subunit of the chitin polymer, which is found throughout the environment in organisms such as zooplankton and crustaceans. The expression data in the current study demonstrated up-regulation of chitobiase when shifting from 33 to 37 °C. Warmer temperatures could simulate these internal conditions and trigger the up-regulation of chitobiase activity to ensure stabilization on GlcNAc-containing cells within the host. Little has been reported on the effect of combinations of temperature, salinity, and chitin on chitobiase gene expression in *V. parahaemolyticus*.

The complete degradation of chitin to GlcNAc requires a sequential reaction of chitinase and chitobiase (Wadsworth and Zikakis, 1984; Cody, 1989). Chitinases attack free strands of chitin polymer and release intermediate-sized chitin oligosaccharides. Chitinases cleave bonds at the C1 and C4 positions of consecutive GlcNAc molecules in chitin. These oligosaccharides are then hydrolyzed to N,N-diacetylchitobiose (chitobiose), which is then hydrolyzed by chitobiase into two molecules of GlcNAc. Because of the consecutive use of these enzymes in chitin degradation, it is plausible that the activity of chitinase would be followed by chitobiase activity. This association may help describe analogous activity of chitobiase to that of chitinase in bacteria. A recent study of *Bacillus amyloliquefaciens* SM3 demonstrated a significant correlation between chitinase and temperature; chitinase activity in the presence of chitin decreased when temperatures were decreased from 37 °C to 20 °C (Das et al., 2012). Thus, the current findings implicate chitobiase expression at 37 °C as one means of acclimating to an extracellular stressor such as abrupt exposure to the mammalian gut or ballast-mediated transport from an area of low copepod density to an area of high copepod density.

3.5.3 VPA0459 (collagenase)

The expression ratio results of the current study indicate that collagenase production may be higher in the presence of chitin substrates than in the absence of chitin. Production of collagenase molecules may be impacted in a chitin-rich scenario such as a near-shore site or a mammalian gut. For example, when vibrios residing in ballast water are transported to a geographic location in which chitin presence is higher than in the original location, there may be a significant decrease in collagen-degrading

ability of vibrios. With respect to temperature, VPA0459 was up-regulated in the presence of chitin when shifted from 33 °C to 37 °C but not to other temperatures tested. Thus, collagenase expression was only significantly impacted when vibrio cells were warmed but not when the cells were chilled, which indicates collagenase does not play a role in acclimation to the stress of cold temperatures but does play a role in acclimation to 37 °C, a condition found in mammalian hosts. These findings are consistent with results of studies of vertebrates regularly exposed to *V. parahaemolyticus* in the marine environment. Warm-blooded vertebrates have an internal temperature of approximately 37 °C and contain GlcNAc on internal cells (Shimokawa et al., 2011). The internal environment of these vertebrates is conducive to an increase of collagenase production in the presence of GlcNAc substrates and a temperature of 37 °C. Such an increase of collagenase production would render *V. parahaemolyticus* better adapted to invading mammalian tissues.

3.5.4 VP1892 (methyl accepting chemoreceptor used in chemotaxis)

VP1892 expression ratios were affected by temperature. There was a nonlinear relationship between expression and temperature in the absence of particles, and there was peak expression at 25 °C. Thus, expression ratios were lowest at the more extreme temperatures examined in these studies, and optimal expression occurred at 25 °C. The lack of chemoreceptor production following shifts to the colder temperature was not surprising, as it is expected that refrigeration would cause a decrease in motility. However, the results following shifts to 37 °C were surprising. These results indicate shifting from 33 °C to mammalian body temperature does not elicit a response in motility. In the presence of chitin particles at 35 gpL, there was increased

chemoreceptor production as temperature increased. This would indicate higher activity when in association with marine mammals in the open ocean, and this could theoretically render the *V. parahaemolyticus* more infectious because of ability to colonize.

Fold expression data indicated that chitin alone did not cause a significant change in VP1892 expression. However, when the data were binned into categories, chitin caused a significant up-regulation in VP1892 expression following a 10 °C shift and a significant down-regulation following a 25 °C shift. Chitin also caused a significant up-regulation at 35 gpL, but not at 20 gpL. Thus, chitin played a role in regulating the expression of this chemotaxis gene under specific conditions in the current study.

The up-regulation of VP1892 when *V. parahaemolyticus* cells were shifted from 33 °C to 10 °C may be associated with communication via chemotactic signals to other cells in the surrounding environment in an attempt to flocculate for protection from unfavorable temperatures (Bible et al., 2012). Stress induced by shifting to sub-optimal temperatures, such as when oysters are relayed or placed on ice, may cause cells to up-regulate chemotaxis genes to aid in survival under less than favorable circumstances. Relatively high concentrations of *V. parahaemolyticus* have been detected in sediment in winter months (Kaneko and Colwell, 1978; Belas and Colwell, 1982), thus indicating a capacity for acclimation to cold in the short term and genetic adaptation throughout its natural history. Increased chemotaxis under cold conditions may indicate a form of communication between bacteria that would allow them to attach to sediment particles for protection under cold shock conditions.

