ABSTRACT

DRAKE, STEPHENIE LYNN. The Ecology *Vibrio vulnificus* and *Vibrio parahaemolyticus* from Oyster Harvest Sites in the Gulf of Mexico. (Under the direction of Dr. Lee-Ann Jaykus).

The *Vibrionaceae* are environmentally ubiquitous to estuarine waters. Two species in particular, *V. vulnificus* and *V. parahaemolyticus*, are important human pathogens that are transmitted by the consumption of contaminated molluscan shellfish. There is limited information available for the recent risk assessments; accordingly, the purpose of this study was to address some of these data gaps in the *V. vulnificus* and *V. parahaemolyticus* risk assessments. The objectives of this study were to (i) quantify the levels of total estuarine bacteria, total *Vibrio* spp., and specific levels of non-pathogenic and pathogenic *V. vulnificus* and *V. parahaemolyticus* over the harvest period; and (ii) determine if length of harvest time affects the levels of *V. vulnificus*.

Oyster and water samples were harvested seasonally from 3 U.S. Gulf Coast sites over 2 years. Environmental parameters were monitored during harvesting. Both surface and bottom water samples (1 L) were taken at the beginning of harvesting and at the end of harvesting. Oyster samples (15 specimens for each time point) were taken at 0, 2.5, 5.0, 7.5, and 10 hrs intervals after being held at ambient temperature during harvesting. Samples were processed for many different bacteria. For enumeration of total *V. parahaemolyticus*, pathogenic *V. parahaemolyticus*, and *V. vulnificus* was done using colony lift hybridization (*tlh*, *tdh*+ and/or *trh*+, and *vvhA* gene targets, respectively).

MPN methods were also used to obtain estimates of pathogenic *V. parahaemolyticus* (*tdh*+ and/or *trh*+) counts. Representative *V. parahaemolyticus* and *V. vulnificus* isolates were subjected to phenotyping; *V. vulnificus* isolates were also subjected to genotyping. Different statistical analysis were used to establish relationships where appropriate.

The first manuscript describes the field study which examined the distribution and variation in the levels of *V. parahaemolyticus* and *V. vulnificus* (both total and pathogenic strains) in shellfish and their overlay waters, and established the effect of seasonal and environmental/ecological factors on these distributions. The best estimate of growth for *V. parahaemolyticus* (*tlh*) in oysters based on water temperature was 0.054 log10 per ^{o}C, while the best estimate for growth for *V. vulnificus* (*vvhA*) in oysters based on water temperature was 0.068 log10 per ^{o}C. Estimated relationships between *tlh* and *vvhA* growth rates and air temperature were consistent with the risk assessments. No statistical relationship could be established between pathogenic *V. parahaemolyticus* (*tdh*+ and/or *trh*+) growth rates and air temperatures.

In the second study, we specifically looked at the effect of extended boat deck storage of commercially harvested oysters held at ambient air temperature on the levels of *V. vulnificus*. During summer, increases in *V. vulnificus* were as high as 1.4 log10 (CFU/g) after 10 h storage at ambient air temperatures; for spring/fall oysters, a 1.0 log10 increase was observed; and in winter, increases were <0.5 log10. Statistically significant ($p<0.05$)

correlations between ambient air temperature and increases in *V. vulnificus* counts were noted.

Taken together, this research provides information which can be used to fill key data gaps in the current risk assessments for *V. vulnificus* and *V. parahaemolyticus*. These data can be used in future iterations of the risk assessments to help better predict risk and inform policy as risk managers seek to reduce the disease burden associated with these important foodborne pathogens.

The Ecology *Vibrio Vulnificus* and *Vibrio Parahaemolyticus* from Oyster Harvest Sites in the Gulf of Mexico

by
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BIOGRAPHY

Stephenie Lynn Drake was born February 6, 1979 in Sunnyside, Washington. She grew up in the city of Wenatchee in the foothills of the Cascade Mountains. She graduated from Wenatchee High School in 1998, where she was very active in FFA and 4-H. She then moved to Starkville, Mississippi to escape shoveling snow and to start her undergraduate college education at Mississippi State University. While at MSU, she was very active in the Food Science club especially as president for 3 years. She completed an undergraduate research project under the direction of Dr. Marshall. She received second place in the undergraduate research competition at the Institute of Food Technologists/ Meeting in June, 2002. She obtained her Bachelor of Science degree in Food Science in 2002.

The confidence gained during her undergraduate research project was invaluable when it came time for her to begin her own graduate research under the direction of Dr. Jaykus. She received second place for her oral presentation in the Food Microbiology Division at the Institute of Food Technologists' meeting in July, 2003 and at the 2004 meeting. She was also a developing scientist finalists for her presentation at the International Food Protection Association meeting in 2004.

After graduating, Stephenie did a summer internship with the Food and Drug Administration/Center for Food Safety and Applied Nutrition at Dauphin Island, Alabama

under the direction of Dr. Angelo DePaola. During this time, she analyzed seafood samples for *Vibrio* species using real-time PCR and colony hybridization methods.

The time spent working in Dr. DePaola lab positioned her well to transition back into her doctoral program at North Carolina State University with Dr. Lee-Ann Jaykus. The techniques learned in Dr. DePaola lab would be used frequently in her research.

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LITERATURE REVIEW

AN OVERVIEW OF *VIBRIO VULNIFICUS AND VIBRIO PARAHAEMOLYTICUS*

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1.1. INTRODUCTION

In the United States, contaminated seafood is responsible for 26.5% of all foodborne disease outbreaks (Mead and others 1999) with the majority of these illnesses associated with the consumption of raw bivalve molluscan shellfish (Cook 1991). Bivalves, including oysters, clams, mussels, and cockles, are filter-feeding organisms that pump seawater through their digestive systems to obtain oxygen and food and, in this process, accumulate and concentrate microorganisms. These organisms can be harmless commensals as well as pathogens, the most significant of which are the human enteric viruses and the pathogenic *Vibrio* species. Since shellfish are frequently consumed whole and raw, they can serve as passive carriers of foodborne disease agents.

1.2. *VIBRIO* SPECIES DESIGNATIONS

The genus *Vibrio* is in the family *Vibrionaceae*, which also includes the genera *Aeromonas*, *Plesiomonas*, and *Photobacterium* (Atlas 1997). All vibrios are ubiquitous in the marine environment and all species except *Vibrio cholerae* and *Vibrio mimicus* require sodium chloride supplementation of media for growth. There are 30 species in the genus *Vibrio*; thirteen of these are pathogenic to humans, including *V. cholerae*, *V. mimicus*, *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. cincinnatiensis*, *V. hollisae*, *V. vulnificus*, *V. furnissii*, *V. damsela*, *V. metshnikovii*, and *V. carchariae*. All of the pathogenic vibrios have been reported to cause foodborne disease, although *V. cholerae* O1, *V.*

parahaemolyticus, and *V. vulnificus* are considered the most significant agents. Members of the *Vibrio* genus are straight or curved Gram-negative, nonspore- forming rods, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length (McLaughlin 1995). However, when they are grown in the laboratory, they frequently revert to straight rod morphology (Atlas 1997). Vibrios are motile by a single polar flagellum and are aerobic or facultatively anaerobic. Most species produce oxidase and catalase and ferment glucose without producing gas (McLaughlin 1995). *V. vulnificus* is similar phenotypically to *V. parahaemolyticus* (Oliver 1989). The two most distinctive characteristics of *V. vulnificus* are fermentation of lactose and production of β-D-galactosidase and these biochemical tests for them can be used to distinguish it from the related *V. parahaemolyticus* (Hollis and others 1976).

1.3. CLASSIFICATION OF *V. VULNIFICUS* STRAINS

Historically, *V. vulnificus* strains have been classified by biotyping, a technique based on a combination of different phenotypic, serologic, and host range characteristics. Biotype 1 can be found in warm marine waters and was initially thought to be the only biotype associated with human infection (Blake and others 1980). Biotype 1 strains are pathogenic to humans, have different immunologically distinct lipopolysaccharide (LPS) types, and are indole positive (Biosca and others 1996). Biotype 2 was first thought to be pathogenic only to eels (Tison and others 1982), but this was later disputed based on human clinical evidence (Veenstra and others, 1992; Amaro and Biosca, 1996). In addition, Amaro and others (1992)

compared the two biotypes, finding that biotype 2 strains were able to adhere to human and fish cell lines and were highly cytotoxic. In addition, biotype 2 strains were more virulent for mice ($LD_{50} = 10^5$-10^6 CFU) when compared to biotype 1 strains ($LD_{50} = 10^8$ CFU). In general, biotype 2 strains have the following characteristics: pathogenic to both humans and eels; expression of a common LPS type; and negative indole reaction (Biosca and others 1996). In 1996, *V. vulnificus* biotype 3 was first described when it was associated with an outbreak involving 62 Israeli patients with either wound infection or septicemia (Bisharat and others 1999; Bisharal and others, 2005). To date, human disease caused by biotype 3 has not been associated with food consumption.

There appears to be a relationship between different 16S rRNA sequences and the virulence of *V. vulnificus* and this has been used as a means of strain typing as well. The sequence of 16S rRNA is highly conserved among all organisms and is commonly used to discern the evolutionary relationships among prokaryotes. Various regions within the rRNA genes evolve at slightly different rates, resulting in alternating regions of nucleotide conservation and variability (De Rijk and others 1992; Van de Peer and others 1996). Nilsson and others (2003) reported differences in rRNA sequences between clinical and environmental *V. vulnificus* strains. These data showed that two 16S rRNA types (designated A and B) contain a 492 bp-amplified region which has an *Alu*I cleavage site after nucleotides 202 and 244, and a *Hae*III cleavage site after nucleotides 168 and 372. The difference between types A and B is that the type A sequence has an additional *Alu*I cleavage site after

nucleotide 140, whereas type B has an additional *Hae*III site after nucleotide 147. In general, the B sequence is more highly associated with clinical strains and the A sequence is associated with environmental isolates. Additionally, Lin and Schwarz (2003) found more type A strains to be isolated in June and July, while more type B strains were isolated in September. More recently, a 200 bp amplicon originally identified by random amplified polymorphic DNA (RAPD) analysis was also reported to be primarily associated with clinical ("C") *V. vulnificus* strains and a conventional PCR assay was developed to detect these strains (Rosche and others 2005). An independent group using the above PCR assays reported that there was a 100% correspondence between *rrs* type B and "C" isolates (Chatzidaki-Livanis and others 2006).

1.4. PATHOGENICITY OF *V. VULNIFICUS*

V. vulnificus virulence is multifaceted and not well understood. Indeed, many virulence factors have been reported for this organism, including (i) a polysaccharide capsule; (ii) various extracellular enzymes; (iii) exotoxins; and (iv) the ability to obtain iron from transferrin (Linkous and Oliver 1999; Gulig and others 2005). The absence of estrogen has also been cited as a host factor linked to increased risk of infection (Linkous and Oliver 1999).

The presence of a capsule, which is also related to colony opacity, is probably the best known virulence factor for *V. vulnificus*. *V. vulnificus* is an extracellular pathogen that

relies on its polysaccharide capsule to avoid phagocytosis by host defense cells (Linkous and Oliver 1999; Strom and Paranjpye 2000; Gulig and others 2005). The transformation of encapsulated isolates to the nonencapsulated form is dependent on growth phase and temperature, which in turn affect bacterial cell morphology. For instance, Wright and others (1990) found an increase in the expression of capsular polysaccararide (CPS) during the logarithmic growth phase and a decrease during the stationary phase of growth for a clinical isolate of *V. vulnificus*. Also, there was significant expression of CPS observed for cells grown at 30°C as compared to those grown at 37°C. Encapsulated isolates have opaque colony morphology but can undergo a reversible phase variation to the translucent colony phenotype, which is correlated with reduced CPS production (Wright and others 1990; Strom and Paranjpye 2000). Wright and others (1990) reported that nonencapsulated strains (clinical) produced by transposon mutagenesis had a lethal dose over four times higher than that of the encapsulated strains. Research has shown that infection with *V. vulnificus* elicits an antibody response specific to the capsule (Foire and others 1992) and *V. vulnificus,* like other bacteria, relies on the capsule to resist host defenses during systemic disease.

There is evidence that several extracellular enzymes play a role in *V. vulnificus* pathogenicity. Moreno and Landgraf (1998) reported that the enzymes lecithinase, lipase, caseinolytic protease and DNase were present in >90% of the *V. vulnificus* strains screened, all of which were isolated from seafood samples. The protease may be particularly important, as Oliver and others (1986) found that 91% of the clinical and environmental

strains of *V. vulnificus* screened produced a protease that was capable of breaking down native albumin, hypothesizing that this protease might be involved in promoting systemic infection. A separate metalloprotease containing a zinc atom is able to degrade a number of biologically important host-associated proteins, including elastin, fibrinogen, and plasma protease inhibitors (Miyoshi and others 1995). The most dramatic pathological action of the metalloprotease is its vascular permeability-enhancing action (Shinoda and Miyoshi 2000).

The exotoxin hemolysin/cytolysin produced by *V. vulnificus* has been the most studied virulence marker. Hemolysin/cytolysin, encoded by a gene designated *vvh*A (other abbreviations are *cth* and *hha*), is a heat-labile enzyme that lyses mammalian erythrocytes and is cytotoxic to a variety of mammalian tissue culture cell lines (Gray and Kreger 1985; Strom and Paranjpye 2000). The *vvhA* protein displays 65% and 60% amino acid sequence similarity to the *V. cholerae* El Tor hemolysin and *V. cholerae* non-O1 cytolysin, respectively (Yamamoto and others 1990; Wright and Morris 1991; Strom and Paranjpye 2000). Gray and Kreger (1986) reported antibodies specific to the *V. vulnificus* hemolysin in the blood of infected mice, suggesting that the enzyme plays a role in pathogenicity. Later, Gray and Kreger (1987) demonstrated that mice injected with hemolysin developed skin damage similar to that of infected humans. Lee and others (2004) found that 20% normal pooled human serum significantly inhibited hemolytic and cytotoxic activities of the *vvhA* protein, suggesting that it could be inactivated *in vivo* and that its activity might be compromised by serum constituents such as cholesterol. When these same investigators

inoculated mice intraperitoneally with 10^7 CFU of a clinical *V. vulnificus* isolate, they observed the expression of the *vvh*A gene product in bacterial cells isolated from host livers, suggesting that the protein itself is produced *in vivo* and in association with particular tissues.

The amount of iron available in the host is an important factor influencing the lethality of *V. vulnificus*. Wright and others (1981) showed that the intraperitoneal LD_{50} was reduced from 10^6 CFU to 1 CFU in iron-treated mice. Later, Reyes and others (1987) classified both clinical and environmental strains of *V. vulnificus* into categories of virulent and avirulent, with the former demonstrating a lethal infectious dose of $<10^5$ CFU/mL, while the latter failed to kill suckling mice at doses $>10^9$ CFU/mL, although route of administration was an important mitigating factor. Morris and others (1987) found that none of the *V. vulnificus* strains (clinical and environmental) tested were capable of growth in iron-limited media in the presence of 30% saturated transferrin; however, some strains were able to grow in the presence of 100% saturated transferrin. These investigators hypothesized that the increased saturation of transferrin, either through an excess of iron or through a relative decrease in the amount of transferrin, may be associated with the pathogenesis of *V. vulnificus* (Morris and others 1987; Brennt and others 1991). Transferrin is an iron transport protein and, because free iron is virtually absent in the body, pathogenic bacteria like *V. vulnificus* may have evolved mechanisms to scavenge iron from the iron transport proteins (Strom and Paranjpye 2000). Alternatively, they may use iron-scavenging siderophores and proteins that can serve as iron donors [such as phenolate and hydroxamate (Simpson and

Oliver 1983) and hemoglobin, methemoglobin, and hematin (Helms and others 1984)] (Gulig and others 2005). Stelma and others (1992) used the iron-overloaded mouse model to characterize the virulence of various *V. vulnificus* strains of clinical and environmental origin, finding that iron-overloaded mice died after challenge with lower doses ($<10^2$ CFU) of a virulent strain as compared to higher doses ($>4.0 \times 10^3$ CFU) of an avirulent strain. Starks and others (2000) found that three clinical strains and three attenuated isolates of *V. vulnificus* from oysters or seawater caused identical skin lesions in subcutaneously inoculated iron dextran-treated mice; however, the inocula required for identical frequency and magnitude of infection were at least 350-fold higher for the environmental strains. The investigators' data suggested that the difference between these clinical and environmental strains might be related to their ability to grow in the host and/or susceptibility to host defenses. In addition, Starks and others (2000) reported that clinical and environmental strains of *V. vulnificus* required 10^5-fold higher inocula to cause an identical disease process in normal mice as compared to those treated with iron dextran. However, DePaola and others (2003), who evaluated strains of *V. vulnificus* obtained from market oysters and from oyster-associated primary septicemia cases, found that 88% of all the strains characterized were virulent when subcutaneously inoculated into iron dextran-treated mice, suggesting little strain-to-strain variability in the infection process when animals cannot appropriately metabolize iron. Recently, Choi and others (2006) conducted a study on the cyclic AMP-cAMP receptor protein (CRP) complex by creating a *crp* deletion mutant to study the role

this complex plays in *V. vulnificus* virulence. They found that *V. vulnificus* growth decreased under iron-limited conditions. The vulnibactin-mediated iron-uptake system was suppressed along with the transcription of the *vis* and *vuuA* genes, and growth was suppressed on transferrin-bound iron and in cirrhotic ascites. Furthermore, all the defects of the *crp* mutant were restored by in-trans complementation of the wild-type *crp* gene. These data suggest that the CRP complex plays an important role in iron utilization (Choi and others 2006).

Epidemiological evidence suggests that men are more susceptible to *V. vulnificus* infection than women. For instance, Shapiro and others (1998) reported that 86% of the reported cases of *V. vulnificus* infection occurred in men. Eighty-five percent of individuals who develop endotoxic shock from *V. vulnificus* are males (Oliver 1989; Merkel and others 2001). Although this may be due to the fact that men are more likely to consume raw oysters, or that men are more likely to have underlying liver disease, a recent study by Merkel and others (2001) offers an alternative explanation related to the protective effect of estrogen. In this study, the investigators showed that male rats injected with *V. vulnificus* lipopolysaccharide (LPS) had an 82% fatality rate whereas normal female rats treated identically had a fatality rate of only 21%. When these female rats were ovariectomized, thereby lowering their estrogen levels, fatality rates increased to 75% (Merkel and others 2001). When ovariectomized female mice were treated with subsequent estrogen replacement therapy, a decrease in mortality rates was observed, making the mortality rates of hormonally-treated ovariectomized females similar to those of the nonovariectomized female

mice (38% and 21%, respectively). Furthermore, gonadectomized male mice died at the same rate as nongonadectomized males. However, when gonadectomized male mice were treated with estrogen, a decrease in the mortality rate occurred (from 80% down to 50% mortality, respectively). Protection in these male mice increased with increasing estrogen dose. Taken together, the data of Merkel and others (2001) suggest that estrogen provides protection against *V. vulnificus* endotoxic shock.

1.5. CLASSIFICATION OF *V. PARAHAEMOLYTICUS* STRAINS

Historically, the species *V. parahaemolyticus* has been further classified based on serotype, which is discussed below. More recently, classifications have been made based on the presence of particular genes, some of which correlate with pathogenicity. For general species delineation, the thermolabile hemolysin (*tlh*) gene is used. *V. parahaemolyticus* strains are considered "pathogenic" if the thermostable direct hemolysin *(tdh*) and/or TDH-related hemolysin (*trh*) gene(s) are present. These genes, and their relationship to pathogenicity, are discussed in greater detail below.

1.6. PATHOGENICITY OF *V. PARAHAEMOLYTICUS*

Many virulence factors are thought to play a role in the pathogenicity of *V. parahaemolyticus*, including those associated with beta-hemolysis, adherence factors, various enzymes, and the products of the *tdh, trh,* and *ure* genes. Historically, *V. parahaemolyticus*

pathogenicity has been associated with the Kanagawa phenomenon (KP) which is observed as beta-hemolysis on Wagatsuma agar. Virtually all clinical isolates of *V. parahaemolyticus* are KP-positive, whereas only 1 to 2% of environmental strains are KP-positive (Sakazaki and others 1968; Miyamoto and others 1969; Nishibuchi and Kaper 1995). It is now known that the Kanagawa reaction is caused by the thermostable direct hemolysin (TDH) protein (Nishibuchi and Kaper 1995), so named because it is not inactivated by heat (100 ^{o}C for 10 min) and because its hemolytic activity is not enhanced by the addition of lecithin, suggesting direct activity on erythrocyctes (Sakurai and others 1973; Nishibuchi and Kaper 1995). Kaper and others (1984) were the first to clone the gene encoding the TDH protein (designated *tdh* 1) from *V. parahaemolyticus* strain WP1, which was clinical in origin. This group subsequently used probes derived from this gene to identify *tdh* genes in other *V. parahaemolyticus* strains. Later, Hida and Yamamoto (1990) found that *V. parahaemolyticus* strain WP1 actually contained a second and distinct *tdh* gene, designated *tdh* 2. A survey conducted by Nishibuchi and Kaper (1990) showed that all KP-positive (clinical) *V. parahaemolyticus* strains do indeed contain two *tdh* genes, whereas *V. parahaemolyticus* strains that show weak hemolysis on Wagatsuma agar and are considered to be only KP-intermediate have only one *tdh* gene. When looking at KP-negative strains, most of which are of environmental origin, 16% of these strains contained one copy of the *tdh* gene, while the rest of the KP-negative strains did not have the *tdh* gene, suggesting that most KP-negative strains cannot produce the TDH protein (Nishibuchi and others 1985; Nishibuchi and Kaper

1995). Occasionally, isolates of other *Vibrio* spp., including *V. hollisae*, *V. cholerae* non- O1, and *V. mimicus,* have been found to carry the *tdh* gene (Nishibuchi and Kaper 1995).