It is unknown why there was significant down-regulation of the chemotaxis gene expression when shifted from 33 °C to 25 °C in the current study. These findings are in contrast with previous studies targeting other *Vibrio* species in which chemotaxis increased at 25 °C (Rahman et al., 2003; Larsen et al., 2004). There was, however, a 10-fold down-regulation of VP1892 expression in a previous study by (Gode-Potratz et al., 2011). These studies were conducted in heart infusion media that contained 15 gpL of NaCl, a salinity that is below that tested in the current study. Further investigations are needed to more thoroughly describe the specific relationship between VP1892 and down-regulation of gene expression at 25 °C.

VP1892 was significantly up-regulated in the presence of chitin at 35 gpL but not at 20 gpL; however, expression ratios at 20 gpL were significantly higher than at 35 gpL. Thus, when examining the impact of chitin on VP1892 expression, chitin impacted VP1892 expression under oceanic conditions but not under estuarine conditions. However, when examining the effect of salinity alone on VP1892 expression ratios (i.e., VP1892 expression normalized to the internal control gene *pvsA*), there was a significantly higher expression level under estuarine conditions than under oceanic conditions. A possible explanation for this difference could likewise be related to bacterial communication in response to environmental conditions. At a salt concentration of 35 gpL, representative of the open ocean, increased communication when in contact with chitinous substrates may increase survival of *V. parahaemolyticus* in a sparsely populated environment, given that vibrios fare better in an attached state than in a planktonic state (Keyhani and Roseman, 1999). With lower concentrations of chitinous organisms in open ocean waters than in estuaries, it would be beneficial for *V.*

parahaemolyticus to communicate through chemotaxis when one or a few bacteria happen to come in contact with a chitinous substrate onto which they could send out signals to attract other cells to form a biofilm. This may occur during ballast water dumping at sea before reaching coastal waters. Introduction to an open ocean environment may require bacteria to increase chemotaxis to communicate when limited particles are present for adsorption. Conversely, with higher concentrations of chitin in an estuary than in the open ocean (Keyhani and Roseman, 1999), the impact of chitin on intra- and inter-species communication may be more subdued.

3.5.5 VPA1598 (attachment to GlcNAc containing substrates)

VPA1598 expression ratios were significantly higher in the presence of chitin, and expression ratios increased with temperature. The distribution of *V. parahaemolyticus* in the water column in the summer months and in the sediment in winter months was associated with chitinous material (Belas and Colwell, 1982). Increased VPA1598 gene expression at higher temperatures and in the presence of chitin may help to explain the survival of *V. parahaemolyticus* in specific niches under variable salinity associated with seasonality. Adsorption of *V. parahaemolyticus* in an estuarine environment is critical for the survival of the bacteria and for chitin degradation as part of the carbon cycle within the estuary. Because of this, it is likely that production of chitin-binding proteins would increase in an estuarine environment in which large concentrations of chitin particles would be present. Future studies comparing the expression of the chitin-binding protein, VPA1598, which is similar to GlcNAc-containing proteins found internally in marine mammals and humans, may lead to results that help

to explain the role temperature plays in the adsorption of *V. parahaemolyticus* to GlcNAc-containing cells.

It has been reported previously that pathogenic bacteria have a decreased tendency to attach to inert particles as opposed to organic substrates (Iordache et al., 2008). The reasoning behind this theory is that vibrio strains enter a viable but non-culturable state (VBNC) (i.e., when exposed to conditions that are not suitable for successful survival, they enter a dormant state) in which they remain metabolically active but cannot be cultured on growth media (Iordache et al., 2008). This state is not likely in the case of the present study because of the timeframe of the experiments. A VNBC state in pathogenic bacteria is reached after days of exposure to adverse environments (Oliver, 2010). Because this study was conducted for 3.5 hours, there was not enough exposure time for the bacteria to reach a VBNC state.

In conclusion, the responses of *V. parahaemolyticus* to environmental conditions were diverse. This study represents one of few that have examined gene expression in *V. parahaemolyticus*, and it is the first study to examine gene expression in an environmental *tdh*+ isolate in response to a combination of changes in temperature, salinity, and chitin using real-time qPCR. There were several surprises in this study. It was unexpected that the impact of temperature and chitin on the expression of the genes investigated would be so minor. Although this study takes some steps in the direction of understanding how *V. parahaemolyticus* responds to its surrounding environment, knowledge gaps remain.

Results from these studies are an indication of the response of *V. parahaemolyticus* to the beta form of chitin, which is the most common form found in

crustaceans. Possible future studies could involve experiments with the alpha and gamma forms of chitin, which are found in squid pens and diatoms, and squid stomachs, respectively (Souza et al., 2011). Differences in vibrio responses have been described in the presence of different types of chitin (Svitil et al., 1997; Wong et al., 2012).