Regardless of the importance of the Kanagawa factor and the TDH protein, KP-negative strains of *V. parahaemolyticus* have occasionally been associated with outbreaks of gastroenteritis. Honda and others (1987, 1988) reported that some KP-negative strains of *V. parahaemolyticus* associated with illness in humans produced a TDH-related hemolysin (designated TRH) which was similar but not identical to the TDH protein. The TRH protein was first found in O3:K6 strains. Furthermore, this new hemolysin, which was mostly associated with environmental *V. parahaemolyticus* isolates, was responsible for significant lethality in the mouse model when the animals were challenged by intraperitoneal injection (Sarkar and others 1987). The gene corresponding to this protein was designated *trh*. There is about a 69% similarity in nucleotide sequence when comparing the *trh* and *tdh* genes, suggesting that they evolved from a common ancestor (Honda and others 1988; Nishibuchi and others 1989). In addition, evidence exists that there are multiple forms of the *trh* gene among some *Vibrio spp.* which differ in nucleotide sequence and whose corresponding proteins differ in hemolytic activity, but which appear to be derived from a common ancestor (Kishishita and others 1992). Some clinical isolates were shown to contain both the *tdh* and *trh* genes; whereas most environmental isolates do not contain *tdh* or *trh* genes (Xu and others 1994). In a series of deletion mutation experiments, investigators deleting all or part of the *trh* gene observed that the hemolytic activity of the protein was lost; however, the

mutants were still somewhat active in cytotoxity assays and caused partial fluid accumulation in ligated rabbit small intestines (Ming and others 1994). These data suggest that virulence factors in addition to TRH and TDH are involved in the pathogenicity of *V. parahaemolyticus*. However, the CDC recently noted that *V. parahaemolyticus* strains lacking both the *tdh* and *trh* genes were associated with more severe cases of *V. parahaemolyticus* infection, many of which required hospitalization (Yu and others 2006).

Early work suggested that "adhesiveness" appears to play an important role in *V. parahaemolyticus* pathogenicity. Hackney and others (1980) found that all clinical and environmental strains of *V. parahaemolyticus* that they tested were capable of adhering to human fetal intestinal (HFI) cells, although the degree of adherence was variable. Strains isolated from patients were observed to have high adherence capability regardless of their Kanagawa reaction, whereas Kanagawa-negative strains isolated from seafood exhibited the weakest adherence. Yamamoto and Yokota (1989) reported that the ability of *V. parahaemolyticus* clinical isolates to adhere to human small intestinal mucosa correlated roughly with hemagglutinin levels in human or guinea pig erythrocytes.

Many enzymes are thought to play a role in the pathogenicity of *V. parahaemolyticus.* Baffone and others (2001) examined several enzymatic (lipase, gelatinase, and hemolysin), biological (adhesiveness, cytotoxicity, and enterotoxicity), and enteropathogenic activities of *V. parahaemolyticus* strains isolated from seawater, finding that virtually all strains tested had lipase and gelatinase activity, whereas only 10% were positive for hemolysin activity.

As many as 80% and 90% of the *V. parahaemolyticus* isolates screened had adhesive and cytotoxicity capabilities, respectively. Furthermore, 30% of the *V. parahaemolyticus* strains were pathogenic to white mice using the ileal loop assay, while 60% of strains were lethal to adult mice using the whole animal bioassay.

It has been suggested that urea hydrolysis may be used as a marker to predict potentially virulent strains of *V. parahaemolyticus*. Abbot and others (1989) first reported this phenomenon, finding that the urease-positive phenotype was associated with the O4:K12 serotype. Kaysner and others (1994) reported that *tdh*-positive isolates of clinical and environmental origin were also urease-positive, while Osawa and others (1996) reported that all clinical and environmental strains carrying the *trh* gene tested positive for urease. Iida and others (1997) found that the *ure* gene was responsible for urease production in *V. parahaemolyticus* and that the *ure* and *trh* genes were genetically linked, as demonstrated by restriction endonuclease digestion. A later study revealed close proximity of the *tdh*, *trh*, and *ure* genes on the chromosome of pathogenic (clinical) *V. parahaemolyticus* strains (Iida and others, 1998). These data suggest the presence of a pathogenicity island, which may have occurred as a consequence of gene transfer, because the GC content of the *tdh* and *trh* genes is considerably lower than the mean GC content of the genomic DNA of *V. parahaemolyticus*.

The means of transfer of this putative pathogenicity island has motivated recent research endeavors. One hypothesis is the role of filamentous phage in gene transfer. For

instance, Southern blot hybridization has demonstrated the integration of a filamentous phage genome into chromosomal DNA of *V. parahaemolyticus* (Chang and others 1998) and others have shown filamentous phage specifically associated with the pandemic *V. parahaemolyticus* strains (O3:K6, O4:K68, and O1:K untypeable) (Iida and others 2001; Chang and others 2002). Gene transfer by plasmid is another means by which *V. parahaemolyticus* could have obtained genes associated with pathogenicity. For instance, it is well documented that the *tdh* gene is found in many *Vibrio* species (Nishibuchi and others 1985; Honda and others 1986; Nishibuchi and others 1990; Nishibuchi and others 1996). Some investigators favor plasmid-mediated gene transfer between *V. parahaemolyticus* and *V. cholera* non-O1 *tdh* genes (Honda and others 1986; Baba and others 1991), while others do not (Nishibuchi and Kaper 1990; Nishibuchi and others 1985). Nonetheless, there is evidence that the *tdh* genes of many *Vibrio* species are flanked by insertion sequence-like elements (Baba and others 1991; Terai and others 1991), suggesting that the *tdh* genes may be derived from a common ancestral source and may be readily transposed within chromosomes. Lin and others (1993) reported that *V. parahaemolyticus* AQ3815 contains a *toxRS* operon, a regulatory gene that controls the expression of the *tdh* gene, similar to *V. cholerae*.

Recent sequencing efforts have aided in elucidation of the relationships between *Vibrio* species, which contain two circular chromosomes (Yamaichi and others 1999). Tagomori and others (2002) compared the genetic maps of KP-positive *V. parahaemolyticus*

strain KX-V237, *V. parahaemolyticus* AQ4673, and *V. cholerae* N16961, finding that the genomes of KX-V237 and AQ4673 were very similar. The large chromosomes of KX-V237 and *V. cholerae* N16961 were similar, although the small chromosomes were less so. Similarly, Makino and others (2003) found that, when comparing sequences associated with the *V. parahaemolyticus* genome to those of *V. cholerae*, there were apparently many rearrangements within and between the two chromosomes. The genes for the type III secretion system (TTSS) were identified in the genome of *V. parahaemolyticus*, but not in *V. cholerae.* The TTSS is a central virulence factor for diarrhea-causing bacteria such as *Shigella, Salmonella,* and enteropathogenic *Escherichia coli*. These data suggest that TTSS might be a mechanism associated with *V. parahaemolyticus* infection, one considerably different from the mechanism of disease caused by *V. cholerae*. In a recent study, Ono and others (2006) showed that *V. parahaemolyticus* RIMD2210633 contains two sets of the gene clusters (TTSS1 and TTSS2) that encode for the TTSS.

1.7. SEROVARS OF *V. PARAHAEMOLYTICUS*

Serotyping of *V. parahaeomolyticus* is done using antibodies specific to O (somatic) and K (capsular) antigens; all *V. parahaemolytiucs* strains share a common H (flagellar) antigen. To date, 12 O antigen types and over 70 K antigen types have been described, though many strains remain untypable (FDA BAM 2001). Furthermore, five of the K

antigens have been found to occur with either of two O group antigens, yielding 76 recognized serotypes (Table 1).

In 1996, a unique serovar (O3:K6) of *V. parahaemolyticus* abruptly appeared in Calcutta, India (Okuda and others 1997). A total of 134 strains of *V. parahaemolyticus* collected between 1994 and 1996 during active surveillance among hospitalized patients in Calcutta, India were classified as serovar O3:K6. The so-called Calcutta O3:K6 strain was very different from other O3:K6 strains isolated from Asian travelers between 1982 and 1993; however, the Calcutta O3:K6 strain was indistinguishable from other O3:K6 isolates obtained between 1995 and 1996 from Southeast Asian countries. This suggested that a unique O3:K6 clone may have become prevalent worldwide in the late 1990s (Okuda and others 1997; Bag and others 1999). In addition to the appearance of this new O3:K6 serovar, strains of serovars O4:K68 and O1:K untypeable (KUT) have been associated with an increased incidence of *V. parahaemolyticus* infections worldwide. Furthermore, these strains (serovars newly emerged O3:K6, O4:K68, and O1:K untypeable) appear to be highly similar by restriction fragment length polymorphism-pulsed field gel electrophoresis (RFLP-PFGE) and arbitrarily primed polymerase chain reaction (AP-PCR) (Okura and others 2003). After the appearance of these pandemic strains in India, they spread to many Asian countries. In Vietnam, from 1997 to 1999, 49% of 523 *V. parahaemolyticus* strains isolated from hospitalized patients were pandemic strains. During this survey, there was an obvious transition of prevalence between the pandemic strains, with O3:K6, O4:K68, and O1:K25

serotypes being more prevalent during 1997, 1998, and 1999, respectively (Chowdhury and others 2004). From 1998 to 2000 in Bangladesh and Thailand, 66 strains of *V. parahaemolyticus* were isolated from patients and 14 different serotypes were identified (Bhuiyan and others 2002). Taiwan observed an increase in foodborne outbreaks during 1996 to 1999 with the new *V. parahaemolyticus* serovar O3:K6 accounting for 50.1-83.8% of annual *V. parahaemolyticus* infections (Chiou and others 2000). Wong and others (2000) compared O3:K6 strains from India, Japan, Taiwan, and Korea by RFLP-PFGE and found 13 different patterns. Cluster analysis revealed 2 distinct cluster groups; one group contained all strains isolated before 1996 and a second group consisted of strains isolated after 1996. This was the first report that demonstrated that the new O3:K6 strains from Korea, Taiwan, Japan, and India were genetically related. Chowdhury and others (2000) showed that some strains with serotypes O4:K68 and O1:KUT have RFLP-PFGE patterns similar to the pandemic O3:K6 strains, suggesting that they may have originated from the Calcutta O3:K6 pandemic strain. In a recent review, Nair and others (2007) postulated that other serotypes with identical genotypes and molecular profiles to those of O3:K6 emerged from a single O3:K6 serotype. These were collectively referred to as "serovariants" of O3:K6. These serovariants appeared to have diverged from the O3:K6 isolates by alteration of the O and K antigens, and they constitute what are now considered as pandemic O3:K6 strains.

During 1998 there were *V. parahaemolyticus* outbreaks in the United States associated with pandemic O3:K6 strains (DePaola and others 1998). Matsumoto and others

(2000) compared pandemic O3:K6 strains from North America and Asia using molecular methods and demonstrated that the North American strains were indistinguishable from the Asian strains. This was a significant finding as never before had *V. parahaemolyticus* been considered pandemic (Matsumoto and others 2000). The pandemic O3:K6 serotype was implicated in two outbreaks in Chile in 1998 and 2004 (Gonzalez-Escalona and others 2005). Quilici and others (2005) reported the presence of the pandemic *V. parahaemolyticus* O3:K6 serovar in France and, during this same time frame, Martinez-Urtaza and others (2005) described a pandemic O3:K6 outbreak in Spain, suggesting that this serovar had spread to Europe. Ansaruzzaman and others (2005) reported the first appearance of the pandemic serovars of *V. parahaemolyticus* in sub-Saharan Africa, with 42 cases of *V. parahaemolyticus* in Beira, Mozambique, from February to May 2004. Of the 42 isolates, 32 belonged to the O3:K6 serotype, two belonged to the O4:K68 serotype, and the remaining 8 isolates did not belong to any of the known pandemic serovars. In 2005, Fuenzalida and others (2006) described the largest *V. parahaemolyticus* outbreak ever reported (about 11,000 cases),caused by the pandemic O3:K6 strains and associated with Chilean shellfish consumption. However, analysis of shellfish isolates showed only 3/50 samples positive for *V. parahaemolyticus* contained detectable levels of pandemic O3:K6 strains. Nonpathogenic *V. parahaemolyticus* was isolated from the majority of samples and was separated into 14 distinct groups by direct genome restriction enzyme analysis (DGREA); these were clearly distinguishable from the pandemic clone.

1.8. EPIDEMIOLOGY

There are three major clinical manifestations of *Vibrio* infection: wound infection, primary septicemia, and gastroenteritis. Although both *V. vulnificus* and *V. parahaemolyticus* cases occur sporadically, the former are almost always sporadic while the latter can also occur in outbreak settings. Desenclos and others (1991) used the case control study design to estimate the annual incidence of all *Vibrio* infection at 95.4 per million for raw oyster consumers with liver disease, 9.2 per million for raw oyster consumers without liver disease, and 2.2 per million for those who do not consume raw oysters. Another case control study conducted by Hlady and Klontz (1996) reported disease manifestation proportions of 51%, 24%, and 17% for gastroenteritis, wound infection, and septicemia, respectively. Fatality rates were only 1% for gastroenteritis, but were 5% for wound infection and 44% for septic disease. Sixty-eight percent of gastroenteritis and 83% of primary septicemia cases were associated with raw oyster consumption. Ninety-one percent of the primary septicemia cases and 86% of the wound infections occurred in April through October, with 48% of those with primary septicemia reporting pre-existing liver disease (Hlady and Klontz 1996). Possibly, as a consequence of recent climate events such as El nino, which caused the water temperatures to be warmer than normal, about 20% of all *V. vulnificus* primary septicemia cases since 2000 have occurred in November (M.Glatzer, personal communication, 2006).

1.8.1 Gastroenteritis

When *V. vulnificus* and *V. parahaemolyticus* are isolated from stool alone, they are characterized as causing gastroenteritis (Strom and Paranjpye 2000). Gastroenteritis caused by *V. vulnificus* and *V. parahaemolyticus* may go unreported since the disease is not usually life-threatening and symptoms are typically not severe enough to warrant medical attention. In a study conducted by Hlady and Klontz (1996), *V. parahaemolyticus*, *V. cholerae*, *V. hollisae*, *V. mimicus*, and *V. fluvialis*, as well as *V. vulnificus,* were all associated with the gastrointestinal disease syndrome. *V. parahaemolyticus* is the vibrio most often associated with gastroenteritis. In fact, *V. parahaemolyticus* seafood-borne gastroenteritis is the leading cause of foodborne disease outbreaks in Taiwan and Japan (Pan and others 1997). Chiou and others (2000) reported that 542 out of 850 outbreaks in Taiwan between 1995 and 1999 were caused by *V. parahaemolyticus*; with 40 serovars (primarily O3:K6) represented. Su and others (2005) reported that, during 1995 to 2001, there were 2,057 cases of *V. parahaemolyticus* in northern Taiwan; the majority (99.4%) of *V. parahaemolyticus* strains could be identified by K serotyping, with 55.2% representing the K6 serovar.

1.8.2 Gastroenteritis oubreaks caused by *V. parahamolyticus*

Historically, *V. parahaemolyticus* has been associated primarily with sporadic disease in the U.S. however large gastroenteritis outbreaks have occurred. Early on, post-cooking contamination of crustaceans was associated with outbreaks. In the late 1990's, there was a

shift toward links to the consumption of raw oysters. In a 1981 outbreak in Washington and Oregon, raw oysters from Willipa Bay, WA were implicated. All 5 isolates obtained from the feces of individuals showing gastrointestinal symptoms hydrolyzed urea, were KP-positive, and belonged to serotype O4:K12 (Nolan and others 1984). In 1988, *Vibrio* surveillance began in four Gulf Coast states (Alabama, Florida, Louisiana, and Texas) and by the end of that year, 34 *V. parahaemolyticus* cases had been reported with one case of septicemia, 26 cases of gastroenteritis, and 6 wound infections (Levine and others 1993). Between 1988 and 1997, a total of 345 cases of *V. parahaemolyticus* infection were reported to the CDC by the Gulf Coast *Vibrio* Surveillance System. Of these cases, 59% were gastroenteritis, 34% were wound infections, and 5% were septicemia (Daniels and others 2000).

In 1997, a culture-confirmed outbreak of *V. parahaemolyticus* occurred in North America and resulted in 209 cases, all attributable to the consumption of oysters harvested from coastal waters of California, Oregon, Washington, and British Columbia. Many different serotypes were isolated from patients, some of which matched those identified from oyster samples (CDC 1998). The following year, another multi-state outbreak associated with the consumption of raw oysters harvested from the Galveston Bay, TX occurred. In this case, *V. parahaemolyticus* infections were reported in 296 Texas residents and 120 individuals from 12 other states. Subsets of the clinical isolates collected were all identified as the *V. parahaemolyticus* pandemic serotype O3:K6, which contained the *tdh* gene.

Although none of the oyster isolates had RFLP-PFGE patterns matching the clinical strains, the RFLP-PFGE patterns of the Galveston Bay and the Asian *V. parahaemolyticus* pandemic O3:K6 strains were shown to be distinct but closely related (Matsumoto and others 2000). Consumption of shellfish or crustaceans harvested from Long Island, NY waters were implicated in another *V. parahaemolyticus* outbreak in 1998. In this case, 12 *V. parahaemolyticus* clinical isolates were identified as the pandemic O3:K6 serotype (CDC 1999). In 2006, another *V. parahaemolyticus* outbreak occurred in New York, Oregon, and Washington, with a total of 177 cases of which 72 were confirmed. In this outbreak, the strains implicated were not of the pandemic serotype. Contaminated oysters and clams harvested from Washington and British Columbia sites were linked in the traceback investigation (CDC 2006).

Following the outbreaks in Washington, Texas, and New York in 1997 and 1998, DePaola and others (2000) tested shellfish from the same location as the outbreaks for total *V. parahaemolyticus* and pathogenic (*tdh* and/or *trh*) strains. These investigators recovered *V. parahaemolyticus* in 77% of the Pacific Northwest oyster samples tested, with pathogenic strains detected at densities of <10 MPN/g in only 15% of the 1997 samples, and no pathogenic strains detected in the 1998 samples. However, all Texas oyster samples tested positive for *V. parahaemolyticus*, most with densities ranging between 100 and 1,000 MPN/g; one sample had a density of 23,000 MPN/g. Only two samples tested positive for pathogenic strains. New York samples had total *V. parahaemolyticus* densities ranging from

<10 to 120 MPN/g but no samples tested positive for pathogenic strains. These data show that the levels of *V. parahaemolyticus* vary widely in different harvesting locations, and that the proportion of pathogenic strains is generally quite small and they are frequently nondetectable.

In July, 2004, an outbreak of *V. parahaemolyticus* occurred in Alaska; 62 people were reported as having gastroenteritis associated with consumption of raw oysters harvested from Alaskan waters and served during a cruise. Nine stool samples were confirmed as positive for *V. parahaemolyticus* and 8 isolates were sent to the CDC for typing; *V. parahaemolyticus* O6:K18 was identified as 7 of the 8 clinical isolates. All oyster samples taken from an implicated cruise ship were positive for *tdh*, and 4 different serotypes (O6:K18, O1:K9, O5:K17, and O10:K68) were represented. Ninety-six oyster samples were taken from Alaskan farms and 31 samples were positive for *V. parahaemolyticus*. All samples positive for *V. parahaemolyticus* came from farms in the Prince William Sound and southeastern Alaska. In this case, 11 serotypes were identified, but all O6:K18 isolates came from a single farm. The RFLP-PFGE patterns obtained for the clinical and oyster isolates were highly related. The RFLP-PFGE pattern of O6:K18 isolates observed in this outbreak was similar to that of the O6:K18 isolates found in Pudget Sound, suggesting possible spread of these strains by such routes as discharge of ballast water, migration of marine animals, or sea birds. Interestingly, all oysters were harvested when the mean daily water temperature was 15^{o}C or

greater; previously, it was thought that Alaskan waters were too cold to harbor *V. parahaemolyticus* (McLaughlin and others 2005).

1.8.3 Wound infections

V. vulnificus and *V. parahaemolyticus* are most often associated with wound infections, although Hlady and Klontz (1996) reported that other *Vibrio* species can occasionally be responsible for this disease syndrome. Wound infections are defined as those cases where a patient incurred a wound before or during exposure to seawater, seafood drippings, or punctures from fish spines or bones, and from which *V. vulnificus* or *V. parahaemolyticus* was subsequently cultured from that wound, blood, or an otherwise normally sterile site (Strom and Paranjpye 2000). The majority of wound infections, whether caused by *V. vulnificus* or *V. parahaemolyticus*, occur in fisherman and seafood processors. In a study conducted by Strom and Paranjpye (2000), 69% of wound infections appeared to be related to occupational exposures among oyster shuckers and commercial fishermen.

1.8.4 Atypical infections

There have been some atypical infections caused by *Vibrio* spp. reported in the literature. Vartain and Septimus (1990) were the first to describe osteomyelitis caused by *V. vulnificus* in a person who scraped his leg on a rock in brackish water. The patient initially developed a wound infection; 13 weeks later the bone was infected. An ocular infection

caused by *V. vulnificus, Plesiomonas shigelloides,* and *Shewanella putrefaciens* occurred when a fisherman was struck in the eye with a fishhook (Butt and others 1997). Johnson and Arnett (2000) reported a case of septic arthritis in a patient who consumed oysters the day before onset and *V. vulnificus* was isolated from blood and synovial fluid around an arthritic wrist. A fatal case of *V. vulnificus*-associated meningoencephalitis occurred in a patient who consumed raw fish and had a history of chronic liver disease (Kim and others 2003). Penland and others (2000) reported 17 cases of trauma-associated ocular infections, including 7 caused by *V. vulnificus,* 5 by *V. alginolyticus*, 3 by *V. parahaemolyticus,* and one each by *V. albensis* and *V. fluvialis*. The first case of a *V. vulnificus* ocular infection not associated with trauma was recently reported in Korea and linked to raw fish consumption (Jung and others 2005).