Results of this study may also be an indication of the role temperature plays in mammalian hosts with respect to *V. parahaemolyticus* infection in the presence of varying environmental parameters. Changes in regulation of gene expression indicate changes in bacterial responses, which could lead to an increase in environmental distribution and potential pathogenicity. This line of reasoning is further reason for the need for increased monitoring of *Vibrio* spp. populations during acute changes in temperatures and salinities. Such changes could be correlated with infection by exposure.

The ecology of *V. parahaemolyticus* may be a direct reflection of how these bacteria are capable of surviving in nature. As a result *V. parahaemolyticus* are capable of adapting to alternate environments with which they may come into contact. Such environments include changes related to icing, depuration, relaying, global transport via ballast water, and colonization of mammalian hosts. Gene expression profiles will help to give insights about the response of *V. parahaemolyticus* to abrupt changes in its surrounding conditions. It is hoped that by furthering understanding *V. parahaemolyticus* responses to changing environmental stressors, this research may lead to a better understanding of *V. parahaemolyticus* stressor responses, and in conjunction, its role relative to marine mammals and human health.

CHAPTER 4: CONCLUSIONS

Since the discovery of the interactions between naturally occurring vibrios and their environment, scientists have explained how vibrios correlate and respond to various environmental parameters, including temperature, salinity, turbidity, seasonality, dissolved organic carbon, primary productivity, and other environmental factors. These relationships are very difficult to model because of the variation in their individual and combined contributions to vibrio abundance and distribution. The current study attempts to make progress in the direction of a fuller understanding of how vibrios respond under certain conditions. The strength in these studies lies in the combination of environmental parameters and examination of gene expression. Allowing for the influence of more than one controlled environmental parameter at a time gives a more precise and real world evaluation of the trends and responses of *V. parahaemolyticus* in the environment. The parameters introduced in these studies were used to determine the effects of environmental determinants that may otherwise be difficult to study in field studies alone. The results from this study could be used in the construction of an ecological model demonstrating *V. parahaemolyticus* response to varying temperature, salinity, and substrate.

These studies have application to real-world settings. Combining the results from chapters 2 and 3 indicate the interactions between chitin and *V. parahaemolyticus* in the estuarine and marine environments are complicated. For example, there was decreased adsorption at 10 °C presumably due to cell death and shock; there was also significant up-regulation of chemoreceptor due to chitin. This indicates the response to the shock of shifting from 33 to 10 °C was induction of chemotaxis as indicated by the up-

regulation of VP1892 which encodes the *V. parahaemolyticus* methyl-accepting chemoreceptor. Chitin caused up-regulation at the coldest temperature tested indicating acclimation to colder temperatures, e.g., during oyster relaying or icing when the receptor could play a role in migrating toward substrates onto which they could endure the cold shock. Since the chemoreceptor was up-regulated following the shift from 10 to 35 gpL, chemotaxis would be highly efficient in the presence of chitin in the open ocean, but chitin's role in an estuary would not be as significant. This may be important because chitin concentrations are very low in the open ocean; thus, the impact of chitin in this environment may be more relevant than when chitin is abundant because the bacteria can use chitin as a carbon source. The role of zooplankton in chemotaxis is significant under diverse environmental conditions. For example, under cold, starvation conditions, *V. parahaemolyticus* may be more prone to attach to chitin-containing organisms since *V. parahaemolyticus* can use chitin as a carbon source.

VPA1548 encodes the *V. parahaemolyticus* lateral flagellin gene. Following the shift from 10 to 20 gpL, there was increased expression ratios as temperature increased. This would indicate more lateral flagellin production and thus more swarming when shifted to warmer conditions in an estuary.

When controlling for temperature and salinity, collagenase (VPA0459) expression was higher in the presence of chitin than in the absence of chitin. With respect to the substrate effects, chitin caused increased expression of collagenase at 37 °C but not at the other temperatures tested. This indicates association with GlcNAc and collagen in warm-blooded animals could be important steps in the progression of *V. parahaemolyticus* colonization and adaptation to eukaryotic hosts.

Plausible reasons for decreased concentrations of adsorbed *V. parahaemolyticus* following a shift from 33 to 10 °C could indicate changes in gene expression causing physiological changes and death in bacterial cells, which could result in the prevention of attachment.

Additional studies could also include the introduction of nutrients, which may affect adsorption properties. As previously reported, when adsorption was measured in the presence of two differing medias containing different nutrient conditions and ionic strengths, adhesion rate was higher with higher nutrient condition and ionic strength (Walker, 2005).

The lack of additional nutrients in the tested microcosms may also have had an effect on attachment of *V. parahaemolyticus* to particles, particularly chitin. A study conducted to follow the breakdown of chitinous materials by marine bacteria indicated that media consisting of only chitin in seawater led to a slower growth (which was not necessarily an indication of chitin degradation), and that the same bacteria in media containing chitin and additional nutrients decomposed chitin more rapidly (Hock, 1940). This same phenomenon is applied in environmental bioremediation, where addition of external nutrients, including nitrogen and phosphorus, increase the biodegradation of oil (Ladousse and Bernard, 1991; Floodgate, 1995). These observations indicate a possible need for future studies with microcosms that include external nutrients that could facilitate attachment to and/or breakdown of chitin components.