1.8.5 Primary septicemia caused by *V. vulnificus*

Primarly septicemia caused by *V. vulnificus* is usually associated with the consumption of raw shellfish and is defined as a systemic illness characterized by fever and shock and in which *V. vulnificus* is isolated from blood or an otherwise sterile site (Strom and Paranjpye 2000). Although most often caused by *V. vulnificus*, Hlady and Klontz (1996) showed that *V. cholerae* non-O1 and *V. parahaemolyticus* can cause septic disease. Fortunately, these infections are relatively rare and, on average, there are 32 *V. vulnificus* culture-confirmed primary septicemia cases reported to CDC annually, with nearly all of

these associated with the consumption of oysters harvested from the Gulf of Mexico**.** Because this does not include cases for which there is no food history, the CDC estimates approximately 100 primary septicemia cases per year in the U.S (Mead and others 1999). The Korean CDC estimates 40 to 70 confirmed cases annually in that country (Korea Center for Disease Control and Prevention 2004). This apparent higher incidence of *V. vulnificus* infections in Korea may be the result of greater exposure due to high consumption of raw seafood or a higher prevalence of predisposing factors. It is well recognized that there are specific risk factors for the development of *V. vulnificus* sepsis (Hlady and Klontz 1996). Not only is raw oyster consumption a risk factor, but underlying liver diseases, including cirrhosis, damage to the liver due to alcoholism, and chronic hepatitis, are strong predictors for fatal outcomes of *V. vulnificus* sepsis, with 80% of those who die from the infection falling into these risk groups (Shapiro and others 1998; Strom and Paranjpye 2000).

1.9. CULTURAL-BASED METHODS OF ISOLATION AND DETECTION

Multiple methods are recommended for the detection and/or enumeration of *Vibrio* species. The FDA Bacteriological Analytical Manual (BAM) (2001) cites standard procedures for the recovery of *V. vulnificus* and V. *parahaemolyticus* from raw molluscan shellfish. For enumeration, most probable number (MPN) analysis (Figure 1.1) or direct plating on non-selective media followed by DNA colony hybridization are the two techniques most frequently used (Figure 1.2). Briefly, MPN analysis for the enumeration of

either organism is done by 10-fold serial dilution of shellfish samples in phosphate-buffered saline (PBS), followed by inoculation of dilutions in alkaline peptone water (APW), typically in triplicate. APW is incubated at 35-37 ^{o}C for 18-24 h and tubes positive for growth are streaked onto modified cellobiose-polymyxin B-colistin (mCPC) agar (for isolation of *V. vulnificus*) and/or thiosulfate-citrate-bile salts-sucrose (TCBS) agar (for isolation of *V. parahaemolyticus*). The mCPC and TCBS plates are incubated for 18-24 h at 39-40°C and 35-37°C, respectively, followed by examination for typical colonies. For biochemical identification, three or more typical colonies from each agar type are subjected to oxidase, arginine-glucose slant (AGS), ornithine decarboxylase, O/129 Vibriostat sensitivity, and the ONPG tests (Table 1.2). Alternatively, biochemical profiles can be obtained using API 20E (bioMerieux Inc., Hazelwood, MO) strips. As an alternative to biochemical identification, the FDA BAM suggests the use of species-specific alkaline phosphatase-labeled DNA probes (Figure 1.2) or PCR (Figure 1.3). Probes targeting the cytolysin gene (*vvhA*) are used for the identification of *V. vulnificus*, while those targeting sequences for the thermolabile hemolysin gene (*tlh*) can be used to identify *V. parahaemolyticus*. Identification of the "virulent" *V. parahaemolyticus* strains can be done by hybridization or PCR targeting the *tdh* (BAM 2001) and/or *trh* genes (Nordstrom and others 2006).

BAM methods are recommended for official analysis but may not reflect the latest technology or optimal methodology for detection of *V. vulnificus* and *V. parahaemolyticus* in naturally contaminated shellfish. Investigators have compared a variety of methodological

alternatives (Alam and others, 2001), including different dilution and enrichment buffers (Azanza and others, 1996; Hagan and others, 1994) and plating media (Oliver, 1981; Hoi and others, 1998; Oliver and others 1992; Cerda-Cuellar and others, 2000). Direct plating remains difficult because of the large amount of natural microflora which may also grow on selective media. Micelli and others (1993) developed an alternative method for direct plaing of *V. vulnificus* from oyster homogenates. Using their so-called *V. vulnificus* enumeration (VVE) medium which contained Oxgall, sodium cholate, sodium taurocholate, and potassium tellurite, they reported reduction of 61-99% of marine-associated background microflora without adversely affecting the recovery of *V. vulnificus*. Detection limits were as few as 10 culturable *V. vulnificus* cells in 100 g of shellfish and compared favorably to MPN enrichment approaches with a shorter time to result. Recently, a chromogenic medium (Bio-Chrome Vibrio medium, BCVM, BioMedix, Pomona, CA) was developed to differentiate *V. parahaemolyticus* from other *Vibrio* species (Hara-Kudo and others 2001) and its efficacy has since been validated (Duan and Su, 2005; Su and others, 2005). .

1.10. MOLECULAR-BASED DETECTION METHODS

1.10.1 DNA Hybridization

Molecular-based methods, which rely on detection of specific gene targets by a variety of methods, have aided in the rapid identification and discrimination of *Vibrio* species

from one another. See Tables 1.2 and 1.3 for details about gene targets and primers/probes for detection. Nishibuchi and others (1985) were the first to report a specific DNA probe for the detection of *V. parahaemolyticus*, which targeted the *tdh* gene but cross-reacted with some KP-negative strains. Soon thereafter, Nishibuchi and others (1986) evaluated four synthetic oligodeoxyribonucleotide probes corresponding to different regions of the *tdh* gene and demonstrated that under stringent hybridization conditions, two of the probes were capable of distinguishing KP-positive from negative or weakly positive strains. Lee and others (1992) developed a different oligonucleotide probe targeting the *tdh* gene and found that this probe identified 89 of 95 *V. parahaemolyticus* isolates. McCarthy and others (1999) reported that an alkaline phosphatase-labeled probe targeting the thermolabile hemolysin (*tlh*) gene correctly identified all 124 strains vibrio strains tested. Gooch and others (2001) used alkaline phosphatase (AP)-labeled *tlh* and digoxigenin-labeled *tlh* probes for DNA probe colony hybridization to enumerate *V. parahaemolyticus* after direct plating onto T_1N_3 (1% tryptone, 3% NaCl, 2% agar) medium, finding similar results to those obtained using the BAM MPN method. At low *V. parahaemolyticus* densities, the MPN method was more sensitive (3 MPN/g for a 0.1 g) than direct plating methods (10 CFU/g for a 0.1g sample). Nordstrom and DePaola (2003) reported that spread-plating on T_1N_3 after APW enrichment followed by colony hybridization using AP-labeled *tdh* probes was superior for the recovery of pathogenic *V. parahaemolyticus* when compared to a more conventional streak plate method. Ellison and others (2001) used the BAM-MPN and a direct plating procedure

followed by DNA probe colony hybridization using an AP-labeled *tlh* probe (direct-VPAP) to determine *V. parahaemolyticus* levels in retail oysters from Florida. Although the correlation between methods was good, the direct-VPAP method was more rapid and precise.

Wright and others (1993) developed an AP-labeled DNA probe (VVAP) targeting the cytolysin (*vvh*A) gene of *V. vulnificus* which effectively differentiated the organism from other *Vibrio* species. DePaola and others (1997) applied VVAP for DNA colony hybridization following direct plating of Gulf of Mexico oysters onto *V. vulnificus* agar (VVA) and designated this method as direct-VVAP. The direct-VVAP and the BAM MPN methods were compared for enumeration of *V. vulnificus* levels in Gulf Coast oysters. The methods were in agreement >90% of the time and the direct-VVAP approach was more rapid and precise than BAM MPN, although it did have a higher limit of detection (DePaola and others 1997). Cerda-Cuellar and others (2000) developed a probe specific to the 16S rDNA gene of *V. vulnificus* and successfully used it to distinguish this organism from other species of the *Vibrio* genus. For enumeration of *V. vulnificus* and *V. parahaemolyticus* in water samples, a hydrophobic grid membrane filtration (HGMF) technique has been applied in conjunction with cultural (DePaola and others 1988) and molecular (Kaysner and others 1994) detection approaches. For example, Banerjee and others (2002) demonstrated that enumeration of *V. parahaemolyticus* and *V. vulnificus* from water samples could be achieved in one day by DNA probe colony hybridization of HGMF colony lifts using digoxigenin-

labeled probes specific for *tlh* and *vvh*A genes of *V. parahaemolyticus* and *V. vulnificus*, respectively.

1.10.2 Polymerase Chain Reaction (PCR)

Conventional PCR and real-time PCR have also been used to identify *V. parahaemolyticus* (Table 1.3) and *V. vulnificus* (Table 1.4). Brauns and others (1991) detected culturable and nonculturable *V. vulnificus* by PCR amplification using primers flanking the cytotoxin-hemolysin (*vvhA*) gene. In this case, as little as 72 pg and 31 ng of DNA from culturable cells and nonculturable cells, respectively, could be detected. Lee and others (1995) developed a species-specific PCR assay to differentiate *V. parahaemolyticus* from *V. alginolyticus* using a DNA region (pR72H) that is present in *V. parahaemolyticus* and absent in *V. alginolyticus.* The sensitivity of the PCR was approximately 1 CFU using purified chromosomal DNA in the amplification reactions, with a high degree of specificity. Karunasagar and others (1996) developed a PCR assay targeting the *tdh* gene, reporting detection limits $>10^4$ CFU/g of *V. parahaemolyticus* when applied to lysates prepared directly from fish homogenates. Improved detection sensitivity (<10 CFU/mL) was obtained by performing PCR after an 8 -h enrichment in APW. Dileep and others (2003) compared conventional cultural methods and PCR targeting the *toxR* gene for the detection of *V. parahaemolyticus* in various seafood products; these investigators found that PCR performed better if it was preceded by a 6-h culture enrichment.

A number of multiplex PCR assays have been developed for detection of the pathogenic vibrios. Brasher and others (1998) designed a multiplex PCR assay to simultaneously detect *V. vulnificus, V. cholerae,* and *V. parahaemolyticus* (species-specific) based on amplification of regions corresponding to gene targets *vvhA, ctx*, and *tlh*, respectively. When applied to artificially inoculated oyster homogenates, these investigators were able to detect $<10^1$-10^2 CFU/g after a 6-h enrichment. Wang and others (1997) developed a PCR method able to detect 13 different foodborne pathogens, including *V. cholerae* (*ctx*), *V. parahaemolyticus* (pR72H fragment), and *V. vulnificus* (*vvhA*), with detection limits of 40, 4, and 100 cells per reaction, respectively. Bej and others (1999) designed a multiplex PCR assay to detect total and pathogenic strains of *V. parahaemolyticus* using *tlh, tdh,* and *trh* genes as targets. This assay gave the expected reactions on 111 isolates of *V. parahaemolyticus* and the investigators found that, in a few cases, the presence of the *tdh* gene was not associated with the Kanagawa phenomenon. The investigators reported that the detection limit for all 3 genes was between 10^1- 10^2 CFU per 10 g when the assay was applied to seeded oysters that were pre-enriched for 6-h (Bej and others 1999).

The open reading frame (ORF8), derived from a filamentous phage (f237), has been exclusively associated with pandemic *V. parahaemolyticus* strains (Nasu and others, 2000). The ORF8 sequence is distinct from other sequences in the database, but the phage itself is similar to the CTX phage that carries the genes that encode for cholera enterotoxin (*ctx*AB), an important virulence marker of *V. cholerae* (Waldor and others 1996). Interestingly, the

ORF8 sequence was only detected by colony hybridization using a digoxigenin-labeled DNA probe in pandemic O3:K6 strains isolated after 1996 (Nasu and others 2000). Iida and others (2001) used the same method to evaluate 96 *V. parahaemolyticus* strains and found 53 isolates positive for the ORF8 sequence. These 53 isolates were represented by the O3:K6, O4:K68, and O1:KUT pandemic strains, but not in nonpandemic strains of any other serovar. Although these two studies used hybridization rather than PCR, Myers and others (2003) later develeloped a PCR assay targeting ORF8 specifically for the detection of the pandemic *V. parahaemolyticus* O3:K6 clone. The specificity of this PCR assay was confirmed only DNA from pathogenic *V. parahaemolyticus* O3:K6 pandemic isolates (after 1996) could be amplified, while the primers did not amplify the older (prior to 1996), non-O3:K6 *V. parahaemolyticus* strains, other *Vibrio* spp., or any other non-*Vibrio* spp. screened. Myers and others (2003) detected 10^3 CFU pandemic *V. parahaemolyticus* O3:K6 /100 mL of seeded Gulf waters. At about the same time, Yeung and others (2003) used oligonucleotide primers for ORF8 with conventional PCR and correctly identified 39 *V. parahaemolyticus* pandemic isolates out of 78 total *V. parahaemolyticus* isolates, all of which contained the *tlh* gene.

Sequences corresponding to the *tox*RS operon have also been used as the target for PCR assays to identify pandemic *V. parahaemolyticus* (Table 3). The *tox*R is a regulatory gene of toxigenic *V. cholerae* (Miller and others 1987), but Lin and others (1993) found a *tox*R gene in *V. parahaemolyticus*. This gene had homology to the *tox*R of *V. cholerae*, which appears to promote the expression of the *tdh*2 gene and, to a lesser extent, the *tdh*1

gene. Matsumoto and others (2000) found that pandemic strains of *V. parahaemolyticus* have sequence substitutions at 7 base positions within the *tox*RS operon (*tox*RS/*new*). They developed a PCR method that targeted two of the base positions unique to the pandemic O3:K6 strain, resulting in an assay capable of differentiating the pandemic clone, including divergent serotypes from the old O3:K6 strains, from other nonpandemic strains. Okura and others (2004) developed a PCR assay to identify the pandemic group of *V. parahaemolyticus* using a marker derived from the group-specific sequence of an arbitrarily primed-PCR fragment that encodes for a "hypothetical protein". These PCR assays identified only the pandemic strains and further differentiated 82 *V. parahaemolyticus* strains (38 pandemic and 44 nonpandemic).

The performance of PCR assays based on ORF8 and *tox*RS/*new* sequences for differentiating pandemic *V. parahaemolyticus* strains has been examined by Osawa and others (2002). The investigators found that the ORF8 assay detected only the pandemic clone, while the *tox*RS/*new* assay detected all pandemic clone isolates and 4 strains isolated between 1982-1988, the latter of which were also untypeable by RFLP-PFGE. However, Bhuiyan and others (2002) disputed these findings when they reported that the ORF8 assay failed to identify 8 pandemic O3:K6 strains and one O4:K68 strain. Okura and others (2003) found *tox*RS/*new* sequences in four O3:K6 strains that did not contain *tdh* and ORF8 absent in 3 pandemic O3:K6 strains, while Chowdhury and others (2004) found ORF8 missing in 10% of the pandemic strains they tested. These studies indicate that neither *tox*RS nor ORF8

can be relied upon exclusively to differentiate pandemic *V. parahaemolyticus* from nonpandemic strains.

1.10.3 Real-Time PCR

Real time-PCR allows for the confirmation of amplicon identity while the amplification reaction is progressing, thereby by-passing time-consuming electrophoresis and hybridization methods. The method is considered quantitative by some, although when applied to detection of pathogens in food samples, this has yet to be realized. Real time-PCR has recently been applied to the detection and identification of *Vibrio parahaemolyticus* (Table 1.3). Blackstone and others (2003) were the first to report such an assay when they developed a method to detect pathogenic *V. parahaemolyticus* by targeting the *tdh* gene in a TaqMan format. When applied to enrichments of naturally contaminated oysters, the real-time PCR method was significantly more sensitive when compared to a streak plate/probe method. In addition, the real-time assay was faster and less resource-intensive. Davis and others (2004) developed a TaqMan multiplex real time-PCR method targeting the *tlh, tdh,* and *trh* genes of *V. parahaemolyticus* using Taqman probes with different labels. This assay was used to identify *V. parahaemolyticus* as the etiological agent in a foodborne disease outbreak associated with consumption of contaminated mussels. Kaufman and others (2004) found a strong correlation between cycle threshold and log concentration when using a real-time TaqMan PCR method targeting the *tlh* gene to detect *V. parahaemolyticus* in oyster

mantle fluid. Recently, a Taqman real-time PCR assay targeting the *toxR* gene was developed to quantify total *V. parahaemolyticus* in shellfish and seawater (Takahashi and others 2005). These investigators found the method to be specific for *V. parahaemolyticus* and reported a correlation between cycle threshold and $\log_{10}$ of *V. parahaemolyticus* cell number. This real-time PCR method was compared to the MPN cultural method for detection of *V. parahaemolyticus* in blue mussel and short-neck clams; 3 of the 10 samples which contained < 5 MPN/g by the cultural method were not detected by PCR, while 5 of the 10 samples gave similar results with both methods (Takahashi and others 2005). Cai and others (2006) developed a Taqman real-time PCR method targeting the *gyr*B gene, which is well-conserved in *V. parahaemolyticus* and has a single gene copy. The method had a detection limit of 1 CFU per PCR reaction when applied to pure culture and 6-8 CFU per PCR reaction in spiked raw oyster. The method was used to evaluate 300 seafood samples and 97 were PCR-positive for *V. parahaemolyticus*; only 78 samples were positive using a conventional culture method. Ward and Bej (2006) developed a TaqMan multiplex real-time PCR method targeting the *tlh,* ORF8, *tdh,* and *trh* genes of *V. parahaemolyticus* for identification of the organism in shellfish. This method identified total and pathogenic *V. parahaemolyticus* with detection limits of 1 CFU/g of oyster after overnight enrichment (16 h).

Real–time PCR has also been used for identification of *V. vulnificus* (Table 1.4). Campbell and Wright (2003) developed a TaqMan real-time PCR assay targeting the cytolysin gene (*vvhA*) of *V. vulnificus* and found this method to be specific after examination

of 28 *V. vulnificus* strains and 22 non-*V. vulnificus* strains; the detection limit was 72 fg/µL of genomic DNA. When compared to the colony lift hybridization using the VVAP gene probe, the two methods correlated well and had similar sensitivity (Campbell and Wright 2003). Panicker and others (2004) developed a SYBR Green-based real-time PCR method targeting the hemolysin (*vvh*) gene of *V. vulnificus* and applied it to the detection of the organism in shellfish and Gulf waters. They reported no cross-reactivity with other *Vibrio* and non-*Vibrio* bacterial strains. The minimum detection limit of the assay was 10^2 CFU *V. vulnificus*/g of oyster tissue homogenate, or 10^2 CFU/10 mL water, as applied to samples without prior cultural enrichment. Improved detection limits (1 CFU/g) were obtained when samples were enriched for 5 h. The entire method took only 8 h, including sample processing, enrichment, and real-time PCR. Panicker and Bej (2005) compared three sets of oligonucleotide primers for detection of the *V. vulnificus vvhA* gene in the TaqMan real-time PCR format. Two of the 3 primer sets (set 1: F-vvh785/R-vvh990 and set 2: F-vvh731/R-vvh1113 primers with P-vvh874) were specific for *V. vulnificus*. Detection limits of 1 pg/µL of purified DNA, 10^3 CFU/mL of pure culture, and 1 CFU/g of oyster (after a 5-h enrichment) were achieved. Recently, Wang and Levin (2006) reported a Taqman real-time PCR assay that discriminated between viable and nonviable *V. vulnificus* cells using the DNA intercalating agent ethidium monoazide (EMA).

1.11. STRAIN TYPING METHODS

Many different methods have been applied to *V. parahaemolyticus* and *V. vulnificus* strain typing. In an early study, Tamplin and others (1996) reported a high degree of variation in RFLP-PFGE profiles of 53 clinical and 78 environmental isolates of *V. vulnificus*. Ryang and others (1999) reported similar genetic diversity using RFLP-PFGE to type clinical *V. vulnificus* strains in Korea. Both studies reported slightly less diversity for other typing methods such as ribotyping (Tamplin and others 1996) and random amplified polymorphic DNA (RAPD) analysis (Ryang and others 1999). RFLP-PFGE has been used to identify the vehicle of *V. vulnificus* infection and to study the relationship between patient isolates. Overall, infection appears to result from the proliferation of a single strain, although clinical strains from different patients are frequently unique (Jackson and others 1997). Warner and Oliver (1999) used RAPD analysis to differentiate various *Vibrio* species, finding a great degree of heterogeneity in banding patterns, even within a specific species (*V. vulnificus* in particular). Arias and others (1998) recommended RAPD PCR for the differentiation of phenotypically atypical *V. vulnificus* strains as a simpler and slightly less discriminatory method, while recommending ribotyping for finer discrimination between isolates. Others confirmed the great degree of diversity seen with RAPD PCR, noting little correlation between strain source and RAPD pattern (Lin and others 2003). The same can be said for arbitrarily-primed (AP)-PCR (Vickery and others 2000). It is also clear that biotype designations do not always correlate with phylogenies generated by molecular typing

methods (Gutacker and others 2003). A promising new *V. vulnificus* typing method, repetitive extragenic palidormic PCR (rep-PCR), has recently been reported (Chatzidaki-Livanis and others, 2006).

Much of the typing work for *V. parahaemolyticus* has been done by Wong and colleagues. These investigators examined 130 *V. parahaemolyticus* isolates from Taiwan by RFLP-PFGE, finding 14 RFLP-PFGE types and 39 patterns; domestic clinical isolates were clustered into 4 types and showed little similarity to foreign clinical strains and domestic environmental strains (Wong and others 1996). When they used RFLP-PFGE to group 315 *V. parahaemolyticus* isolates from contaminated seafood, 96 patterns and 22 types were obtained. There was little relationship between RFLP-PFGE type and strain origin (Wong and others 1999). Wong and others (2000) also used RFLP-PFGE to characterize Taiwanese clinical isolates, reporting 57 patterns grouped into 19 types, with 5 of these types containing 76% of the isolates and a clear and distinct type for the pandemic O3:K6 strains. The ability of RFLP-PFGE to differentiate between pandemic O3:K6 and non-O3:K6 isolates was confirmed by Yeung and others (2002) and Wong and others (2000). Marshall and others (1999) compared RFLP-PFGE, enterobacterial repetitive intergenic consensus sequence (ERIC) PCR, ribotyping, and RFLP-PFGE on patient and environmental isolates associated with a 1997 *V. parahaemolyticus* outbreak in Canada and found no single method to be superior. In general, ERIC PCR and ribotyping were less discriminatory, whereas RFLP-PFGE was extremely discriminatory. Likewise, Wong and others (2001) evaluated three

PCR-based *V. parahaemolyticus* typing methods, finding ribosomal gene spacer sequence (RS)-PCR a more practical method than ERIC PCR because it generated fewer bands and patterns. More recently, Hara-Kudo and others (2003) concluded that, based on RFLP-PFGE, TDH-negative isolates were rather distant from TDH-positive isolates, and that TDH-positive strains were closely related to one another, regardless of serovar. DePaola and others (2003) used ribotying and serotyping to characterize *V. parahaemolyticus* isolates derived from clinical, environmental, and food sources and found no relationship between serogroup and ribogroup. Certain serogroups and ribogroups contained both clinical and environmental isolates, while others just contained environmental isolates, implying that certain serotypes or ribotypes may be more relevant to human disease. Isolates from the Pacific Coast of North America appeared to be a distinct population from those found near the Gulf and Atlantic Coasts.