It is important not to just investigate adsorption but also to understand the physiology behind *V. parahaemolyticus* trends, as vibrios are equipped with a large

cache of tools with which to adapt to changing environments. Increasing concentrations of *V. parahaemolyticus* on organisms regularly used for human consumption will make it necessary to more fully understand the implications of postharvest approaches to reduce risk of infection such as icing, depuration, and relaying. Understanding the physiology of *V. parahaemolyticus* as indicated by gene expression with temperature, salinity, and substrate interactions leads to a better understanding of *V. parahaemolyticus* response to stressors. Investigating the regulation of genes involved in metabolism, adsorption, pathogenicity, and chemotaxis, in association with changing environmental parameters and their interactions, allows for further understanding of *V. parahaemolyticus* ecology. Understanding of abrupt changes in environmental parameters, individually and in combination, impacting *V. parahaemolyticus,* leads to further knowledge of their responses to environmental stressors and the implications this may have on mammalian consumption and infection.

Future studies correlated with the findings of this research could also include differing concentrations of bacteria in association with particles. A range of concentrations would allow for a better understanding of adsorption as related to bacterial concentrations and growth. The removal of possible temperature shock due to rapid temperature shifts from inoculum temperatures at 33 °C to respective test incubation temperatures could be obtained by maintaining equal inoculum and test temperatures throughout the experiment. Follow-up studies could also use metagenomic approaches to look at gene profiles collectively when exposed to a wider range of conditions that mimic estuarine and mammalian host conditions, and different sources of chitin such as intact chitin-containing animals.

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APPENDIX A: GENERAL SUPPLEMENTAL INFORMATION

A.1 Hybridization

To detect the presence of *V. parahaemolyticus* by direct plating / colony hybridization (DP/CH), Whatman 541 ashless filters (Whatman, Kent, ME) were used to lift bacterial colonies from plates, as previously described (Johnson et al., 2010). The filters were probed using alkaline phosphatase-conjugated oligonucleotide probes (DNA Technology A/S, Risskov, Denmark) specific for *tdh, trh*, and *tlh.* Positive colonies were determined by the presence of purple colonies on probed filters.

A.2 Real-Time PCR

To determine the number of total (*tlh*+) and potentially pathogenic (*tdh*+ and *trh*+) *V. parahaemolyticus,* most probable number (MPN) estimates were determined in triplicate for oyster enrichments using real-time PCR, as previously described (Nordstrom et al., 2007; Johnson et al., 2010). Probes and equipment were used as previously described (Johnson et al., 2010) (Table A.1).

The *tlh*, *tdh*, and IAC primers and the *tlh* and IAC probes were purchased from Integrated DNA Technologies (Coralville, IA), and the *tdh* probe was obtained from Applied Biosystems (Foster City, CA). Each 25-µl reaction consisted of a final concentration of 2.25units/µl Platinum Taq Polymerase (Life technologies, Grand Island, NY), 1X Buffer, 5mM $MgCl^2$, 10mM mixed dNTPs, 0.075µM forward and reverse *tlh* and IAC primers, 0.2µM forward and reverse *tdh* and *trh* primers, 0.15µM *tlh* and IAC probes, 0.075 *tdh* and *trh* probes, and 11.18µl nuclease free PCR water. Each reaction included 2µl IAC template and 2ul sample template. The two-step thermal profile

employed throughout the study consisted of an initial 1 min denaturation step at 95 °C followed by 45 cycles of 5s denaturation at 95 °C and a 45s combined annealing/extension step at 59 °C. Fluorescence data were collected at the end of each amplification cycle.

Table A.1: Primers and probes

	Name	Sequence
DP / CH	AP-*tdh*	5'-XGG TTC TAT TCC AAG TAA AAT GTA TTT G-3'
	AP-*tlh*	5'-XAA AGC GGA TTA TGC AGA AGC ACT G-3'
	AP-*trh*	5'-XAC TTT GCT TTC AGT TTG CTA TTG GCT-3'
	AP-*vvh*	5'-XGA GCT GTC ACG GCA GTT GGA ACC A-3'
Real-time PCR	tl_884F	5'-ACTCAACACAAGAAGAGATCGACAA-3'
	tl_1091R	5'-GATGAGCGGTTGATGTCCAAA-3'
	*tdh*_89F	5'-TCCCTTTTCCTGCCCCC-3'
	*tdh*_321R	5'-CGCTGCCATTGTATAGTCTTTATC-3'
	*trh*_20F	5'-TTGCTTTCAGTTTGCTATTGGCT-3'
	*trh*_292R	5'-TGTTTACCGTCATATAGGCGCTT-3'
	IAC fwd	5'-GACATCGATATGGGTGCCG-3'
	IAC rev	5'-CGAGACGATGCAGCCATTC-3'
	tlh probe	TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT -3'-BHQ2
	tdh probe	6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ
	trh probe	TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ
	IAC probe	Cy5-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-BHQ2

DP / CH, direct plating / colony hybridization; AP, alkaline phosphatase; X denotes alkaline phosphatase conjugated 5' amine- C6; IAC, internal amplification control.

Table A.2: Growth following incubation for 3.5 hours under various conditions.

Percent growth	Substrate	Temperature	[Salt]
48	Plastic	10	20
47	Plastic	10	20
27	Plastic	10	20
48	Plastic	10	35
44	Plastic	10	35
103	Plastic	21	20
202	Plastic	21	35
184	Plastic	21	35
233	Plastic	25	20
315	Plastic	25	20
668	Plastic	25	35
582	Plastic	25	35
918	Plastic	25	35
611	Plastic	25	35
2348	Plastic	25	35
266	Plastic	30	20
204	Plastic	30	20
113	Plastic	30	20
110	Plastic	30	20
344	Plastic	30	35
254	Plastic	30	35
244	Plastic	30	35
130	Plastic	37	20
100	Plastic	37	20
65	Plastic	37	20
1249	Plastic	37	35
793	Plastic	37	35
195	Plastic	37	35
608	Plastic	37	35
2235	Plastic	37	35
1492	Plastic	37	35
128	Chitin	10	20
95	Chitin	10	20
28	Chitin	10	20
109	Chitin	10	35
75	Chitin	10	35
43	Chitin	10	35
104	Chitin	21	20
143	Chitin	21	20
72	Chitin	21	20
199	Chitin	21	35

(Table A.2. Continued)

Percent growth	Substrate	Temperature	[Salt]
1393	Chitin	21	35
104	Chitin	25	20
67	Chitin	25	20
115	Chitin	25	20
251	Chitin	25	35
678	Chitin	25	35
508	Chitin	25	35
210	Chitin	25	35
303	Chitin	30	20
201	Chitin	30	20
233	Chitin	30	20
475	Chitin	30	35
448	Chitin	30	35
156	Chitin	30	35
267	Chitin	30	35
969	Chitin	30	35
132	Chitin	37	20
120	Chitin	37	20
397	Chitin	37	35
283	Chitin	37	35
349	Chitin	37	35
145	Chitin	37	35
25	Beadless	10	20
24	Beadless	10	35
90	Beadless	10	35
106	Beadless	21	20
102	Beadless	21	35
110	Beadless	25	20
329	Beadless	25	35
128	Beadless	30	20
272	Beadless	30	35
817	Beadless	30	35
44	Beadless	37	20
90	Beadless	37	35
169	Beadless	37	35

A.3 Copy number calculations

A.3.1 Calculation of the molar mass of double-stranded *tdh* product.

The mass of the atomic components of each individual nucleotide was calculated manually. A molecular weight of 18 g/mole was subtracted to account for the loss of a water molecule that results from the hydrolysis of nucleotide polymerization. These calculations resulted as follows:

dAMP 331.226-18=313.226 g/mole
dCMP 307.198-18=289.198 g/mole
dGMP 347.223-18=329.223 g/mole
dTMP 322.209-18=304.209 g/mole

The molecular weight was calculated based on the sequence of the *V. parahaemolyticus tdh* gene, which is 233 bp, which includes 73 adenines, 68 thymines, 47 cytosines, and 45 guanines. Multiplying the number of adenines by the molecular weight of each dAMP gives 73 * 313.226 = 22863.6 g/mol. Multiplying the number of thymines by the molecular weight of each dTMP gives 68 * 304.209 = 20685.6 g/mol. Multiplying the number of cytosines by the molecular weight of each dCMP gives 48 * 289.198 = 13592.4 g/mol. Multiplying the number of guanines by the molecular weight of each dGMP gives 45 * 329.223 = 14814 g/mol. The sum of these 4 molecular weights was 71955.6 g/mol, which when divided by 233, gave a final average of 71955.6 / 233 = 308.8223 for ssDNA (single-stranded DNA) and 618 g/mol for dsDNA (double-stranded DNA). Thus, the molar mass of a single *tdh* molecule is 618 g/mole.

DNA consists of adenine-thymine base pairs and cytosine-guanine base pairs. Adenines and guanines, both purines, are two-ringed nitrogenous bases. Cytosines and thymines, both pyrimidines, are single-ringed nitrogenous bases. Thus, a base pair

almost always consists of one purine and one pyrimidine. The combined mass of an A-T base pair is 313 + 304 = 617 g/mole. The combined mass of a G-C base pair is 329 + 289 = 618 g/mole. Although A and T have different masses, when examining a full-length DNA product, on average each nucleotide in an A-T base pair is 617 / 2 = 308.5 g/mole. Likewise, on average each nucleotide in a G-C base pair is 618 / 2 = 309 g/mole. This is the case in *V. parahaemolyticus*, of which almost half is G-C base pairs (45.4%). Thus, in these studies, the value of 309 * 2 = 618 g/mole was used to calculate single base pair mass, using the following equation:

$$m = n * \frac{1\ mole}{6.023 * 10^{23}\ molecules} * \frac{618\ g}{mole} = \frac{n * 1.026^{-21}\ g}{bp}$$

where the molecules are individual base pairs, m = mass, n = length of DNA in bp, 6.023 *10^{23} = Avogadro's number, 618 g is the molar mass of double-stranded DNA.