In recent outbreaks, a new method based on direct genome restriction enzyme analysis (DGREA) has been used to group *V. parahaemolyticus* isolates. The method involves digestion of bacterial DNA with a six-base restriction endonuclease that generates 30-40 fragments of sizes ranging from 500 to 2,500 bp in length. These are separated using polyacrylamide gel electrophoresis and banding patterns visualized by silver nitrate staining. Fuenzalida and others (2006) found that DGREA was able to discriminate different clones of *V. parahaemolyticus*, with cluster analysis identifying 16 different groups; only two groups

corresponded to the pandemic O3:K6 isolates. DGREA results had discriminatory power similar to that of RFLP-PFGE.

1.12. ECOLOGY

Vibrio species are ubiquitous in estuarine waters and can frequently be isolated in high numbers from bivalves, crustaceans, finfish, sediment, and plankton (Kelly 1982: Oliver and others 1982; Tamplin and others 1982; O'Neil and others 1992; DePaola and others 1994). In general, higher densities of the organisms are found in oyster digestive tissue (Tamplin and Capers 1992; DePaola and others 1997) as compared to muscle tissue. Considerable oyster to oyster variability in vibrio levels have been noted. For example, Kaufman and others (2003) observed occasional "hot" oysters containing *V. parahaemolyticus* levels >10-fold higher than those of oysters harvested at the same time and within a 1 m^2 proximity (Kaufman et al., 2003).

Both organisms have been isolated from U.S. waters as far north as the Great Bay of Maine (*V. vulnificus*) (O'Neil and others 1992), Alaska (*V. parahaemolyticus*) (McLaughin and others 2005) and Long Island, New York (*V. parahaemolyticus*) (Tepedino 1982). Lower densities of *V. vulnificus* and *V. parahaemolyticus* were isolated from Pacific, Canadian, and North Atlantic waters where the water temperatures were generally cooler year-round; higher densities were found in Mid-Atlantic, Chesapeake Bay, and Gulf of Mexico waters where the water temperatures were warmer year-round (Kaysner and others 1987; O'Neil and others

1992; DePaola and others 1994; Cook 1994; Wright and others 1996; Motes and others 1998).

1.12.1 Seasonal temperature and salinity

Both temperature and salinity play important and interrelated roles in the levels of *Vibrio* spp. Kelly and Stroh (1988) reported that *V. parahaemolyticus* was found in Pacific Northwest coastal waters only during the summer months, when water temperatures were above 17 ^{o}C and salinities were below 13 ppt. Further research by Kaspar and Tamplin (1993) demonstrated that at salinities between 5 and 25 ppt, *V. vulnificus* levels increased; however, when salinities were 30, 35, and 38 ppt, *V. vulnificus* levels decreased by 58, 88, and 83%, respectively. The same trend was reported by Motes and others (1998), who observed lower numbers of *V. vulnificus* at salinities above 28 ppt, which is typical of some Atlantic coastal sites in North and South Carolina. This high salinity may explain in part why *V. vulnificus* cannot be isolated routinely in oysters harvested from waters off these shores. High *V. vulnificus* levels, however, were found in oysters harvested from intermediate salinities between 5 and 25 ppt (Motes and others 1998).

Regardless of the role of salinity, temperature probably has the most important effect on the prevalence and levels of the pathogenic vibrios. The growth of *V. vulnificus* is favored

by relatively high temperatures and the organism has an optimum growth temperature of 37 °C (Kelly 1982). Kaspar and Tamplin (1993) reported that *V. vulnificus* grew in the temperature range of 13 °C to 22 °C. Wright and others (1996) were able to culture *V. vulnificus* from estuarine waters of the Chesapeake Bay collected at temperatures as low as 8 °C; however, *V. vulnificus* was not recovered at temperatures lower than 12.5 °C from Gulf of Mexico waters (Simonson and Siebeling 1986). In general, *V. vulnificus* is isolated infrequently from surface water samples from the Gulf of Mexico in January through March, when water temperatures are below 20 °C (Kelly, 1982). Peak recovery of *V. vulnificus* occurs in September, and there is substantial seasonal variation in prevalence and levels of the organism. Studies have demonstrated that during the summer months, *V. vulnificus* levels were similar (about 10^4 CFU/g) in oysters harvested from the Gulf of Mexico and Mid Atlantic states, but the levels were considerably higher in the Gulf of Mexico for other seasons (Cook and others 2002). Virulent strains of *V. vulnificus* have been found on the West Coast, although not as frequently or in as high numbers as from Gulf and Atlantic Coast waters (Kaysner and others 1987). Likewise, clams harvested from the Northeastern U.S. Coast and all U.S. West Coast waters had comparatively lower levels of *V. vulnificus* (Brenton and others 2001), perhaps due to the lower mean temperatures of these waters.

Seasonal and regional variation in the prevalence and levels of *V. parahaemolyticus* has also been noted. As is the case for *V. vulnificus*, the levels of *V. parahaemolyticus* in

Gulf Coast oysters also peaks during the summer, followed by a gradual reduction in the colder months of the year (Motes and others 1998). DePaola and others (1990) compared seasonal levels of *V. parahaemolyticus* in Pacific, Gulf, and Atlantic Coast waters and oyster samples. The data showed strong correlations between water temperature and *V. parahaemolyticus* levels. The Gulf Coast had the warmest mean water temperature (22 ^{o}C) and highest mean *V. parahaemolyticus* levels of 11,000 CFU/100 g (oysters) and 44 CFU/100 mL (water), while the Pacific coast water was the coldest (15 ^{o}C) and was associated with lower levels of *V. parahaemolyticus* (2,100 CFU/100 g for oysters and 2 CFU/100 mL for water). Kaufman and others (2003) reported total *V. parahaemolyticus* levels immediately after harvest during June, July, and September to range from 200 to 2,000 CFU/g in 90% of the oysters tested. Cook and others (2002) detected *V. parahaemolyticus* in 94.2% of shellfish taken from waters that were above 25 ^{o}C, but the organism was present in only 14.9% of shellfish samples harvested from waters that were below 10 ^{o}C. Gooch and others (2002) found that when water temperature at harvest was above 20 ^{o}C (April through December), the mean density of *V. parahaemolyticus* was 13,000 CFU/100 g, whereas, when water temperatures were below 20 ^{o}C (January through March), the mean density was approximately one $\log_{10}$ lower, at 1,500 CFU/100 g. DePaola and others (2003) reported similar seasonal trends in total *V. parahaemolyticus* for two sampling sites in Alabama. They also found that pathogenic *V. parahaemolyticus* (*tdh*+) strains constituted a higher percentage of the *V. parahaemolyticus* population when water temperatures and total *V.*

parahaemolyticus levels were lower. The levels of pathogenic strains (*tdh*-positive) ranged from 10 to 20 CFU/g in 40% of the oysters harvested during June and July but pathogenic strains were nondetectable in oysters harvested in September. However, after storage at 26 ^{o}C for 24 h, pathogenic *V. parahaemolyticus* was detected at levels of >100 CFU/g in some oysters collected in June and July but remained nondetectable in oysters collected in September (Kaufman and others 2003).

1.12.2 The role of aquatic wildlife and zooplankton

Aquatic birds may be a vector for *Vibrio* spp., especially during the winter months. For example, *V. cholerae* has been isolated from aquatic birds at low levels and studies have reported the presence of non-O1 *V. cholerae* in ducks (Bisgaard and Kristen 1975) and gulls (Lee and others 1981) during the winter, when *Vibrio* spp. were not found in the water column. However, there is less information available regarding the role of aquatic birds in the persistence and/or spread of *V. parahaemolyticus* and *V. vulnificus*. In one study, non-O1 *V. cholerae*, *V. parahaemolyticus*, and other lactose-positive vibrios were isolated from bird feces (Roberts and others 1984). A later study by Buck (1990) reported *Vibrio* spp. in association with gulls and pelicans, while Miyasaka and others (2005) found a higher percentage of *V. parahaemolyticus* positive samples (55.4%) compared to *V. vulnificus*

positive samples (14.1%) in wild aquatic birds in Japan during the winter months. In virtually all instances, the level of vibrios in bird populations was quite low.

Although *V. vulnificus* levels are higher in the estuarine environment during the warm summer months, the organism persists throughout the year. There are many ways in which *V. vulnificus* and *V. parahaemolyticus* can survive. Vanoy and others (1992) and Wright and others (1996) found *V. vulnificus* in plankton, suggesting that this bacterium may inhabit habitats similar to *V. cholerae* and *V. parahaemolyticus*. *V. vulnificus* also persists in marine sediment, suggesting winter survival in the floc zone at the sediment interface; when conditions are more conducive for growth (summer months), *V. vulnificus* will then colonize plankton (Vanoy and others 1992). DePaola and others (1994) isolated *V. vulnificus* throughout the winter months from the intestines of estuarine fish from the Gulf of Mexico, at densities higher than those found in oysters, sediment, or seawater.

Indeed, the relationship between zooplankton and *Vibrio* spp. may explain the year-round persistence of the vibrios. It is well documented that *Vibrio* spp. make up a significant portion of the natural microflora of zooplankton, especially zooplankton with chitinous exoskeleton such as copepods (Huq and others 1983, Sakar and others 1983, Chowdhury and others 1989, Carli and others 1993). Huq and others (1983) found higher numbers of vibrios associated with zooplankton than were found in the surrounding water column. A study conducted by Watkins and Cabelli (1985) demonstrated that only chitin and net zooplankters (live or dead) supported the growth of *V. parahaemolyticus* in estuarine water. Heidelberg

and others (2002) found a diverse group of bacteria associated with zooplankton, with higher levels of bacteria associated with zooplankton during the cooler months of the year. However, the majority of these organisms were *V. cholerae, V. mimicus* and *V. vulnificus*.

Extracellular proteins produced by *V. vulnificus* are also important in the organism's ability to survive in the estuarine environment and perhaps cause disease in infected hosts. For example, *V. vulnificus* exports a chitinase that may be used by the bacterium to colonize and adhere to the chitin exoskeletons of zooplankton. The metalloprotease and hemolysin may allow the organism to colonize and multiply in molluscan shellfish by breaking down tissue at the site of colonization, promoting release of necessary nutrients (Strom and Paranjpye 2000).

1.12.3 Bacteriophages

Bacteriophages are abundant in the marine environment, and those specific for the pathogenic *Vibrio* spp. are no exception (Suttle and others 1990, Boehme and others 1993, Jiang and Paul 1994). For example, Moebus and Nattkemper (1983) isolated 366 phages from the Altantic, 362 of which initiated infection in bacteria belonging to the *Vibrionaceae* family. Furthermore, 280 of these phages were specific for the *Vibrio* spp. Pelon and others (1995) isolated nine phage strains specific for *V. vulnificus*, with patterns of susceptibility varying with specific *V. vulnificus* strain. Based on these results, the same investigators (Luftig and Pelon, 1996) attempted to use these 9 bacteriophage strains to reduce *V. vulnficus*

populations in estuarine water, finding that *in vitro* exposure to 1.0 mL of the pooled phage reduced *V. vulnificus* levels by 5 $\log_{10}$. DePaola and others (1997) identified phages infecting *V. vulnificus* in estuarine waters, sediments, plankton, crustacea, and the intestines of finfish and molluscan shellfish harvested from the Gulf Coast. The latter habitat had the highest abundance of phages, however, the lowest densities of phages were in the hemolymph and mantle fluid of oysters. Estimates of abundance ranged from 10^1 to 10^5 PFU/g of oyster tissue.

As is the case for *V. vulnificus* bacteriophages, those infecting *V. parahaemolyticus* are abundant and diverse, having been isolated from the coastal waters of Laos, Hawaii, Florida, and the Pacific and Atlantic coasts of North America (Sklarow and others 1973, Baross and others 1978, Kellogg and others 1995, Nakasone and others 1999, Hardies and others 2003, Comeau and others 2005). However, *V. parhaemolyticus* bacteriophages were not detected in the sediment and only found at low levels in waters off the coast of British Columbia (Comeau and others 2006). Koga and others (1982) isolated 18 bacteriophages infectious to *V. parahaeomlyticus* and reported 4 different morophological groups. Furthermore, there appeared to be no correlation between O and K serotype of *V. parahaeomlyticus* strains and host range of phages (Koga and others 1982). Like *V. vulnificus* phages, Comeau and others (2005) found 13 phages specific for *V. parhaemolyticus* to be consistently higher (0.5×10^4 to 11×10^4 virus/cm^3) year round in oysters compared to sediment and water. In a later study, the same investigators (Comeau

and others, 2006) demonstrated that *V. parahameolyticus* phages infect between 4 and 13 *V. parahaemolyticus* strains with a unique host range pattern. There also appears to be a relationship between host range and season (Comeau and others 2005). Using these same 13 phages to control *V. parahaemolyticus* in the environment, the investigators achieved reduction of the organism by 74%, 62%, and 30% in sediment, oysters, and in the water column, respectively. However, bacteriophage treatment has yet to be realized as a practical method to control *Vibrio* contamination in oysters or their waters.

1.12.4 Starvation and the viable but non-culturable (VBNC) state

Marden and others (1985) were the first to characterize the behavior of marine bacteria to starvation. In fact, starvation is one of several stresses (in addition to cold temperature and suboptimal pH) (Gauthier, 2000) that can induce the so-called viable but nonculturable (VBNC) state. Starvation combined with cold stress may be particularly effective in inducing VBNC (Linder and Oliver, 1989). This term describes bacterial cells that do not form colonies on high-nutrient solid media, but are considered alive because metabolic activity can still be detected (Oliver 2000; Gauthier 2000). The VBNC state can be contrasted to cell injury in that injured cells lose their ability to grow on selective media, but can still be cultured on nutrient-rich media; VBNC cells cannot be cultured at all. Significant effort has gone into characterizing the VBNC state as related to the survival and virulence of *V. cholerae* and *V. vulnificus* (Oliver and Bockian 1995; Wong and others 2004;

Asakura and others 2007). There is, however, evidence that *V. parahaemolyticus* also enters the VBNC state (Jiang and Chai, 1996; Wong and Wang, 2004).

There are physiological manifestations associated with entry into the VBNC state. Morphologically, *V. vulnificus* cells in the VBNC state are small cocci (0.3 μm), whereas after resuscitation the cells become rod-shaped (3 μm in length and 0.7 in width) (Linder and Oliver 1989; Nilsson and others 1991). VBNC cells also clump, suggesting the production of exopolysaccharides resulting in an outer membrane that is "blebbed" (Johnston and Brown 2002). Blebbing is a modification to the outer membrane that is frequently associated with bacterial resistance mechanisms (Jones and others 1989). Oliver and Colwell (1973) observed that, as temperature decreased, there was a proportional increase in the amount of unsaturated fatty acids in the cell membrane of *V. vulnificus*. Indeed, the palmitic (C_{16}) plus palmitoleic ($C_{16:1}$) fatty acid content was decreased by 57%, whereas short-chain fatty acid content increased from 5.4% to 29.0% as cells were entering the VBNC state (Linder and Oliver 1989). Wong and others (2004) found differences in the activities of two enzymes and in the fatty acid profiles of *V. parahaemolyticus* ST550 cells based on culturability status. During the first week of exposure to starvation conditions, an increase in $C_{15:0}$ fatty acid content and a decrease in $C_{16:1}$ content was observed. Also, the enzyme superoxide dismutase became nondetectable in the VBNC state, while the cellular concentration of glucose-6-phosphate dehydrogenase did not change upon entry into the VBNC state.

Clearly, the VBNC state is a mechanism for bacteria to survive adverse conditions and there is evidence that stress conditioning impacts both induction of and speed at which cells enter the VBNC state. For example, Bryan and others (1999) observed that *V. vulnificus* entered the VBNC state when the temperature was shifted from 35 °C to 6 °C; however, when the culture was subjected to 15 °C prior to further temperature downshift, the cells remained culturable. Oliver and others (1991) reported that when *V. vulnificus* cells were pre-starved for 24 h at room temperature and subsequently exposed to 5 °C, they failed to enter the VBNC state, whereas cells starved for the same period at 5 °C did enter the VBNC. When cells were starved for only 1, 2, and 4 h before exposure to 5 °C, the cells also entered the VBNC state, but at a slower rate.

Vibrio species which have entered the VBNC state can usually be revived within 3 days after a temperature shift to 21 °C. A leading theory to explain VBNC is that it is associated with increased sensitivity to hydrogen peroxide. This was first reported by Whiteside and Oliver (1997) who noted that VBNC cells of *V. vulnificus* could not be resuscitated after temperature upshift to 22 °C if suspended in nutrient-rich broth, but could be resuscitated in minimal media, such as artificial seawater (ASW), most likely due to the presence of peroxide byproducts occurring during media sterilization. It is well documented that injured cells frequently demonstrate an increased sensitivity to the toxic effects of hydrogen peroxide, a phenomenon which can be ameliorated by media supplementation with sodium pyruvate or catalase (Baird-Parker and Davenport, 1965; Rayman and others, 1978).

Bogosian and others (2000) were the first to supplement media with catalase or pyruvate to promote the recovery of *V. vulnificus*, noting that higher culturable cell counts were observed after such supplementation. More recently, Kong and others (2004) constructed a deletion mutant of *V. vulnificus* which lacked catalase (*oxyR*) activity. When compared to the wild-type strain, the investigators showed that low temperature inhibited catalase activity, which likely contributed to loss of culturability. The loss of the superoxide dismutase activity in *V. parahaemolyticus* strains having entered the VBNC state provides further evidence for increased sensitivity to hydrogen peroxide (Wong and others, 2004).

Some have speculated that the VBNC state does not really exist, but instead some viable cells remain and when the sample is subjected again to a more favorable environment, those residual viable cells replicate and become detectable on microbiological media. Bogosian and others (2000) conducted a series of experiments to address this issue. Specifically, they demonstrated that when warmed to room temperature, VBNC cells which could be cultured on pyruvate-supplemented media were able to use the nutrients provided by the dead cells to support the formation of more than one progeny cell. However, when the hydrogen peroxide-sensitive cell population declined to nondetectable levels on pyruvate-supplemented media, leaving only nonculturable cells present, warming did not lead to cell growth (Bogosian and others, 2000).

The relationship between the VBNC state and virulence also is of great interest. Colwell and others (1996) fed VBNC cells of *V. cholerae* to human volunteers and observed

an absence of disease but low levels of fecal shedding. Linder and Oliver (1989) reported that VBNC cells of *V. vulnificus* lost virulence in the mouse model; however, a low level of inoculum (5 x 10^4 cells) by the intraperitoneal route was used in these experiments. In a later study, Oliver and Bockian (1995) showed that intraperitoneal injection of mice with a total of 10^5 VBNC cells of *V. vulnificus* was lethal. In an effort to determine if VBNC cells remained pathogenic, 3 strains (*V. alginolyticus*, *V. parahaemolyticus* (environmental origin), and *V. parahaemolyticus* ATCC 43996) were induced into the VBNC state, followed by intragastric inoculation of 8 Balb/C mice for each bacterial strain. In this experiment, isolation and confirmation was obtained in 25% of the mice challenged with *V. alginolyticus*, 37.5% of mice challenged with *V. parahaemolyticus*, and 50% of mice challenged with *V. parahaemolyticus* ATCC 43996. In addition, when the strains were first inoculated in the mouse model, they caused fluid accumulation and expressed virulence characteristics (hemolysin production, adhesiveness, and cytotoxicity). However, when the strains were reisolated from the mice, grown in BHI broth, and then injected into the rat ileal loop, virulence factor expression (hemolysin production, adhesiveness, and cytotoxicity) was lost. Nonetheless, after two consecutive passages in the rat ileal loop model, virulence characteristics were reactivated. This is important because it suggests that VBNC cells retain their ability to express proteins associated with pathogencity, although such expression may be transient and/or unpredictable (Baffone and others 2003).

1.12.5 Stress Response: pH and Refrigerated Storage

It is well documented that vibrios in the VBNC state are more resistant to sublethal stressors. For example, *V. parahaemolyticus* cells induced into the VBNC state by exposure to cold temperatures were observed to be more resistant to thermal inactivation (42 and 47 ^{o}C), low salinity, and acid inactivation (pH 4) (Wong and Wang 2004). Koga and Takumi (1995) reported that *V. parahaemolyticus* cells in the starved state were more resistant to other environmental stresses such as heat (47 ^{o}C) and osmotic pressure.