A.3.2 Calculation of sample copy number using the single base pair molar mass plus DNA concentration in genomic DNA samples.

The *V. parahaemolyticus* genome contains 5,165,770 bp. According to the above equation, each bp in *tdh* has a mass of 1.026 * 10^{-21} g. Thus, multiplication of the genome size by the mass of a single bp yielded a value of 5.3000 * 10^{-15} g. The genomic mass of 5.3000 * 10^{-15} g contains 2 copies of *tdh* per genome. The genomic DNA for the samples used in the experiments described in Chapter 2 was analyzed using a Nanodrop instrument, and the original genomic DNA concentration was 70.45 ng/µL. Thus, if 5.3000 * 10^{-15} g is representative of 2 copies of *tdh*, then 70.45 ng/µL is representative of 2.5618 * 10^{7} copies of *tdh* per µL. This value was used to generate a

standard curve, and the standard curve was used to convert the Ct values for samples to copy numbers.

A.3.3 Standard curve to determine sample copy numbers.

A standard curve (Figure A.1) for the absolute quantification of DNA was generated by preparing ten-fold serial dilutions of DNA extracted from a fresh culture of a *tdh*+ *V. parahaemolyticus* strain, *V. parahaemolyticus* 342, which was isolated from oysters in 2010 off the coast of Louisiana (Figure 1 and 8). The qRT-PCR was carried out for *tdh* gene on a Cepheid SmartCycler II system. The standard curve was graphically represented by plotting Ct values vs. DNA copies per µL.

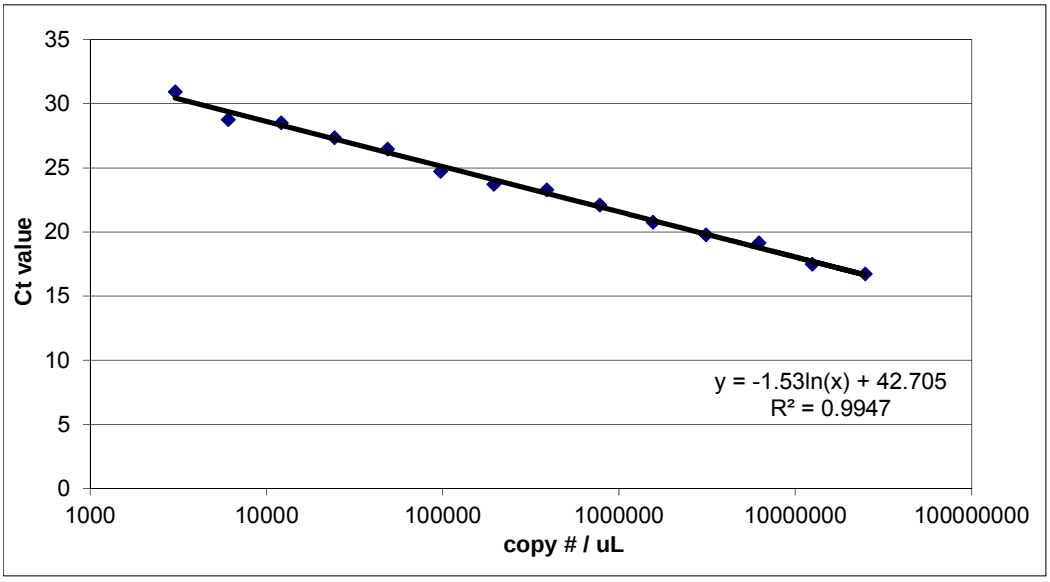


Figure A.1: Standard curve showing Ct values vs. DNA copy number.

APPENDIX B: SUPPLEMENTAL INFORMATION FOR CHAPTER 2

B.1 Wagatsuma Agar

Additional testing of Kanagawa positive *V. parahaemolyticus* strain, *V. parahaemolyticus* 342, was carried out on Wagatsuma agar. Wagatsuma agar was prepared following *The Handbook of Microbiological Media for the Examination of Food* (Atlas, 2006). Below is a list of reagents used in preparation of media (Table B.1). Wagatsuma agar was prepared by mixing all components except rabbit blood cells into a 1000ml solution and adjusting pH to 8.0. Solution was then placed in a steam bath for 30 min. Once solution cooled to 45 to 50 °C, 50ml of rabbit red blood cells were added. Solution was then mixed and placed in petri dishes.