Such stress response may be of concern when using processing methods intended to reduce the levels of vibrios in raw or minimally processed molluscan shellfish. With regards to the effect of pH, Karem and others (1994) demonstrated that when *Aeromonas hydrophila* suspended in broth was shifted from pH 7.2 to 5.0 (conditioned), the cells survived longer when exposed to a further pH downshift to 3.5. It appears that *A. hydrophila* exhibits an adaptive acid-tolerance response capable of protecting cells at pH values as low as 3.5. Wong and others (1998) found that *V. parahaemolyticus* was more acid-tolerant in the broth model when first conditioned by a pH downshift from 7.5 to 5.0. Koga and others (1999) found acid-adapted *V. parahaemolyticus* cells had an increased resistance to heat (47 °C), crystal violet, bile, and deoxycholic acid, as compared to nonadapted cells. In addition, these investigators noticed a change in the composition of the outer membrane protein of acid-adapted cells. Koo and others (2000) reported strain-to-strain differences in acid-tolerance for *V. vulnificus*, although there did appear to be a pH value (somewhere around pH 2.0)

below which all strains were inactivated. Bang and Drake (2004) reported that 3 strains of *V. vulnificus* had increased acid-resistance in broth acidified with citric acid (pH 3.5) after prior adaptation at pH 5.5, regardless of strain and duration of adaptation time; the same phenomenon was not observed when acetic acid was used as the acidulant. These same investigators demonstrated that acid adaptation involves induction of specific proteins. Freeze-thaw resistance and cold storage survival were improved with prior exposure to citric acid (pH 5.0) for 10 h, but this effect was strain-specific (Bang and Drake 2004). Most recently, Wong and Lui (2006) found that *V. vulnificus* cells adapted by exposure to acid (pH 4.4) or heat (41 ^{o}C) were not cross-protected when exposed to low salinity (0.04% NaCl) conditions.

Although we know that prolonged exposure to nutrient-depleted media and cold temperatures can induce the VBNC state, some investigators became interested in the response of *Vibrios* to so-called cold stress. Indeed, some organisms are able to adapt and persist at very low temperatures when previously conditioned by exposure to less-cold temperature. Bryan and others (1999) suggested that cold-adaptive or protective proteins produced by *V. vulnificus* may enhance survival and tolerance to cold and freezing temperatures. They also hypothesized that iron plays a role in adaptation at cold temperature, since the removal of iron from the growth medium prior to cold adaptation reduced viability by 2 $\log_{10}$ CFU/mL. It was demonstrated that 40 different proteins were synthesized at higher levels by *V. vulnificus* upon exposure to cold stress (McGovern and Oliver 1995). Lin

and others (2004) found that when *V. parahaemolyticus* was cold-shocked at 20 °C or 15 °C for 2 or 4 h, the cells demonstrated better survival upon subsequent exposure to low temperature of 5 °C or -18 °C, or to crystal violet, but were more susceptible to high temperature (47 °C), hydrogen peroxide, and lactic and acetic acids, when compared to unconditioned cells. Bryan and others (1999) showed that a culture of *V. vulnificus* demonstrated better survival during frozen storage (-78 °C) when freezing was preceded by cold shock. Bang and Drake (2002) also found improved survival of *V. vulnificus* under cold temperature storage when cells underwent a cold temperature pre-conditioning step.

1.13. TECHNIQUES TO ELIMINATE *VIBRIO* SPECIES FROM OYSTERS

Currently, shellfish harvesting waters are classified using the coliform or fecal coliform index. Unfortunately, since *Vibrio* species are ubiquitous to the marine environment, the levels of the traditional fecal indicators do not correlate with the presence or levels of the environmental vibrios, and hence the fecal coliform index is not useful for controlling these organisms (Tamplin and others 1982). This was confirmed by O'Neil and others (1992) who found no correlation between fecal coliform and *V. vulnificus* levels. However, Watkins and Cabelli (1985) found an indirect relationship between *V. parahaemolyticus* levels and pollution in the Naraganssett Bay and hypothesized that this was a result of nutrient stimulation. Ruple and Cook (1992) observed correlation between the

fecal coliform level and *V. vulnificus* during the warmer months (May-Sept.), but this relationship did not hold up during the cooler months of the year.

1.13.1 Commercial heat shock

Currently, the commercial heat shock process is used as a processing aid, primarily in North and South Carolina, to facilitate the shucking of shellstock oysters (Hesselman and others 1999). This process involves submerging about 70 chilled oysters in wire baskets into a heat-shock tank containing approximately 850 L of potable water at a temperature of 67 °C for about 5 min, depending on oyster size and relative oyster condition. After heat-shocking, the oysters are cooled by spraying for 1 min with potable water prior to shucking and washing. Hesselman and others (1999) found that this commercial heat-shock process reduces *V. vulnificus* levels by 2 to 4 $\log_{10}$. No reduction in *V. vulnificus* levels were observed in oysters that were merely washed. Ruple and Cook (1992) showed that while commercial heat-shock processing of oysters did not reduce the levels of *V. vulnificus*, immediate storage on ice did reduce the levels by 1 to 2 $\log_{10}$ CFU/g.

Cook and Ruple (1992) demonstrated that low-temperature pasteurization (50 °C) for 10 min reduced *V. vulnificus* and *V. parahaemolyticus* counts from 10^5 MPN/g to nondetectable levels in inoculated shellstock oysters; this was confirmed by Andrews and others (2000). In a later report, Andrews and others (2003) demonstrated that a combined hot-water/cold-shock "pasteurization" process with a temperature of 50-52 °C reduced *V.*

parahaemolyticus 03:K6 (10^6 CFU/g of oyster) in shellstock oysters to nondetectable levels within 22 min, without changing the sensory properties of the product.

1.13.2 Depuration and relaying

Depuration is the process of controlled purification whereby shellfish are placed in disinfected, recirculating or flow-through seawater and allowed to actively filter-feed, typically for 24 to 48 h. The use of this practice is quite limited in the U.S. but extensive in Europe. Disinfectants commonly used in depuration waters are chlorine, ozone, and ultraviolet light. Groubert and Oliver (1994) used a *V. vulnificus* strain (CVD713), which was genetically transformed to carry a stable TnphoA transposon encoding kanamycin resistance and alkaline phosphatase activity, to demonstrate that oysters allowed to filter-feed in artificially contaminated waters were able to reduce, to non-detectable levels, accumulated *V. vulnificus* within 48 h of the onset of depuration. Interestingly, however, the level of naturally occurring *V. vulnificus* in these oysters was not reduced by depuration. Eyles and Davey (1984) also reported that depuration did not produce a substantial reduction in *V. parahaemolyticus* levels in shellfish, but Nordstrom and others (2004) were able to achieve better reduction in *V. parahaemolyticus* levels after overnight tidal submersion, compared to intertidal exposure, in Hood Canal, WA. While this is technically not depuration, it does suggest that, under certain conditions, *V. parahaemolyticus* can be eliminated. Tamplin and Capers (1992) found that recirculation of depuration waters through

UV light at above 23 °C was an ineffective control because *V. vulnificus* was able to multiply in oyster tissues under these conditions. However, when the seawater was maintained at 15 °C, *V. vulnificus* could not be detected in seawater, nor did multiplication of *V. vulnificus* occur in the oyster.

Relaying is another purification method that involves moving shellfish from a restricted harvesting area to an open area where natural cleansing can occur. Cook and Ellender (1986) found that the temperature and the microbiological quality of the relaying water had an impact on the length of time needed to reduce fecal coliform levels in oysters. Additionally, oysters that were physiologically stressed took longer to cleanse than did unstressed oysters, presumably due to slower metabolic activity. While pathogens such as *Salmonella* can be eliminated within 5 days by relaying (Cook and Ellender 1986), Motes and DePaola (1996) demonstrated that longer relaying periods (17 to 49 days) and high salinity (>30 ppt) were required to decrease *V. vulnificus* levels from 10^3 CFU/g to <10 MPN/g. As with depuration, relaying cannot be relied upon to completely eliminate *V. vulnificus* from shellfish.

1.13.3 GRAS compounds

As a possible aid in controlling *Vibrio* contamination in shellfish, investigators have examined certain preservatives that are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Sun and others (1994) were able to achieve a 2 $\log_{10}$

reduction in the levels of naturally occurring *V. vulnificus* in oysters treated with diacetyl at a concentration of 0.05%, while lactic acid and butylated hydroxyanisole (BHA) compounds at a concentration of 0.05% did not have an effect on *V. vulnificus* levels. Diacetyl appears to affect the permeability of cell membranes and accumulates in the membrane lipid bilayer (Johnson and Steele 2001).

There are also naturally-occurring compounds in oysters that may promote the inactivation of *V. vulnificus* and *V. parahaemolyticus.* For example, oysters contain hemocytes which entrap bacteria within phagosomes, after which an enzymatic degradation process begins (Cheng 1975). Although theoretically this process may be lethal to the vibrios, Genthner and others (1999) reported that oyster hemocytes did not have a significant lethal effect on either opaque or translucent strains of *V. vulnificus*; for *V. parahaemolyticus*, the opaque stains were more resistant to the effect of hemocytes than were the translucent strains. Unfortunately, most oysters found in mid-Atlantic and Gulf Coast waters are infected with *Perkinsus marinus*, an oyster pathogen which produces a a serine protease capable of digesting oyster connective tissues. Tall and others (1999) found that oyster hemocytes treated with the serine protease produced by *P. marinus* were less efficient in controlling the levels of naturally occurring *V. vulnificus* when compared to untreated hemocytes, suggesting that *P. marinus,* may actually suppress the natural ability of oyster hemocytes to eliminate *V. vulnificus.*

1.13.4 Ionizing irradiation

Gamma-irradiation can eliminate *Vibrio* species from shellstock and shucked oysters. *Vibrio* species are among the most radiation-sensitive bacteria; *V. cholerae* and *V. vulnificus* can be eliminated when exposed to doses less than 0.1 kGy (Mallett and others 1991). Novak and others (1966) found that a 0.2 Mrad (2 kGy) dose of gamma radiation could be applied for pasteurization of oyster meat without causing changes in organoleptic quality. After this treatment, total bacterial counts decreased by 99%. Matches and Liston (1971) found that, in most cases, *V. parahaemolyticus* was reduced 4-6 $\log_{10}$ using a dose of 30-40 krad (0.3-0.4 kGy). Andrews and others (2003) showed that ionizing irradiation doses of 1.0 kGy reduced *V. vulnificus* at initial inoculum of 10^7 CFU/g to non-detectable levels as applied to whole shell oysters. Oysters inoculated with *V. parahaemolyticus* 03:K6 (10^4 CFU/g) reached nondetectable levels after treatment with 1.5 kGy. Most oysters survived the treatment and sensory data showed that consumers could not tell a difference between irradiated and nonirradiated oysters. Recently, the FDA approved irradiation as a food additive for seafood, including oysters.

1.13.5 Temperature control and refrigeration

Refrigeration controls the multiplication of *V. vulnificus* and *V. parahaemolyticus* in oysters. Cook and Ruple (1989) investigated the effects of various storage temperatures (10,

22, 30 °C) on oysters and found that members of the *Vibrionaceae* family increased in shellstock oysters stored at 22 and 30 °C, while10 °C storage prevented growth. Cook (1994) also observed that *V. vulnificus* did not multiply in oysters stored at below 13 °C and growth at 18 °C was significantly slower than at ambient air temperature (23 to 34 °C).

Prolonged refrigeration may actually reduce the levels of the pathogenic vibrios. For instance, Cook and Ruple (1992) observed that within 14 to 21 days of refrigerated storage, *V. vulnificus* in shellfish could be reduced to non-detectable levels (< 3 MPN/g). Later Cook and others (2002) estimated that *V. vulnificus* levels declined by 0.041 log unit/day during refrigeration of retail oysters. However, Kaysner and others (1992) demonstrated that, in artificially contaminated shellstock and shucked oysters, *V. vulnificus* survived for 14 days at 2^{o} C. It is generally recognized that, while levels may decline over time, prolonged refrigeration cannot be relied upon to eliminate *V. vulnificus* or *V. parahaemolyticus* from contaminated shellstock.

If the temperature of shellstock is not immediately controlled, growth of vibrios can occur quite rapidly. For example, Cook (1997) observed that the levels of *V. vulnificus* in freshly harvested shellstock oysters held without refrigeration for 3.5, 7, 10.5, and 14 h increased 0.75, 1.30, 1.74, and 1.94 log units, respectively. For this reason, the U.S. National Shellfish Sanitation Program stipulated (in 1993) first refrigeration guidelines for raw molluscan shellfish. These were made more stringent in 1995, with a requirement that shellstock be placed under temperature control within 12-14 h of harvest, depending on the

average monthly maximum water temperature (Cook 1997). More recently, regulations state that commercial shellfish must be refrigerated within 10 h after harvest when water temperature exceeds 27 °C (U.S. Department of Health and Human Services 1999). In 1995, the Interstate Shellfish Sanitation Conference (ISSC) adopted an additional control plan for states that had been confirmed as the originating site of shellstock products associated with 2 or more *V. vulnificus* illnesses. In this case, if water temperature was between 18 °C and 23 °C, shellstock was required to be placed under temperature control within 14 h; if greater than 23 °C and less than 28 °C, the time limit was less than 12 h; and if the water temperature was greater than 28 °C, the time limit was less than 6 h (Associated Press 1996). Once placed under temperature control, shellstock must be iced, or the storage area or conveyance otherwise continuously maintained at 7.2 °C or below, until final sale to the consumer.

Not only is time unrefrigerated on boat docks an issue, but commercial cooling of oyster sacks has been estimated to take an average of 5.5 hours (CFSAN/FDA 2005), during which time *Vibrio* growth can still occur, albeit more slowly. This is contrasted to die-off that occurs during extended refrigerated storage. Taken together, the growth of *V. vulnificus* and *V. parahaemolyticus* that occurs before oysters reach the target refrigeration temperatures of 13^{o}C (*V. vulnificus*) and 8^{o}C (*V. parahaemolyticus*) results in higher levels of these organisms at consumption relative to the levels at harvest. For example, Wright and others (1996) and Motes and others (1998) reported that the levels of *V. vulnificus* in Gulf of

Mexico and Chesapeake Bay oysters at harvest are typically 1 $\log_{10}$ lower than they are at retail. Likewise, Cook and others (2002) observed that *V. vulnificus* and *V. parahaemolyticus* levels in retail oysters originating from the Gulf of Mexico were 1 to 2 $\log_{10}$ greater than at harvest.

1.13.6 Freezing and frozen storage

Cook and Ruple (1992) reported that freezing reduces the levels of *Vibrio* spp. in shellfish, although it does not eliminate the organism, even after frozen storage for up to 12 weeks. A temperature of -20 °C was more effective for inactivating *V. vulnificus* than was 0 °C. At -80 °C, *V. vulnificus* and *V. parahaemolyticus* cell numbers in brain heart infusion broth supplemented with 3% NaCl dropped by one $\log_{10}$ CFU/g during the freezing process and remained stable thereafter for 35 days (Boutin and others 1985). Johnston and Brown (2002) showed that the total cell numbers were the same for freshly cultured *V. vulnificus, V. cholerae,* and *V. parahaemolyticus* both before and after freezing (-20 °C); similar results were obtained for VBNC cells. In a study conducted by Parker and others (1994), the combination of vacuum-packaging and freezing decreased *V. vulnificus* levels in oysters by 3 to 4 $\log_{10}$ CFU/g within 7 days post-freezing, and levels continued to drop throughout frozen storage up to day 70, although complete elimination was never achieved. The combination of vacuum-packaging and freezing controlled *V. vulnificus* levels more effectively than did freezing with conventional packaging (Parker and others 1994). ISSC has adopted freezing

combined with frozen storage as an acceptable means for post-harvest treatment to control *V. vulnificus* and *V. parahaemolyticus*. A number of firms now use this process, which must be validated and HACCP compliant (21 CFR 123).

1.13.7 High hydrostatic pressure

Most microorganisms are baroduric, meaning they can survive under high pressures but normally grow best at atmospheric pressure. High pressure application is a promising emerging technology to control pathogens in certain foods. When using pressure to inactivate microorganisms, the treatment depends on the intensity of the pressure and the length of exposure (Hoover and others 1989). In general, *Vibrio* spp. are extremely sensitive to pressure. Styles and others (1991) demonstrated that *V. parahaemolyticus* is rapidly reduced to nondetectable levels at pressures higher than 1,700 atm when suspended in clam juice. More recent research has used the international system of units (SI) conversion and the megaPascal (MPa) unit in place of atmospheres (atm) (the relationship between the two is 10:1, atm:Mpa). Berlin and others (1999) reported that treatment with hydrostatic pressure of 250 MPa for 10 min at 25 °C reduced *V. vulnificus* in pure culture to nondetectable levels without triggering the VBNC state. However, *V. vulnificus* cells in the VBNC state appear to be more resistant to the lethal effects of high hydrostatic pressure (Berlin and others 1999). Cook (2003) found that *V. vulnificus* strains suspended in phosphate-buffered saline (PBS) were the most sensitive to high pressure (200 MPa), whereas *V. cholerae* strains were more

resistant. Furthermore, strains of the pandemic O3:K6 serotype of *V. parahaemolyticus* were more resistant to pressure than were strains of other serotypes or *Vibrio* spp. For instance, in order to obtain a better than 5 $\log_{10}$ CFU/g reduction of *V. vulnificus* in oysters, a treatment of 250 MPa for 120 s was required, while a treatment of 300 MPa for 180 s was required to obtain a similar reduction in pandemic *V. parahaemolyticus* serotype O3:K6 (Cook 2003). Most recently, Koo and others (2006) found that at 241 MPa, it took 11 and 5 min (including a 3-min pressure come-up time) to achieve a 6 $\log_{10}$ reduction of pandemic *V. parahaemolyticus* O3:K6 and *V. vulnificus*, respectively, in phosphate-buffered saline (PBS). Both *V. parahaemolyticus* and *V. vulnificus* reached nondetectable levels in PBS and oysters at 586 MPa after 8 and 7 min, respectively. Some companies have obtained ISSC approval to use this method for post-harvest processing (A. DePaola, personal communication).

1.13.8 Heat treatment

Heat is a very effective means to eliminate cells of *Vibrio* species and was approved as a post-harvest process by the ISSC in 2003. *V. vulnificus* cells are rapidly and exponentially inactivated at 50 °C or higher (Ama and others 1994). Cook and Ruple (1992) demonstrated that *V. vulnificus* (4.3×10^3 CFU/g) in naturally-contaminated shellfish could be reduced to nondetectable levels by exposing oysters to a temperature of 50 °C for 10 min. Cultures of *V. vulnificus, V. cholerae*, and *V. parahaemolyticus* showed D-values of 12 s, 22.5 s, and 1.75 min, respectively, at 55 °C, and all three organisms, when suspended in

broth, were reduced by more than 7 $\log_{10}$ CFU/mL when treated at 70 °C for 2 min (Johnston and Brown 2002). In broth, *V. parahaemolyticus* was more resistant to heat inactivation at 47 °C when preceded by a heat shock at 42 °C for 30 min; unconditioned *V. parahaemolyticus* cells were readily inactivated at 47 °C (Wong and others 2002). *V. vulnificus* was more resistant to heating when suspended in oyster homogenate than in buffer, presumably due to the protective effects of the suspending matrix (Ama and others 1994). According to Kim and others (1997), *V. vulnificus* morphotype influences thermal death times; opaque strains have higher D and z_D values than do translucent strains, suggesting that the former have increased heat resistance. The D-values for opaque colonies range from 3.44-3.66 min and those for translucent colonies range from 3.18-3.38 min at 47 °C; the range of z_D-values for opaque colonies is 2.45-2.51 °C while the range for translucent colonies is 1.89-2.07 °C.

1.14. RISK ASSESSMENT

Recently, the U.S. Food and Drug Administration (2005) conducted a quantitative risk assessment for *V. parahaemolyticus* (VPRA) in raw oysters in the United States. Since water temperature was considered the major factor affecting *V. parahaemolyticus* density at harvest, different models were constructed for seasons (winter, spring, summer, and fall), regions (Gulf Coast of Louisiana because oyster boats are on the water longer before refrigerating, Gulf Coast excluding Louisiana, mid-Atlantic, Northeast Atlantic, Pacific

Northwest) and by harvesting practice (dredging and intertidal for the Pacific Northwest). The VPRA model predicted the highest levels of total and pathogenic *V. parahaemolyticus* at harvest in the Gulf Coast region due to warmer temperatures. The total levels of *V. parahaemolyticus* at harvest were predicted to be 2.1×10^3, 2.2×10^2, 5.2×10^1, and 9.4×10^2 cells/g oyster for summer, fall, winter, and spring, respectively, for the Gulf Coast region. Although the Pacific Northwest has the coolest water temperature, when harvesting by the intertidal method, the VPRA predicted that it has the second highest levels of pathogenic *V. parahaemolyticus*. This was due to warm air exposure during intertidal harvesting, along with the fact that the ratio of pathogenic to total *V. parahaemolyticus* is higher in this region than in others.

Air temperature was considered second to water temperature in terms of factors influencing the density of *V. parahaemolyticus* in oysters after harvest. This is because the organism's growth rate is temperature-dependent and it continues to multiply after harvest unless shellstock are refrigerated rapidly. During the summer months in the Louisiana Gulf Coast region, the VPRA model predicted pathogenic *V. parahaemolyticus* levels of 720 cells/serving at harvest; at consumption, the levels reached 21,000 cells/serving. In fact, the levels of pathogenic *V. parahaemolyticus* from harvest to consumption increased for all 6 harvest regions/practices in the United States, suggesting the need to provide better control of *V. parahaemolyticus* multiplication immediately after harvest.

Human challenge data in conjunction with the Beta-Poisson model was used to estimate the dose-response relationship. The VPRA suggested that there was a low risk (< 0.001%) of gastroenteritis following the consumption of 10^4 cells of *tdh*+ *V. parahaemolyticus*/serving, and a high (50%) risk when 10^8 cells/serving were consumed. The model was calibrated to the CDC's estimate of 2,800 oyster-associated *V. parahaemolyticus* cases annually in the U.S. However, the Alaskan outbreak investigation suggested that the infectious dose of the Alaska strains may be thousands of times lower (McLaughin and others 2005). The risk assessment predicted the mean annual number of illnesses to be the highest in the Gulf Coast (Louisiana) region with 1,406, 132, 7, and 505 cases occurring in the summer, fall, winter, spring seasons, respectively. The other regions, in descending order of total annual illnesses, were as follows: the Gulf Coast (non-Louisiana) (546 cases), Pacific Northwest (intertidal) (192 cases), Northeast Atlantic (19 cases), Mid-Atlantic (15 cases), and Pacific Northwest (dredging) (4 cases). The Pacific Northwest (intertidal) region had relatively high predictions of illness due to the fact that oysters harvested in intertidal areas are normally exposed to higher temperatures before refrigeration.