Table B.1: Wagatsuma agar reagents

Component	**Amount per 1050ml**
NaCl	70g
Agar	15g
Mannitol	10g
Peptone	10g
K_2HPO_4	5g
Yeast extract	3g
Crystal violet	1g
Washed rabbit red blood cells w/ anticoagulant	50mg
DI water	1000ml

B.2 Surface area calculations

Surface area of magnetized beads were calculated using the following formula:

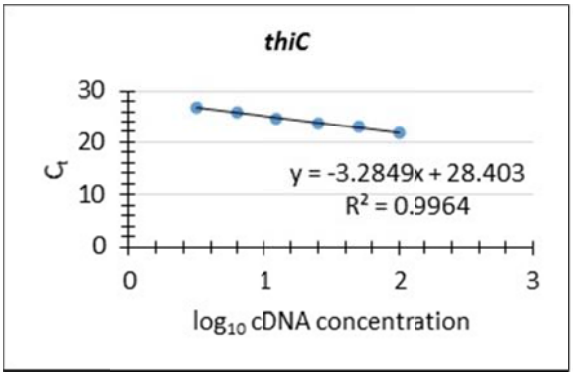
$$SA=4R^2$$

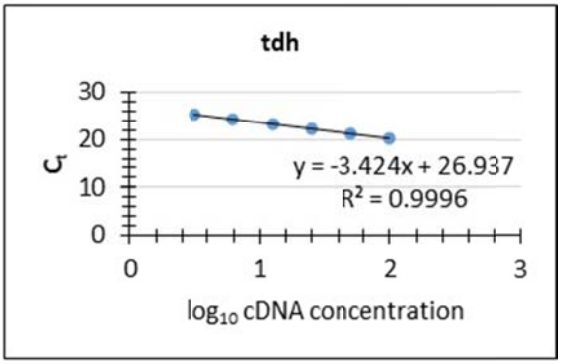
The average size of chitin beads and plastic beads, 60 μM and 58 μM, respectively, were used in surface area calculations.

APPENDIX C: SUPPLEMENTAL INFORMATION FOR CHAPTER 3

C.1 Real-time PCR Efficiencies for relative gene expression

Real-time PCR efficiencies were calculated for all tested genes using a *tdh*+, *trh*+ *V. parahaemolyticus* control strain, VpF11. Standard curves were made by six serial dilutions that were measured by real-time PCR on Applied Biosystems 7900 fast real-time PCR system (Grand Island, NY). A real-time PCR standard curve was graphically represented by plotting Ct values vs. log of input cDNA concentration (Figure C.1). The slope from the linear tread line from these plots was then used to calculate real-time PCR efficiency using the following equation: $E = (10^{-1/slope}-1) \times 100$ (Table C.1).





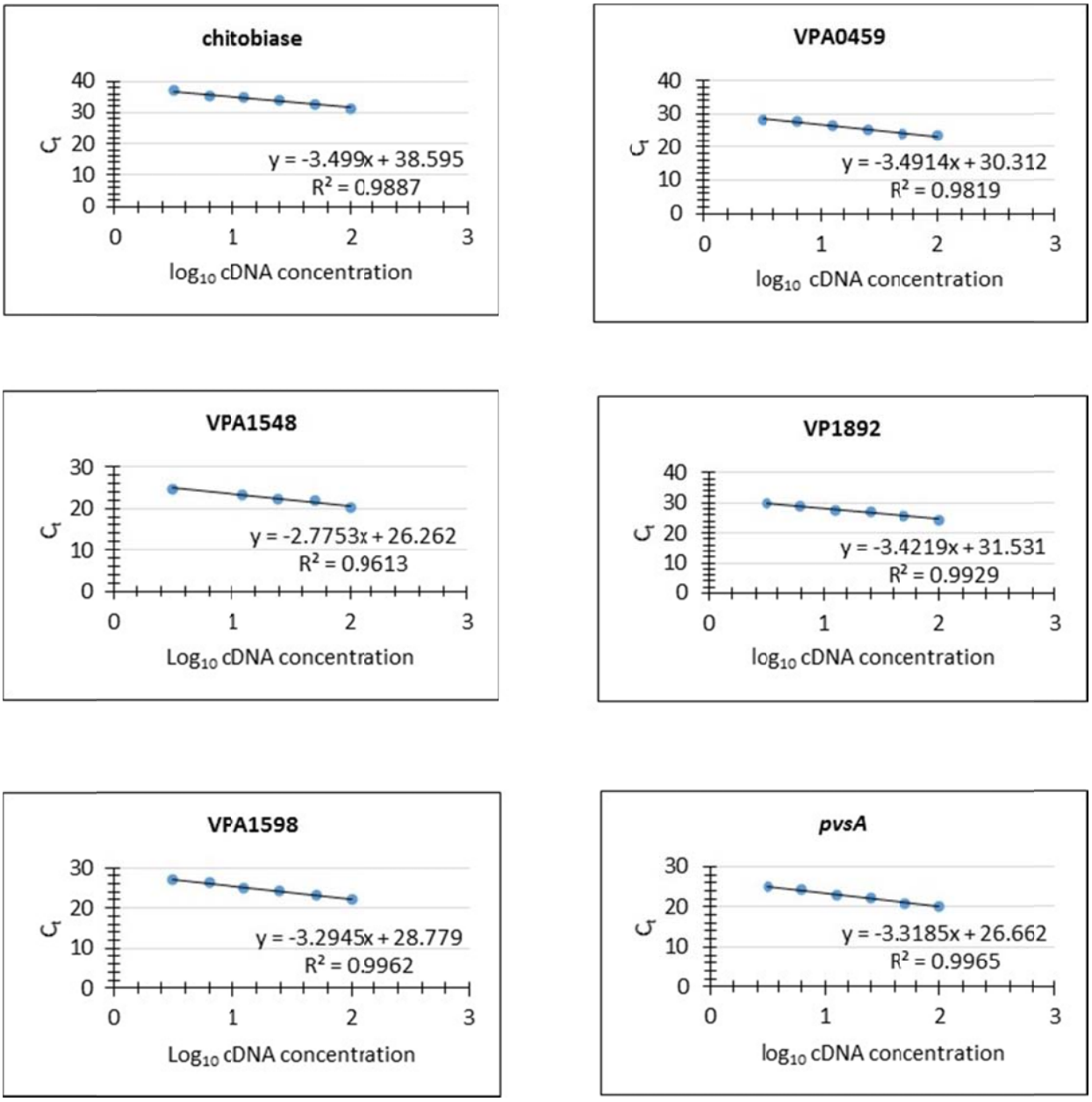


Figure C.1. Standard curves for efficiency calculations

Table C.1: Gene efficiencies

Gene	**Efficiency (%)**
VPA1598	101.1575
VPA1548	129.2564
VP1892	95.99063
thiC	101.5688
VPA0459	93.38289426
pvsA	100.143
tdh	95.90976
chitobiase	93.10608

Real-time PCR amplification efficiencies were calculated for each GOI and for *pvsA* by creating standard curves of serial dilutions and using the slopes of the resulting Ct (threshold cycle) values in the equation $E = (10^{-1/slope}-1) * 100$, where E = efficiency. Efficiency values of >100% were obtained for some genes (Table C.1), and this could be due to the presence of inhibitors or primer dimers in the PCR reaction or variations in the fidelity of oligonucleotide probes.

C.2 Primer design

Primers were designed using Integrated DNA Technologies PrimerQuest. Primers were designed by customizing the design assay to create primers producing an amplicon length of ~100bp.

C.3 Primer testing

Individually designed probes were tested against a using a *tdh* +, *trh*+ *V. parahaemolyticus* control strain, VpF11. Real-time PCR reactions were performed using AgPath-ID™ One-Step RT-PCR Reagents (Life Technologies, Grand Island, NY), including final concentrations of 0.4 µM each of the forward and reverse primers, 1X buffer, 1X enzyme mix, and 0.12 µM probe and 1 µL template in 25 µL reactions. PCR cycling conditions included an initial reverse transcription deactivation step at 95 °C for 15 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, followed by annealing at 60 °C for 1 minute. Quantitative real-time PCR reactions were carried out on an Applied Biosystems 7900 fast real-time PCR system (Grand Island, NY).

C.4 Probe Design

Probes were designed using Integrated DNA Technologies PrimerQuest. Probes were customized under custom design parameters inserting pre-tested forward and reverse primers into the *partial design input*. Final primers were chosen from suggested probe design outputs. Probes were then designed to incorporate 5' 6-FAM - ZEN - 3' Iowa Black® FQ fluorescence and quenchers.

VITA

Vanessa Molina was born on November 25th in Pasadena, Texas. She is the daughter of Iliana Molina and Tomas Molina of La Porte, Texas, and has one sister, Sierra Molina. In May of 2000, she graduated from La Porte High School in La Porte, Texas. In 2004, she received a Bachelor of Science degree in Marine biology from Texas A&M University at Galveston. During her undergraduate tenure, she was a student worker in the Laboratory for Oceanographic and Environmental Research (LOER) at Texas A&M University at Galveston in Galveston, Texas. After graduating with her Bachelor of Science, she continued her research at LOER lab as a Research Assistant. In August 2005, she was offered a graduate teaching assistantship by the Department of Biological and Environmental Sciences at McNeese State University in Lake Charles, Louisiana, where she later graduated with her Master of Science degree in Environmental and Chemical Science. In 2007, she began her Doctor of Philosophy degree in Environmental Toxicology as a Research Assistant in the Department of Environmental Toxicology at Clemson University, Clemson, South Carolina. In 2009, she transferred to Louisiana State University, Baton Rouge, Louisiana, where she was awarded a graduate research assistantship in the Department of Environmental Sciences. She plans to use the knowledge gained in her graduate course work and experience to pursue a career in marine microbiology research.