The VPRA clearly demonstrated that the use of post-harvest treatments (PHT) will reduce the number of illnesses caused by this organism. For example, the model predicted that if a 4.5 $\log_{10}$ reduction of *V. parahaemolyticus* were obtained, the probability of illness would decrease to less than 1.0 case/year in all regions of the United States. Using this same benchmark for inactivation, PHT such as heat, pressure, and freezing were predicted to

reduce the number of cases by >99.99%. Rapid post-harvest chilling of oysters could reduce theoretically reduce *V. parahaemolyticus* illness by 90-99%.

Using a framework and parameters similar to those of the VPRA, the World Health Organization (WHO) and Food and Agriculture Organization (FAO) (2005) conducted a quantitative risk assessment for *V. vulnificus* in raw oysters from the U.S. Gulf Coast (VVRA). Consistent with the previous *V. parahaemolyticus* work, the levels of *V. vulnificus* in oysters at harvest were most influenced by water temperature; they were, however, also influenced by salinity. The highest estimated levels of *V. vulnificus* at harvest were 5.6×10^3 CFU/g during the summer, and the lowest were 8.0×10^1 CFU/gin the winter, for oysters harvested from waters with a salinity of below 30 ppt. The risk assessment model predicted that *V. vulnificus* levels increased substantially during post-harvest storage, with predicted mean levels of 5.7×10^4 and 8.0×10^1 *V. vulnificus*/g in the summer and winter, respectively. A serving size of approximately 196 g of oyster meat would provide an ingested dose of *V. vulnificus* of 1.1×10^7 and 1.6×10^4 in the summer and winter, respectively.

For hazard characterization, the Beta-Poisson dose-response model was used in conjunction with human clinical data. Under current harvest and post-harvest conditions, *V. vulnificus* illnesses were estimated at 0.5, 11.7, 12.2, and 8.0 for winter, spring, summer, and autumn, respectively. If alternative processes were used to reduce *V. vulnificus* levels to 300 CFU/g, 30 CFU/g, and 3 CFU/g, the annual number of cases was estimated at 7.7, 1.2, and 0.16, respectively.

1.15. SUMMARY

V. vulnificus and *V. parahaemolyticus* infections occur worldwide and are associated with significant morbidity and mortality. Although *V. vulnificus* is more abundant than *V. parahaemolyticus* in the Gulf of Mexico during the warmer months, *V. parahaemolyticus* has a greater seasonal and geographic range than does *V. vulnificus*, and it is generally more abundant year-round. Because of their association with seafood, these agents are a significant concern to the shellfish industry and public health agencies. Much research has been conducted regarding the effects of environmental factors, such as water temperature and salinity, on the prevalence and levels of *V. parahaemolyticus* and *V. vulnificus* in water and shellfish. However, less is known about the levels of pathogenic strains of *V. parahaemolyticus* and *V. vulnificus* in oysters and waters, particularly with respect to environmental and seasonal effects.

There are numerous culture-based and molecular methods for the detection of *V. parahaemolyticus* and *V. vulnificus.* These methods have become more efficient over the past 20 years. *V. parahaemolyticus* and *V. vulnificus* can readily be detected and enumerated, but differentiating pathogenic strains from nonpathogenic strains remains a challenge. Most *V. parahaemolyticus* and *V. vulnificus* strains have been shown to be genetically heterogeneous, with the exception of the pandemic strains of *V. parahaemolyticus*. Overall, no precise conclusions can be drawn about pathogenic strains as compared to nonpathogenic strains and questions remain about pathogenicity and the role of recognized and purported virulence

factors. With the ability to sequence the entire genomes of *V. parahaemolyticus* and *V. vulnificus,* we will soon be able to explain how these organisms evolved to survive the changing aquatic environment and to better characterize genes associated with virulence and survivability. This will lead to improved understanding of risk, and hopefully, new and more effective control measures.

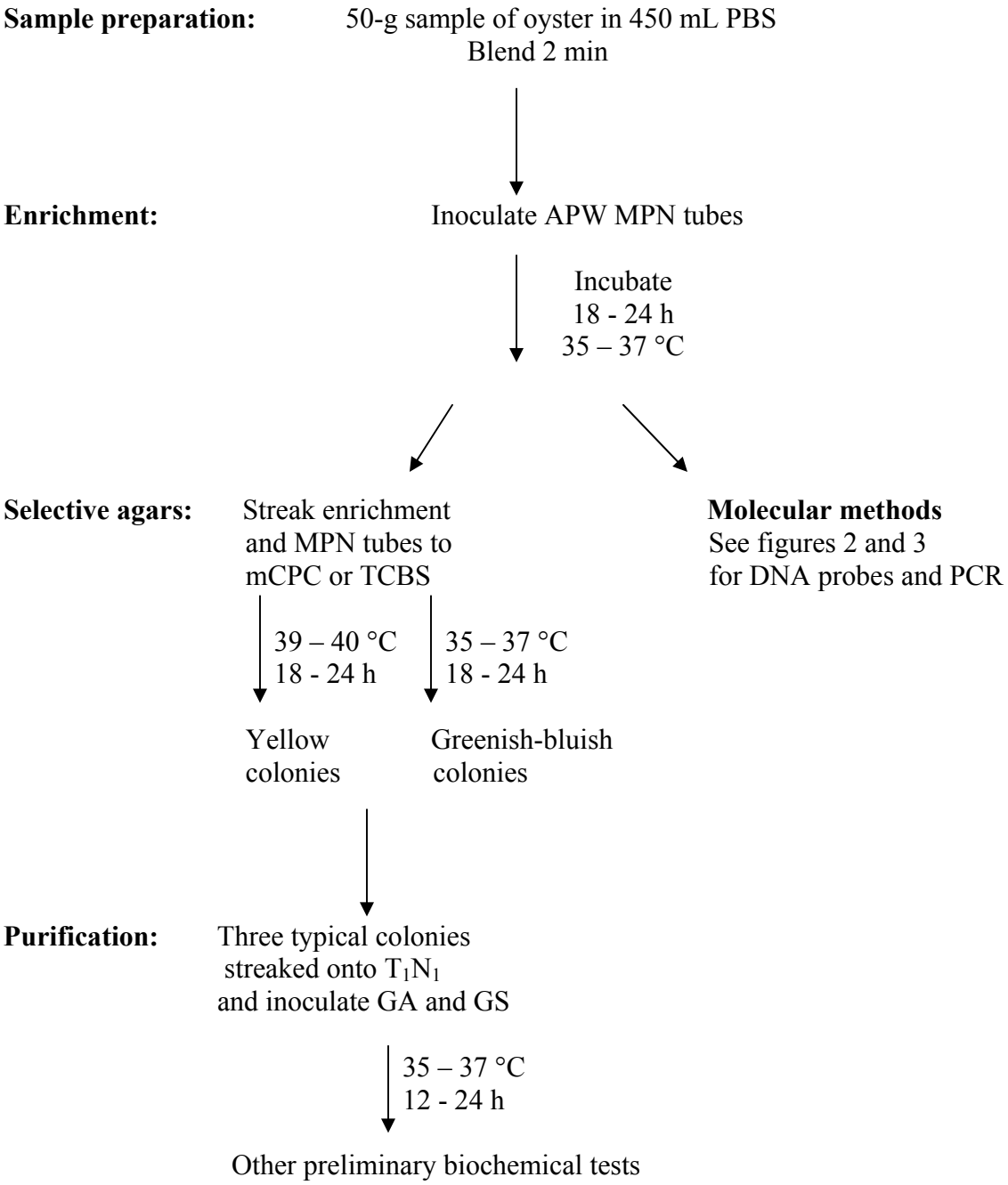

Figure 1.1: Schematic of *V. parahaemolyticus* and *V. vulnificus* identification methods (BAM 2001)

Sample preparation:

200 – 250 g (10 - 12 oysters) oyster sample in equal amounts of PBS making a 1:1 dilution.

↓ Blend 90 sec

Spread plate 0.2 ± 0.01 g of 1:1 dilution on T_1N_3 (*V. parahaemolyticus*) and VVA (*V. vulnificus*) plates and 100 uL of 10^{-2} and 10^{-3} dilution on T_1N_3 and VVA.

↓ Incubate at 35 – 37 °C for 18-24 h

Filter preparation:

Label Whatman #541 filters (*tlh*, *tdh*, or *vvh*A) and place filter, label side down, on the surface of plate. Lysis filters with 1 mL of lysis solution/filter. Microwave filter for 30 s per filter.

↓

Wash filters with ammonium acetate buffer at room temperature for 5 min. Then wash 1x SSC solution

↓

Wash filters with 10 mL of 1x SSC and 20 uL of stock ProK for each filter at 42 °C for 30 min.

↓

Hybridization:

Place 5 filters marked *tlh* and *tdh* (*V. parahaemolyticus*) in a water bath at 54 °C for 30 min and in a water bath at 55 °C for 30 min *vvh*A (*V. vulnificus*) with control strips and add 10 mL of hybridization buffer

Add the individual probe *tlh*, *tdh*, or *vvhA* (final conc. is 0.5 pmol/mL)(sequences can be found in Table 3 and 4) to bag with filters and fresh hybridization buffer and incubate

↓

Rinse *tlh* filters 2 times with 1x SSC/SDS for 10 min in bath at 54 °C
Rinse *tdh* filters 2 times with 3xSSC/SDS for 10 min in bath at 54 °C
Rinse *vvh*A filters 2 times with 1x SSC/SDS for 10 min in bath at 55 °C

↓

Rinse filter 5 times for 5 min each in 1X SSC (10 mL/filter) at room temperature

↓

Add 5 filters to 20 mL of NBT/BCIP solution and incubate 35 °C in the dark.
Reaction is usually complete by 24 h.

↓

Rinse filter 3 times with distilled water (10 mL/filter) for 10 min. Count purple colonies and report as CFU/g. Store filter in the dark.

↓

Confirmation:

Re-line filters with Petri plate and select 5 to 10 colonies that are t*lh*+, *tdh*+, or *vvhA*+ and streak to TCBS or VVA, respectively, and then re-probe with *tlh*, *tdh*, and/or *vvhA*.

Figure 1.2 Schematic of *V. parahaemolyticus* and *V. vulnificus* identification with DNA probes (BAM 2001)

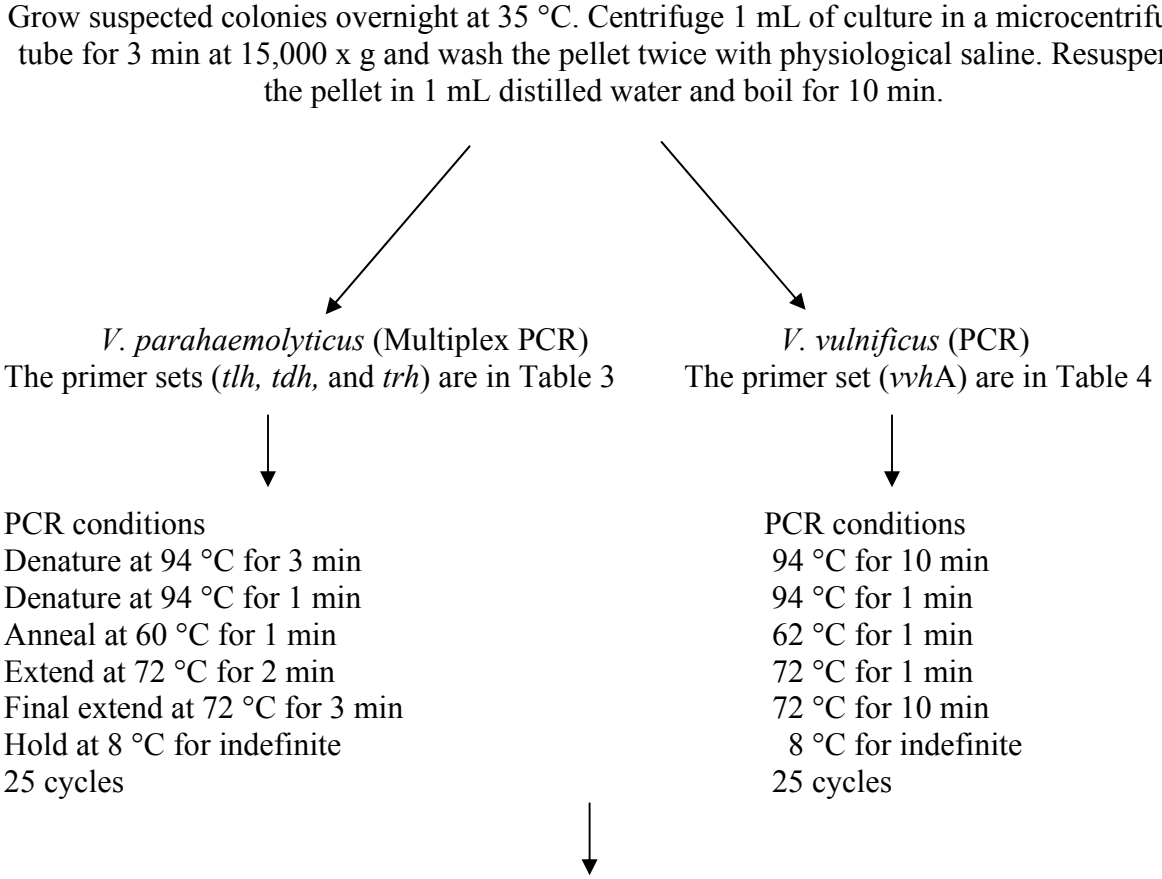

Figure 1.3 Schematic of *V. parahaemolyticus* and *V. vulnificus* Confirmation by PCR (BAM 2001)

Table 1.1 Reported Serotypes of *V. parahaemolyticus* (FDA BAM 2001)

O antigen	**K antigen**
1	1,25,26,32,38,41,56,58,64,69
2	3,28
3	4,5,6,7,27,30,31,33,37,43,45,48,54,57,58,59,65
4	4,8,9,10,11,12,13,34,42,49,53,55,63,67
5	5,15,17,30,47,60,61,68
6	6,18,46
7	7,19
8	8,20,21,22,39,70
9	9,23,44
10	l9,24,52,66,71
11	36,40,50,51,61
12	52

Table 1.2 Preliminary biochemical tests

Test	***V. parahaemolyticus***	***V. vulnificus***
TCBS agar	Green	Green
mCPC agar	No growth	Yellow
CC agar	No growth	Yellow
AGS	KA	KA
Oxidase	+	+
Arginine dihydrase	-	-
Ornithine decarboxylase	+	+
Lysine decarboxylase	+	+
0% NaCl	-	-
3% NaCl	+	+
6% NaCl	+	+
8% NaCl	+	-
10% NaCl	-	-
Growth at 42 ^{o}C	+	+
Sucrose	-	-
D-Cellobiose	V	+
Lactose	-	+
Arabinose	+	-
D-Mannose	+	+
D-Mannitol	+	V
ONPG	-	+
Voges Proskauer	-	-
10 µg O/129	R	S
150 µg O/129	S	S
Gelatinase	+	+
Urease	V	-

KA = slant alkaline/but slightly acidic
V = variable
R = resistant
S = sensitive

(BAM 2001)

Table 1.3 Molecular methods and sequences used to identify *V. parahaemolyticus*.

Gene	Location	Sequence	Application	Reference
tdh	330-350 504-524 685-702 735-754	5'-CCATCTGTCCCTTTTCCTGCC-3' 5'-GGTACTAAATGGTTGACATCC-3' 5'-CCAAGTAAAATGTATTTGG-3' 5'-GCATATGAGAGTGGTAGTGG-3'	DNA Hybridization	Nishibuchi and others (1986) Kaysner and others (1994)
tdh	1,275 bp	5'-GCTAAGTTTGTTGGTGAAGAT-3'	DNA Hybridization	Lee and others (1992)
tlh	904-927	Forward *5'-AAAGCGGATTATGCAGAAGCACTG-3' Reverse 5'-GCTACTTTCTAGCATTTTCTCTGC-3'	DNA Hybridization PCR Multiplex PCR	*McCarthy and others (1999), *Gooch and others (2001), *Ellison and others (2001), *Nordstrom and DePaola (2003) Brasher and others (1998) Bej and others (1999) BAM (2001)
pR72H	140-526	Forward 5'-TGCGAATTCGATAGGGTGTTAACC-3' Reverse 5'-CGAATCCTTGAACATACGCAGC-3'	PCR	Lee and others (1995)
tdh2	85-719	Forward 5'-TTTCATGATTATTCAGTT-3' Reverse 5'-TTTGTTGGATATACACAT-3'	PCR	Karunasagar t and others (1996)
Genomic DNA	Not described	Forward 5'-GAATTCGATAGGGTGTTAACC-3'	PCR	Wang and others (1997)

Table 1.3 Continued

		Reverse 5’-ATCCTTGAACATACGCAGC-3’		
tdh	Not described	5'-GGTTCTATTCCAAGTAAAATGTATTTG-3'	Hybridization	BAM (2001)
*tox*RS/*old*	Not described	Forward 5’-TAATGAGGTAGAAACG-3’ Reverse 5’-ACGTAACGGGCCTACG-3’	PCR	Osawa and others (2002) Okura and others (2003)
*tox*RS/*new*	Not described	Forward 5’-TAATGAGGTAGAAACA-3’ Reverse 5’-ACGTAACGGGCCTACA-3’	GS-PCR	Matsumoto and others (2000) Bhuiyan and others (2002) Osawa and others (2002) Okura and others (2003)
*tox*R	609-958	Forward 5’-GTCTTCTGACGCAATCGTTG-3’ Reverse 5’-ATACGAGTGGTTGCTTGCTGTCATG-3’	PCR	Dileep and others (2003)
ORF8	823-1192	Forward 5’-AGGACGCAGTTACGCTTGATG-3’ Reverse 5’-CTAACGCATTGTCCCTTTGTAG-3’	PCR	Myers and others (2003)
		Probe 5’-FAM-AAGCCATTAACAGTTGAAGGCGTTGA CT-BHQ1	Real-time	Ward and Bej (2006)
ORF8	Not described	Forward 5’-GTTCGCATACAGTTGAGG-3’	Colony hybridization PCR	Nasu and others (2000); Iida and others (2001)

Table 1.3 Continued

		Reverse 5’-AAGTACAGCAGGAGTGAG-3’		Yeung and others (2003) Okura and others (2003)
tlh	Not described	Forward 5’-CGAGAACGCAGACATTACGTTC-3’ Reverse 5’-TGCTCCAGATCGTGTGGTTG-3’ Probe 5’-FAM-TCGCCGCTGACAATCGCTTCTCAT-BHQ1-3’	Real-time PCR	Davis and others (2004) Kaufman and others (2004)
tdh	Not described	Forward 5’-GTAAAGGTCTCTGACTTTTGGAC-3’ Reverse 5’-TGGAATAGAACCTTCATCTTCACC-3’	Multiplex PCR	Bej and others (1999) BAM (2001)
tdh	Not described	Forward 5’-AAACATCTGCTTTTGAGCTTCCA-3’ Reverse 5’-CTCGAACAACAAACAATATCTCATCAG-3’ Probe 5’-FAM-TGTCCCTTTCCTGCCCCCGG-TAMRA-3’	Real-time PCR	Blackstone and others (2003)
tdh	Not described	Forward 5’-CATCTTCGTACGGTTTTCTTTTTACA-3’ Reverse 5’-TCTGTCCCTTTTCCTGCCC-3’ Probe 5’-FAM-TCTCGAACAACAAACAATATCTCATCAGAACCG-BHQ1-3’	Real-time PCR	Davis and others (2004)

Table 1.3 Continued

trh	Not described	Forward 5'-TTGGCTTCGATATTTTCAGTATCT-3' Reverse 5'-CATAACAAACATATGCCCATTTCCG-3'	Multiplex PCR	Bej and others (1999) BAM (2001)
trh	Not described	Forward 5'-GCCAAGTGTAACGTATTTGGATGA-3' Reverse 5'-TGCCCATTTCCGCTCTCA-3' Probe 5'-FAM-ACGCCAGATATTTCGTCAATGTCGA AGC-BHQ1-3'	Real-time PCR	Davis and others (2004)
trh	Not described	5'-ACTTTGCTTTCAGTTTGCTATTGGCT-'3	DNA hybridization	Nordstrom and others (2006)
*gyr*B	Not described	Forward 5'-TGAAGGT-TTGACTGCCGTTGT-3' Reverse 5'-TGGGTTTTCGACCAAGAACTCA-3' Probe 5'-FAM-TTCTCACCCATCGCCGATTCAACCG C-TAMRA-3'	Real-time PCR	Cai and others (2006)
tlh	781-1230	Forward 5'-AAAGCGGATTATGCAGAACTG-3' Reverse 5'-GCTACTTTCTAGCATTTTCTCTGC-3' Probe 5'-TexR-AAGAACTTCATGTTGATGACACT-BHQ2-3'	Hybridization PCR Real-time PCR	BAM (2001) BAM (2001) Ward and Bej (2006)

Table 1.3 Continued

tdh	170-438	Forward 5'-CCATCCATACCTTTTCTTTCTCC-3' Reverse 5'-ACTGTCATATAGGCGCTTAAC-3' Probe 5'-TET-TATTTGTTGTTAGAAATACAACA AT-BHQ1-3'	Real-time PCR	Ward and Bej (2006)
trh	82-287	Forward 5'-GTATAGGTCTCTGACTTTTGGAC-3' Reverse 5'-CTACAGAATTATAGGAATGTTGAAG-3' Probe 5'-Cy5-ATTTTACGAACACAGCAGAAT-Iowa Black RQ-3'	Real-time PCR	Ward and Bej (2006)
*tox*R	Not described	Forward 5'-GACGCAATCGTTGAACCAGAA-3' Reverse 5'-GCAAATCGGTAGTAATAGTGCCAA-3' Probe 5'-VIC-AAAGCACCTGTGGCTTCTGCTG- TAMRA-3'	Real-time PCR	Takahashi and others (2005)

Table 1.4 Molecular methods and sequences used to identify *V. vulnificus*.

Gene	Location	Sequence	Application	Reference
Cytolysin	1857-1880	5'-CTGTCACGGCAGTTGGAACCA-3'	DNA Hybridization	Yamamoto and others (1990), Wright and others (1993)
vvhA	726-1113	Forward 5'-CGCTCACTGGGGCAGTGGCTG-3' Reverse 5'-CCGTTAACCGAACGACCCGC-3'	PCR	Brauns and others (1991)
Cytolysin	3.2 kb	Entire plasmid pCVD702	DNA Hybridization	Kaysner and others (1994)
*vvh*A	Not described	5'-GAGCTGTCACGGCAGTTGGAACCA-3'	DNA Hybridization	BAM (2001)
vvhA	731-1113	Forward 5'-ACTGGGCAGTGGCT-3' Reverse 5'-GCCGTTAACCGAACCA-3' Probe 5'-ROXAACTATCGTGCACGCTTTGGTACCGT-BHQ2-3'	PCR Real-time PCR	Wang and others (1997) Panicker and Bej (2005)
vvhA	785-990	Forward 5'-CAACTTCAAACCGAACTATGAC-3' Reverse 5'-CCAGTCGATGCGAATACGTTG-3' Probe 5'-FAM-AACTATCGTGCA CGC TTTGGTACCGT-BHQ-3'	PCR Real-time PCR EMA real-time	Brasher and others (1998) Panicker and others (2004) Panicker and Bej (2005)

Table 1.4 Continued

*vvh*A	785-1303	Forward 5'-CCGCGGTACAGGTTGGCGCA-3' Reverse 5'-CGCCACCCACTTTCGGGCC-3'	PCR	BAM (2001)
16S DNA	618-641	5'- GTCTGCCAGTTTCAAATGCAGTTC-3'	DNA Hybridization	Cerda-Cuella and others (2000)
vvhA	786-990	Forward 5'-TTATGCTGAGAACGGTGACA-3' Reverse 5'-TTTTATCTAGCCCCAAACTTG-3' Probe 5'--CCGTTAACCGAACCA CCCGCAA-BHQ-3'	Real-time PCR	Campbell and Wright (2003) Panicker and Bej (2005)

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CHAPTER 2

THE ECOLOGY *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* FROM OYSTER HARVEST SITES IN THE GULF OF MEXICO

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2.1 ABSTRACT

The *Vibrionaceae* are environmentally ubiquitous to estuarine waters. Two species in particular, *V. vulnificus* and *V. parahaemolyticus*, are important human pathogens that are transmitted by the consumption of contaminated molluscan shellfish. The purpose of this study was to better characterize the ecology of these organisms in the Gulf of Mexico region in an effort to address data gaps in the 2005 *V. parahaemolyticus* and *V. vulnificus* risk assessments. We conducted a two-year (2006-2007) study in the Louisiana Gulf coast region with seasonal sampling of multiple harvest sites. At each sample time point, environmental data was collected and oyster and water samples collected for microbiological analysis. Water and oyster samples were analyzed for total *Vibrio* spp., aerobic plate count, total estuarine bacteria, and fecal coliforms using standard cultural procedures. Enumeration of total *V. parahaemolyticus* (*tlh*), *V. vulnificus* (*vvh*A), and pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) was done by colony lift DNA hybridization and pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) levels were determined by MPN-PCR. *Vibro* isolates were subjected to various phenotypic and genotyping tests. Data were analyzed using multiple regression analyses to determine which environmental parameters were significantly associated with the presence and concentration of the various microbes. All mathematical models demonstrated that water temperature was the most significant environmental parameter influencing the total numbers of *V. parahaemolyticus* and *V. vulnificus* in oysters. The relative percentage of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) strains, based on

MPN analysis, were comparatively higher when total *V. parahaemolyticus* (*tlh*) counts were lower, which occurred during the cooler seasons. The study provides more information on environmental/ecological factors as they impact the numbers and types of *V. parahaemolyticus* and *V. vulnificus* in commercially harvested Louisiana Gulf Coast oysters, and can be used to fill data gaps identified in previous risk assessment efforts.

2.2 INTRODUCTION

In the U.S., contaminated seafood is responsible for 10-19% of all foodborne disease outbreaks, with many more sporadic cases (Butt et al., 2004). The majority of these diseases are associated with the consumption of contaminated raw bivalve molluscan shellfish (Cook, 1991). Bivalves are filter feeders that concentrate microorganisms in their digestive tracts. Since shellfish are frequently consumed whole and raw, they serve as passive carriers for enteric viruses and pathogenic members of the family *Vibrionaceae*. Of the latter, *V. vulnificus* and *V. parahaemolyticus*, are important human pathogens. *V. parahaemolyticus* is usually associated with a mild gastroenteritis, and each year approximately 2,800 oyster-associated *V. parahaemolyticus* cases occur the U.S (CFSAN-FDA, 2005). Food-borne *Vibrio vulnificus* is associated with a rare disease syndrome (30-40 cases per year) mostly affecting individuals with underlying chronic diseases, but the disease has a high rate of mortality (>50%) (Strom and Paranjpye, 2000; Levine and Griffin, 1993).

Vibrio spp. are ubiquitous in estuarine waters and can frequently be isolated in high numbers from shellfish (Motes et al., 1998). Both temperature and salinity play important and interrelated roles in the levels of *Vibrio* spp. *V. parahaemolyticus* and *V. vulnificus* have been isolated from most if not all U.S. coastal regions (O'Neil et al., 1992; McLaughin et al., 2005; Tepedino, 1982), with lower densities associated with colder locations (Pacific, Canadian, and North Atlantic waters) and higher densities in warmer locations (Mid-Atlantic, Chesapeake Bay, and Gulf of Mexico waters) (Kaysner et al., 1987; O'Neil et al., 1992; DePaola et al., 1994; Cook, 1994; Wright et al., 1996; Motes et al., 1998). The Gulf of Mexico is particularly important, as this region is a key year-round source of oysters for U.S. consumers.

It appears that not all strains of *V. parahaemolyticus* and *V. vulnificus* are pathogenic to humans. For *V. parahaemolyticus*, the presence of the thermostable direct hemolysin (*tdh*) and thermostable direct related-hemolysin (*trh*) genes have been linked to pathogenicity. However, strains possessing these genes make up only a small fraction of the overall *V. parahaemolyticus* population in the marine environment (Kaufman et al., 2003; DePaola et al., 2003).

For *V. vulnificus*, putative virulence factors have been reported, but none are definitively confirmed (Strom and Paranjpye, 2000; Chiang and Chuang, 2003; Gulig et al., 2005). There does, however, appear to be an anecdotal relationship between 16S rRNA sequence and the likelihood of a being associated with disease in humans. This has served as

a means by which to genotype *V. vulnificus*. Specifically, Nilsson et al. (2003) demonstrated two different 16S rRNA types (designated *rrs* types A and B) based on differential restriction enzyme patterns in a 492 bp-amplified region of the gene. In general, the B sequence is more highly associated with clinical strains and the A sequence is associated with environmental isolates. Usually, genotype A strains outnumber genotype B strains in the environment (Nilsson et al., 2003; Vickery et al., 2007), but there appears to be a seasonal shift with a higher relative proportion of genotype B strains appearing late in the summer (Line and Schwarz, 2003). More recently, a gene of unknown function termed the virulence correlated gene (*vcg*) has also been found to be associated with strain virulence. Based on sequence polymorphisms in this gene, a separate genotyping method has been established: strains having one sequence type are primarily of clinical origin ("C," with the *vcgC* sequence) and strains with another are of environmental origin ("E," with the *vcgE* sequence) (Rosche et al., 2005). Investigators have demonstrated near 100% correlation between the two genotyping methods (Chatzidaki-Livanis et al., 2006; Warner and Oliver, 2008). Recent data suggest seasonal changes in the proportions of A (or E) and B (or C) strains (Warner and Oliver, 2008).

In 2005, the Center for Food Safety and Applied Nutrition of the U.S. FDA (2005) released a quantitative risk assessment for *V. parahaemolyticus* (VPRA) in raw oysters harvested from U.S. coastal sites. At approximately the same time, the Food and Agriculture Organization (FAO) of the World Health Organization (2005) released a quantitative risk

assessment for *V. vulnificus* in raw oysters harvest from the U.S. Gulf Coast (VVRA). Both risk assessments found water temperature to be the major factor affecting *V. parahaemolyticus* and *V. vulnificus* densities at harvest and different models were constructed to predict the levels of the organisms as a function of season. Air temperature was considered secondary to water temperature in influencing the density of these organisms. Both risk assessment documents demonstrate that the risk of illness per serving (Gulf coast summer harvests) is most influenced by the total levels of the organism at the time of harvest. This conclusion, however, assumes a stable correlation between total and pathogenic strains of *V. parahaemolyticus* and *V. vulnificus,* and their levels, at the time of harvest. There is a need to better understand the prevalence and proportions of pathogenic *V. parahaemolyticus* strains (based on the presence of the *tdh* gene) in shellfish and their harvesting waters as a function of season. Likewise, the VVRA does not consider differences in strain virulence nor, changes in proportions of A (E) and B (C) strains that may occur over the course of the year.

Clearly, there are limited data which describe the distributions of the different strain types of *V. parahaemolyticus* and *V. vulnificus* based on ecological parameters, as well as seasonal shifts and location. We hypothesize that the density of pathogenic strains is spatially and/or temporally clustered in the environment, with considerable variability in these distributions. This would undoubtedly affect overall risk estimates. Accordingly, this study was undertaken in an effort to better characterize the ecology of *V. vulnificus* and *V. parahaemolyticus* in the Gulf of Mexico region, specifically the Louisiana coast to address

such questions. The two specific study objectives included the following: (i) to quantify the levels of total estuarine bacteria, total *Vibrio* spp., specific *V. parahaemolyticus* and *V. vulnificus*, pathogenic *V. parahaemolyticus* and *V. vulnificus* strain in oysters and overlaying waters; and (ii) to investigate potential associations between environmental/ecological factors and total and pathogenic strains of *V. parahaemolyticus* and *V. vulnificus*.

2.3 MATERIALS AND METHODS

2.3.1 Bacteriological media

All bacteriological media were obtained from Becton, Dickinson and Co. (Sparks, MD) unless otherwise stated and were prepared according to manufacturer's recommendations, or altered accordingly based on requirements of the experimental design.

2.3.2 Sampling and sample collection

Oysters were collected by dredge on commercial oyster harvesting vessels from multiple Louisiana coastal locations, which included a total of 7 different sites sampled over the course of the study. For most seasons, 3 locations were sampled per season at approximately 3-month intervals over a total of two years (January 2006 through December 2007). In a few cases (winter 2006 and fall 2006), only two locations could be accessed. For each sampling event, surface and bottom water temperature and salinity were measured using a YSI model 85 salinometer (Yellow Springs, OH) and ambient air temperature was recorded

using an ACR Smartbutton temperature logger (ACR Systems Inc., Surrey, B.C., Canada), respectively. Water samples were collected in sterile 1-liter wide-mouth bottles (Nalgene, Rochester, NY) according to (APHA, 1970) during each harvesting session from the surface and 1 in above the sediment at the beginning of harvest and at the end of harvest. The first dredge of oysters was placed on the boat deck and oyster samples (15 specimens for each collection point) were taken immediately after this first harvest (time 0) and then after 2.5, 5.0, 7.5, and 10 h intervals after being held on the boat deck. Immediately after collection, the oysters were placed in burlap bags and placed in an ice chest with ice. Upon boat docking, the ice chests containing the oyster samples were shipped to North Carolina State University, by overnight courier where internal shellfish temperature (< 10^{o}C) was verified upon receipt. Microbiological analyses were begun immediately upon receipt. In most instances, this was <24 h after collection, but the time never exceeded 30 h.

2.3.3 Sample Preparation

Water samples were processed without further preparations. The oysters associated with each collection time point were washed with brushing under cold running tap water, drained, shucked, and pooled (150 to 200 g meat and shell liquor). In slight contrast to the APHA method (1970), each pool was separated into 6 oyster aliquots (representing 2 replicates per collection time point). For each aliquot, an equal amount of sterile alkaline peptone water (APW) was added to each sample followed by blending for 2 min. Additional

replications were created by doing duplicate samples of each of the 6 oyster sub-samples for each collection time point. Media inoculations were performed within 15 min of homogenization.

2.3.4 Generic Microbial Methods

Water and oysters were directly plated onto half strength modified salt water yeast extract agar (incubated at 22^{o}C for 24 hrs), thiosulfate-citrate-bile salts-sucrose (TCBS) agar (incubated at 37^{o}C for 24hrs), and modified cellobiose-polymyxin B-colistin (mCPC) agar (incubated 40^{o}C for 24 hrs) for enumeration of total estuarine bacteria, total *Vibrio* spp., and total *V. vulnificus*, respectively. Both water and shellfish samples were tested for fecal coliforms using the decimal dilution MPN method (APHA, 1998).

2.3.5 *Salmonella*

The *Salmonella* VIA test (Tecra International, Pty Ltd, St. Paul, MN), an AOAC-approved method, was used for *Salmonella* screening. Briefly, 50 ml of a 1:1 oyster homogenate was pre-enriched in 225 ml of lactose broth and incubated at 37^{o}C for 24 hrs, followed by enrichment in tetrathionate and Rappaport Vassiliadis broths (Oxoid Ltd., Hampshire, England) with incubation at 42^{o}C for 20 hrs. One ml of each enrichment broth was then transferred to pre-warmed M-broth with incubation at 37^{o}C. After 24 hrs, the *Salmonella* VIA test was performed according to the manufacturer's instructions.

Interpretation of test results was done using the Tecra Color Card to verify proper color change. Presumptively positive samples were streaked on double modified lysine iron agar (Oxoid Ltd), xylose lysine tergitol 4 and brilliant green sulfa agars and incubated at 37°C for 48 hrs prior to visualization for colonies typical of *Salmonella*.

2.3.6 Direct Enumeration of *V. parhaemolyticus* and *V. vulnificus* by colony lift hybridization

The 2000 Direct Plating Procedure outlined by the FDA/Gulf Coast Seafood Laboratory was used for the enumeration of total and pathogenic *V. parahaemolyticus* and total *V. vulnificus* (DePaola, 2004). All media and buffers were prepared in accordance with the FDA Bacteriological Analytical Manual (BAM) (DePaola, 2004). In brief, 0.2 ± 0.01 g of 1:1 homogenate and 100 µl of the 1:10 dilution were spread plated onto the surface of each of 4 and 2 dried T_1N_3 (*V. parahaemolyticus*) or VVA (*V. vulnificus*) plates, respectively. The plates were incubated at 37°C overnight followed by colony lifts done using Whatman #541filters (VWR International, Bristol, CT). Replicate filters were treated for cell lysis by microwave. The filters were neutralized using ammonium acetate buffer, treated with 1X SSC supplemented with 20 µl stock ProK (Sigma Chemical Co., St. Louis, MO) at 42°C for 30 min with shaking, and washed three times at room temperature in 1X SSC. Prehybridization was done at 55°C for 30 min with shaking, followed by hybridization in 10 ml buffer supplemented with 5 pM of *tlh* [5'-XAA AGC GGA TTA TGC AGA AGC ACT G-3', where X = alkaline phosphates conjugated 5'Amine-C6]; *tdh* [5'-XGG TTC TAT TCC

AAG TAA AAT GTA TTT G-3']; *trh* [*5'*-XAC TTT GCT TTC AGT TTG CTA TTG GCT-'3]; or *vvh*A [5'-XGA GCT GTC ACG GCA GTT GGA ACC A-3'] (DNA Technologies A/S, Demark) gene probes for 1 hr at 54°C (*V. parahaemolyticus*) or 55°C (*V. vulnificus*) with shaking. Filters were removed and rinsed 2 times in 1X SSC for 10 min at 55°C. Color development was achieved by the addition of 20 ml of NBT/BCIP solution (Roche Applied Science, Indianapolis, IN) followed by incubation at 35°C with shaking in the dark. Filters were air dried and counted; colonies were visible as dark brown spots. Control strips were used in each run, consisting of *V. vulnificus* (9075-96), a TDH+ strain of *V. parahaemolyticus* (TX2103), a TRH+ strain of *V. parahaemolyticus* (AQ4037), and a TLH+ (lacking the virulence genes) strain of *V. parahaemolyticus* (FIHES98). Presumptively positive strains were identified by aligning the hybridized filters to the plates and 5 - 10 typical colonies were streaked for isolation on VVA and TCBS agars for purification of *V. vulnificus* and *V. parahaemolyticus* isolates, respectively. After overnight incubation at 37°C, all presumptive *tlh, tdh, trh* and *vvh*A isolates were reconfirmed by DNA hybridization with the appropriate gene probe. Isolates were stored indefinitely on slants of trypticase soy agar supplemented with 2% NaCl ($TSAN_2$) with mineral oil overlay at room temperature.

2.3.7 MPN enrichments (*V. parahaemolyticus* only)

Oyster homogenates were prepared by mixing oyster meat with alkaline peptone water (APW) (1:1, w/v) and blending for one min. Thereafter, ten-fold serial dilutions were

prepared for 3-tube MPN analysis at 3 dilutions (10 g, 1.0 g, and 0.1 g) (Zimmerman et al., 2007) A similar 3-tube MPN (100 ml and 10 mll) using 1:10 dilutions of seawater was also done with 10X APW (Zimmerman et al., 2007). All enrichment tubes were incubated at 37^{o}C for 24 hrs after which the contents of each tube was serially diluted by ten-fold and plated onto TCBS incubated at 37^{o}C for 24. These plates were then used in colony lift hybridization (described above) using DNA probes specific for the *tdh* and *trh* genes.

In addition to colony lift hybridization, the enrichments were also screened for pathogenic *V. parahaemolyticus* strains using PCR. Briefly, one ml of the 10g oyster homogenate samples was centrifuged at 9700 g (Sorvall RC-5B, Dupont Company, Wilmington, DE, USA) for 15 min at 4^{o}C. The resulting supernatant was decanted and the remaining pellet resuspended in DNAzol BD Reagent (Invitrogen, Carlsbad, CA, USA) (1:2, w/v) and DNA was extracted as per manufacturer's instructions. The final washed DNA pellet was resuspended in 200 µl sterile deionized water and stored on ice until used. The 1 g and 0.1 g MPN enrichments were boiled for 10 min, chilled on ice, and immediately frozen at 20°C until PCR analysis.

The real-time PCR–MPN method described by Nordstrom et al. (2007) was applied, which consisted of a multiplex assay for the detection of the *tlh*, *tdh*, and *trh* genes of *V. parahaemolyticus*. The primer sets for each gene were synthesized by Integrated DNA Technologies (Coralville, IA), while probes were supplied by either Integrated DNA Technologies (Coralville, IA) (*tlh* probe) or Applied Biosystems (Foster City, CA) (*tdh* and

trh). Sequences were as follows: [for *tlh*: forward primer 5'-ACTCAACACAAGAAGAGATCGACAA-3'; and the reverse primer 5'-GATGAGCGCGGTTGATGTCCAAA-3'; the *tlh* fluorogenic probe 5'-TEXAS RED - CGCTCGCGTTCACGAAACCGT-3'- BHQ2]; [for *tdh*: forward primer 5'-TCCCTTTTCCTGCCCCC-3'; and the reverse primer: 5'-CGCTGCCATTGTATAGTCTTTATC-3'; with the *tdh* fluorogenic probe: 5'-FAM-TCACATCCTACATGACTGTG-3'-MGBNFQ]; [for *trh*: forward primer *trh*: 5'-TTGCTTTCAGTTTGCTATTGGCT-3'; the reverse primer: 5'-TGTTTACCGTCATATAGGCGCTT-3'; with the *trh* fluorogenic probe: 5'-TET-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ]. Real-time PCR amplification was done in a 25-µl volume using the following reaction components (final concentrations shown): 1x PCR Amplification Buffer [10x buffer consisted 200 mM Tris–HCl (pH 8.4) and 500 mM KCl] (Invitrogen, Carlsbad, CA), 9 mM $MgCl_2$, 200 nM of each of the dNTPs (Invitrogen), 300 nM of each of the primers described above, 50 nM fluorogenic probe described above, 1.25 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA), and 2.0 µl of template. Thermal cycling was done using the Smart Cycler® system (Cepheid, Sunnyvale, CA) utilizing the following parameters: 94^{o}C initial hold for 2 min to denature the DNA and activate the hot start *Taq* polymerase, followed by 45 cycles of amplification, with each amplification cycle consisting of denaturation at 94^{o}C for 10 sec followed by a combined primer annealing/ extension step at 60^{o}C for 12 sec. The accumulated fluorescence in each

Smart Cycler® reaction tube was measured by the instrument at the end of each amplification cycle, with positive samples generating a signal of at least 30 fluorescent units above baseline within 50 cycles. This assay included an internal amplification control (IAC) for the detection of possible matrix inhibition. A positive control strain (*tlh*+, *tdh*+, and *trh*+) strain (F11-3A) and a negative control (distilled water) were included in each PCR run.

2.3.8 Phenotypic tests for *V. parahaemolyticus*

V. parahaemolyticus isolates were further characterized for urease production and differing degrees of β-hemolysis. For urease production, isolates were streaked onto Christensen's Urea agar and incubated at 37°C for 24 hrs. If no color change was noted, plates were incubated for an additional 24 hrs. For β-hemolysis, isolates were streaked onto Wagatsuma agar (Remel, Lenexa, KS) and incubated at 37°C for 24 hr. For standardization of the β-hemolysis assay, a 1.0 McFarland standard was made for each strain grown on APW at 37°C for 24 hr. A filter disk was dipped into the 1.0 McFarland standard, placed onto Wagatsuma agar and incubated at 37°C for 24 hr. Finally, the clear zones were measured with an electronic Fowler Caliper to determine degree of hemolysis.

2.3.9 Phenotypic tests for *V. vulnificus*

Sorbitol and mannitol agars were made by adding 10 g of the D-isomer of each sugar, plus 2% NaCl, to phenol red base agar (Remel Inc., Lenexa, KS). Individual colonies from

freshly streaked plates of VVA (24 h) were streaked onto the mannitol and sorbitol agar plates and incubated at 37^{o}C overnight. Colors of yellow or red were classified as positive and negative for sugar fermentation, respectively. All isolates were also screened for ornithine decarboxylase, *o*-nitrophenyl-β-D-galactoside (ONPG), and indole production using tubed media as previously described (DePaola (FDA-BAM), 2004).

2.3.10 Genotypic tests for *V. vulnificus*

Two different genotyping methods were applied to all *V. vulnificus* isolates. Primers and probes used to classify strains by 16S rRNA genotype have been previously reported (Vickery et al., 2007). The primer sets for *V. vulnificus* 16S rRNA typing were synthesized by Integrated DNA Technologies (Coralville, IA), while two TaqMan® fluorogenic probes were supplied by Applied Biosystems (Foster City, CA). The 16S rRNA sequences were as follows: [Vvul16S51 forward primer 5'- CAAGTCGAGCGGCAGCA -3'; and the Vvul1651 reverse primer 5'- TCCTGACGCGAGAGGCC -3'; the genotype A fluorogenic probe 5'-6FAM-TGATAGCTTCGGCTCAA -3'- -MGBNFQ; and the genotype B fluorogenic probe 5'- TET-CCCGTAGGCATCATGC -3'; -MGBNFQ]. A 25 µl reaction volume was used per PCR reaction; final reagent concentrations were as follows: 1 x PCR amplification buffer [10 x buffer consisting of 200 mM Tris-HCl (pH 8.4) and 500 mM KCl] (Invitrogen, Carlsbad, CA), 5 mM $MgCl_2$, 300 nM of each of the dNTPs (Roche, Indianapolis, IN), 200 nM or each primer (described above), 75 nM of each fluoregenic

probe (described above), 1.25 U Platinum™ *Taq* polymerase (Invitroben, Carlsbad, CA), and 2.0 µl of DNA template. Real-time PCR thermal cycling was done using the Smart Cycler® II system from Cepheid (Sunnyvale, CA) with the following cycling parameters: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 10 sec, 62˚C for 35 sec; fluorescence was measured during annealing/extension at 62˚ with the FTTC dye set. Results were analyzed using the default settings, with the exception of the Manual Threshold Fluorescent Units setting which was changed from 30 to 15 units above background. Positive control strains representing *V. vulnificus* 16S rRNA genotype A and B strains, and a negative control (sterile dH_2O) were accompanied each PCR run.

For C and E genotyping, sequence data for the virulence correlated gene (*Vcg)* of *V. vulnificus* (Genebank accessions AY626575 – AY626584, AE016795, BA000037) was aligned using AlleleID (Premier Biosoft, Palo Alto, CA); primers and dual-labeled fluorogenic probes were designed using proprietary algorithms to discriminate the two variants from one another, with each oligonucleotide spanning a variable region in the alignment. The primer sets for each gene were synthesized by Integrated DNA Technologies (Coralville, IA), while probes were supplied by Applied Biosystems (Foster City, CA) (*vcg*E and *vcg*C). Sequences were as follows: [for *vcg*E: forward primer 5'-CTTGGTCTCAGAAAGGCTCAATTG-3'; and the reverse primer 5'-GGTGCTTTCGTTACTGCTCAATG-3'; the *vcg*E fluorogenic probe 5'-6FAM-AGTGATCCCACTCCGCCGACCGC-3'- BHQ1]; and [for *vcg*C: forward primer 5'-

CTGATGGGCGCAGTTCAAAC-3'; and the reverse primer: 5'-TAGCCTGTTCAGATGACACATTAG-3'; with the *vcg*C fluorogenic probe: 5'- TET-ACGAGATCGCTATCGGCAGCTCCT -3'-BHQ1]. Traditional PCR performed according to Rosche et al. (2005) was used to verify the real-time assay performed identically to the traditional assay.

A 25 µl reaction volume was used with final concentrations of 1x PCR buffer (20mM Tris-HCl, 50 mM KCl) (Invitrogen, Carlsbad, CA), 5 mM $MgCl_2$, 300 nM each dNTP (Applied Biosystems, Foster City, CA), 200 nM each primer, 75 nM each probe, 1.25 U Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) and 2 µl template DNA. Templates for all PCR reactions were prepared by boiling 0.5 ml pure culture for 10 min. Thermocycling was done using a Smart-Cycler II system with the following cycling parameters: 95˚C for 60 sec, followed by 40 cycles of 95˚C for 10 sec, 62˚C for 40 sec; fluorescence was measured during annealing/extension at 62˚ with the FTTC dye set. Results were analyzed using the default settings, with the exception of the Manual Threshold Fluorescent Units setting which was changed from 30 to 15 units above background.

2.3.11 Statistical Analysis

The standard correlations of Pearson and Spearman were used to evaluate the relationship between *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvh*A) and the densities of other bacterial populations (APC, total estuarine bacteria, and total *Vibrio* spp.). Replicate

counts were converted to $\log_{10}$ CFU/g (oysters) or ml (water); when counts fell below the assay limit of detection, the data were excluded from the model, so as not to skew the analysis.

Generalized linear mixed-model (GLMM) regressions were done to estimate the distributions of total *V. vulnificus* (*vvh*A), total *V. parahaemolyticus* (*tlh*) and pathogenic *V. parahaemolyticus* (*tdh* and *trh*) densities in oyster and water samples, as well as their relationship to the environmental parameters. Total *V. vulnificus* (*vvh*A) and total *V. parahaemolyticus* (*tlh*) GLMM regression models used only the levels reported at initial harvest (0 hr); while GLMM regression models for pathogenic *V. parahaemolyticus* (*tdh* and *trh*) examined the levels of all time points. Not all environmental variables were evaluated. For instance, conductivity was highly correlated with salinity and dissolved oxygen (DO) was inversely correlated with temperature; therefore, these variables were removed from all models. In univariate and multivariate analyses, the distribution of *V. parahaemolyticus* densities was assumed to be lognormal, with mean $1\log_{10}$ densities being either constant or linearly related to environmental parameters. For univariate GLMM analyses, a log transformation of the scale parameter was used to improve numerical stability and asymptotic properties of statistical estimates and derived confidence intervals. Similarly, for multivariate GLMM analyses, a spherical parameterization of the variance-covariance matrix was used (Pinheiro and Bates, 1996). Plate counts for total *V. vulnificus* and *V. parahaemolyticus* were related to "true" densities assumed by Poisson distributions. PCR-MPN data for *V.*

parahaemolyticus strains having the *tdh* and *trh* virulence genes was related to the "true" densities assumed by Binomial distributions. The fit of all GLMMs relative to the data was evaluated using a statistical deviation as a goodness-of-fit measure, and all fits were found to be adequate. A secondary model (square root model) of ambient air temperature was compared to the standard regression of the estimated growth rates for *V. vulnificus* and *V. parahaemolyticus*. All analyses were conducted using SAS software (Statistical Analysis Software, Version 8, SAS Institute, Cary, North Carolina).

2.4 RESULTS

For all oyster and water samples, fecal coliform counts were always in the acceptable range (of < 14 MPN/100 mL for water; < 1.8 MPN/g or oysters) for open harvesting areas. No water or oyster samples were found to be contaminated with *Salmonella* over the entire two year sampling period. There were many environmental parameters measured during the course of commercial harvesting of oysters, including such variables as ambient air temperature, water temperature (surface and bottom), dissolved oxygen content, salinity, conductivity and pH (Table 2.1). Average values for these parameters did not vary as a function of sample location (data not shown). There were statistically significant seasonal differences in ambient air temperature, water temperature, and salinity.

There were no statistically significant differences in microbiological data (aerobic plate count (APC), total estuarine bacteria, total *Vibrio* spp., total *V. parahaemolyticus* (*tlh*),

and total *V. vulnificus* (*vvh*A)) when comparing the counts obtained in years 1 and 2; therefore, the seasonal data for both years were averaged together. On a seasonal basis, APC and total estuarine bacterial levels in oysters were very similar to one another (Figure 2.1). The numbers of total *Vibrio* spp. were lower than the APC and total estuarine bacterial counts observed for each season; likewise, total *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvh*A) levels were slightly lower than total *Vibrio* spp. counts in oysters. Similar trends were observed for APC, total estuarine bacteria, total *Vibrio* spp., total *V. parahaemolyticus* (*tlh*), and total *V. vulnificus* (*vvh*A) levels in water, although water samples showed a higher degree of variability (Figure 2.2).

In an effort to understand the relationship between environmental parameters and microbiological counts, Pearson's and Spearman's correlation statistics were applied. The $\log_{10}$ total *V. vulnificus* (*tlh*) and *V. parahaemolyticus* (*vvhA*) counts (CFU/ml) obtained from water samples were found to be positively correlated with APC ($\log_{10}$ CFU/ml) for water ($r = 0.67$; $P < 0.05$). This is not unexpected since it is likely that *V. vulnificus* and *V. parahaemolyticus* make up a large percentage of the total aerobic bacterial load in these waters. Log_{10} *V. parahaemolyticus* counts (CFU/g) in oysters were likewise positively correlated with $\log_{10}$ *V. vulnilficus* ($r = 0.62$; $P < 0.05$) counts (CFU/g) in oysters. Estuarine bacterial counts ($\log_{10}$ CFU/g or ml) were also positively correlated with APC ($r = 0.80$; $P < 0.05$ and $r = 0.47$; $P < 0.05$) in both water and oyster samples, respectively. Interestingly, the lack of correlation between estuarine bacterial counts in water or oysters and total *V.*

vulnificus, total *V. parahaemolyticus*, or total *Vibrio* counts (log_{10} CFU/ml for water, or CFU/g for oysters) suggests that total estuarine bacterial counts might not be an appropriate metric to estimate the levels of these organisms. These results conflict with those of Pfeffer et al. (2003) who found that the levels of total estuarine bacteria were correlated with total *Vibrio* spp. counts ($r_s = 0.4.1$; $P < 0.01$) for oysters harvested from the North Carolina coast.

The levels of total *V. parahaemolyticus* (*tlh*) and total *V. vulnificus* (*vvh*A) in water were quite variable (Figure 2.3a). Based on linear modeling (GLMM analysis; 8 different models and 50 combinations), we found that, other than water temperature, no environmental variables were predictive of total *V. parahaemolyticus* (*tlh*) and total *V. vulnificus* (*vvh*A) counts in water. The temperature and salinity model predicted a 0.034 log_{10} CFU *V. parahaemolyticus*/ml increase in overlay waters per degree C increase in temperature using a "best estimate" for the water temperature parameter. For *V. vulnificus*, the same temperature and salinity model predicted a 0.12 log_{10} *V. vulnificus* CFU/ml increase in overlay waters per degree C. Clearly, *V. vulnificus* appeared to be more sensitive to small changes (increases) in water temperature than was *V. parahaemolyticus*.

Total *V. parahaemolyticus* (*tlh*) and total *V. vulnificus* (*vvh*A) levels were similar in oysters for all seasons (Figure 2.3b). Consistent with the VVRA estimates (60-70%), temperature and salinity accounted for about 77% of the variability in our data. Based on the GLMM analysis (12 different models and 60 combinations), we observed that none of the environmental variables (other than water temperature) changed the output of total *V.*

parahaemolyticus (*tlh*) and total *V. vulnificus* (*vvh*A) levels in oysters. The temperature and salinity model predicted 0.054 $\log_{10}$ *V. parahaemolyticus* CFU/g increase in oysters per degree C based on the best estimate for the water temperatures parameter. This growth prediction was lower than the best estimate of 0.10 $\log_{10}$ CFU/g *V. parahaemolyticus* in oyster per degree C for the VPRA. On the other hand, the temperature and salinity model predicted a 0.068 $\log_{10}$ CFU/g *V. vulnificus* increase in oyster per degree C based on the best estimate for the water temperature parameter, which was lower than the VVRA growth prediction of 0.10 log10 *V. vulnificus* CFU/g in oyster per degree C. In a second model, we conducted a standard regression of these estimated growth rates from each site and collection period versus air temperature. A linear relationship between the square root of the growth rate and air temperature is commonly seen in microbial growth, hence the square root model was chosen. This secondary model facilitated comparison of the estimated growth rates for *V. parahaemolyticus* (*tlh*) relative to the VPRA growth rate estimates, although there was some variation in the regression line (Figure 2.4). Likewise, the estimated growth rates for *V. vulnificus* (*vvh*A) compared to air temperature resembled the estimates used in the VVRA, which was initially based on data provided by Cook et al. (1994 and 1997) (Figure 2.5).

Four hundred and sixty nine *V. vulnificus* isolates were obtained over the course of the study; all were positive for indole production, ONPG, and ornithine decarboxylase, consistent with biotype 1 strain designations (Bisharat, et al., 1999). There were various reactions with D-mannitol and D-sorbitol (Chapter 4). Genotyping of *V. vulnificus* isolates

from water and oysters showed more genotype A or E than genotype B or C in general (see Chapter 3 for more details). For *V. vulnificus* isolates obtained from water, neither sampling location nor season appeared to impact the selection of one genotype over another (data not shown). For *V. vulnificus* isolates obtained from oysters, exposure to extended ambient air temperatures on boat decks for 0, 2.5, 5, 7.5, or 10 hr did not significantly affect the selection of one genotype over another (data not shown).

There were significant differences in the numbers of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) as a function of season for oyster samples (Figure 2.6). A total of 661 *V. parahaemolyticus* (*tlh*) and 55 pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) isolates were collected over the duration of the study. Of the pathogenic strains, 43 isolates contained both the *tdh* and *trh* genes. Only one isolate harbored only the *trh* gene and 11 isolates contained only the *tdh* gene. Phenotypically, the 54 isolates containing the *tdh* gene showed strong β-hemolysis on the Watsaguma agar compared to the one isolate with *trh* gene, which showed only weak hemolysis. All of the 44 isolates that harbored the *trh* gene also showed positive results on Christensen's Urea agar which indicates urease production. Urease production is encoded by the *ure* gene and there is evidence that the *trh* and the *ure* genes are linked (Iida et al., 1997).

2.5 DISCUSSION

This study was undertaken in an effort to better characterize the ecology of *V. vulnificus* and *V. parahaemolyticus* harvested from the Louisiana Gulf Coast region. The two specific study objectives included the following: (i) to quantify the levels of total estuarine bacteria, total *Vibrio* spp., specific *V. parahaemolyticus* and *V. vulnificus*, pathogenic *V. parahaemolyticus* and *V. vulnificus* strain in oysters and overlaying waters; and (ii) to investigate potential associations between environmental/ecological factors and total and pathogenic strains of *V. parahaemolyticus* and *V. vulnificus*. Not unexpectedly, our data showed that water temperature was the most important factor influencing the densities of *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvh*A) in oysters and overlying waters.

In fact, our data demonstrated that water temperature was the most important factor influencing the densities of *V. parahaemolyticus* and *V. vulnificus* at harvest, irrespective of other environmental parameters or the densities of competitive microflora, The seasonal and regional variation in the prevalence and levels of *V. parahaemolyticus* and *V. vulnificus* we observed has been previously described (Kelly, 1982; Motes et al., 1998; DePaola et al., 1990; Cook et al., 2002; Kelly and Stroh, 1988; Kaspar and Tamplin, 1993), with higher levels observed in the warmer months, and low or non-detectable levels in the winter. In general, *V. vulnificus* is isolated infrequently from surface water samples from the Gulf of Mexico in January through March, when water temperatures are below 20 °C (Kelly, 1982). Peak recovery of *V. vulnificus* occurs in September, and there is substantial seasonal

variation in prevalence and levels of the organism. Studies have demonstrated that during the summer months, *V. vulnificus* levels as high as 10^4 CFU/g occur in oysters harvested from the Gulf of Mexico (Cook and others 2002). For *V. parahaemolyticus*, Kaufman et al. (2003) reported counts immediately after summer harvest to range from 200 to 2,000 CFU/g, while Gooch et al. (2002) found that when water temperature at harvest was above 20 ^{o}C (April through December), the mean density of *V. parahaemolyticus* was 13,000 CFU/100 g. When water temperatures were below 20 ^{o}C (January through March), the mean density was approximately one $\log_{10}$ lower, at 1,500 CFU/100 g (Gooch et al., 2002).

There were no significant relationships between the isolation of *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) with regards to pH, turbidity, dissolved oxygen, estuarine bacteria, APC, and fecal coliforms. Only water temperature accounted for 77% of the variability in the frequency and numbers of total *V. vulnificus* and total *V. parahaemolyticus*. This finding is consistent with those of other similar but smaller scale ecological studies (Pfeffer et al, 2003; Zimmerman et al., 2007). This suggests that other as yet unidentified variable(s) may have a role in influencing the levels of human pathogenic *Vibrio* species in water and shellfish. It is possible that the presence of host organisms (such as copepods) have an effect on certain *Vibrio* spp. because of the nutritional value offered by their chitinous exoskeletons (Kaneko and Colwell, 1975; Sochard et al., 1979). It is also possible that protozoa or *Vibrio* bacteriophages influence the prevalence and levels of *V. vulnificus* and *V. parahaemolyticus* in ways not yet understood.

A seasonal shift in the proportions of *V. vulnificus* genotypes was observed in this study, and this is discussed in detail elsewhere (Chapter 3). Likewise, pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) densities in oysters were influenced by season. Unfortunately, there are limited quantitative data regarding spatial and seasonal variation in the relative concentrations of the pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) strains in waters and oysters. Previous work to characterize the abundance of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) have been done using labor intensive techniques which consisted of spread-plating on TCBS after APW enrichment followed by colony lift hybridization using alkaline phosphotase labeled *tdh* probe (FDA-CFSAN, 2005; Nordstrom and DePaola, 2003). In studies such as these, the ratios of pathogenic to total *V. parahaemolyticus* could have been affected by differing growth rates in APW or differing plating efficiencies on TCBS when comparing strains with and without virulence factors. Recently, Zimmerman et al. (2007) reported the use of the real-time MPN-PCR format enhances detection sensitivity by allowing for the inoculation of large sample portions (> 3 L of water and >30 g of oysters) and the examination of many *V. parahaemolyticus* cells (4 $\log_{10}$/g) from the APW enrichments. This was also the approach we took in this study.

We were able to detect pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) levels in oysters from all seasons except winter 2006. Zimmerman al. (2007), using the same approach, was able to detect pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) in 44% and 30% of oyster and water samples, respectively, obtained from Alabama harvesting sites. Others have

demonstrated that the levels of *tdh*-positive *V. parahaemolyticus* ranged from 10 to 20 CFU/g in 40% of oysters harvested during June and July, seasons were total levels of *V. parahaemolyticus* are very high. Further, pathogenic strains were non-detectable in oysters harvested in September (Kaufman et al., 2003). DePaola et al. (2003) found that pathogenic strains *(tdh*+) constituted a higher proportion of the total *V. parahaemolyticus* population when water temperatures and total *V. parahaemolyticus* levels were lower. While no clear trends are yet apparent, seasonal changes are likely. This may be due to, at least in part, to the higher *V. parahaemolyticus* (*tlh*) levels relative to the pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) strains. One can speculate that the pathogenic strains were not competing with the non-pathogenic strains for nutrients at high densities of *V. parahaemolyticus* (*tlh*).

Regarding the possibility for differential growth rates of *V. parahaemolyticus* strains as a function of the presence or absence of virulence factors, a recent study by Mudoh et al. (2008) addressed this issue. These investigators used the Baranyi D and linear models to estimate the maximum growth rate (GR) of total *V. parahaemolyticus* (*tlh*) and pathogenic *V. parahaemolyticus* (*tdh*+ and/or *trh*+) strains at various storage temperatures. For instance, they provided GR estimates for *tlh*+ *V. parahaemolyticus* at 5, 10, 15, 25, and 30^{o}C of 0.0005, 0.015, 0.061, 0.12, and 0.17 $\log_{10}$ CFU/h, respectively. On the other hand, the best estimates of GR for *tdh*+ and *trh*+ *V. parahaemolyticus* strains at 5, 10, 15, 25, and 30^{o}C was 0.006, <0.001, 0.16, 0.27, 0.15; and 0.024, 0.006, 0.25, 0.21, 0.16 log MPN/h, respectively. Based on this study, it appears that pathogenic *V. parahaemolyticus* strains multiply more

rapidly at moderate temperatures (15-25^{o}C) compared to nonpathogenic strains, but more information is needed, in particular with respect to the impact of competition on growth rate.

Looking at the VPRA, the total levels of *V. parahaemolyticus* at harvest were predicted to be 2.1x 10^3, 2.2 x 10^2, 5.2 x 10^1, and 9.4 x 10^2 CFU/g for summer, fall, winter, and spring, respectively for oysters harvested from the Gulf Coast region which was similar to levels we found in our study. During summer months in the Gulf Coast region, the VPRA model predicted mean pathogenic *V. parahaemolytius* (*tdh* and/or *trh*) levels of 3.6 CFU/g at consumption; while we observed a range of 0.036 to 11 MPN/g in *V. parahaemolyticus* levels at harvest during the summer and the same ranges in *V. parahaemolyticus* levels after 10 hr at ambient air temperatures during commercial oyster harvesting. This data suggests that there is no clear trend in the presence or levels of pathogenic *V. parahaemolyticus* in oysters. The VVRA estimated levels of *V. vulnificus* at harvest in the U.S. Gulf Coast were 5.6 x 10^3 CFU/g during the summer which is similar to our data. However, the VVRA assumes that all strains of *V. vulnificus* are equally virulent which may not be the case. If *V. vulnificus* genotype B is more often associated with clinical cases then there will be a need for an adjustment in the current dose-response model.

In conclusion, these results demonstrate the seasonal variations in the densities of *V. vulnificus*, total *V. parahaemolyticus,* and pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) in Gulf Coast oysters and overlaying waters. The apparent presence of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) strains during all seasons suggests that the prevalence of

pathogenic *V. parahaemolyticus* strains (*tdh* and *trh*) may be higher than previously believed for Louisiana Gulf Coast oysters. However, there is an obvious seasonal effect on the prevalence of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) which suggests that these pathogenic strains may be poor competitors, particularly in the presence of high numbers of non-pathogenic total *V. parahaemolyticus* strains. The use of total *V. parahaemolyticus* as a surrogate for risk predictions may not be reliable. The seasonal shift in *V. vulnificus* genotypes answers a specific data gap in the VVRA which suggest that all strains are equally virulent. All of the data in this study helps answers specific data gaps or assumptions made in the current VPRA and VVRA.

2.6 ACKNOWLEDGEMENTS

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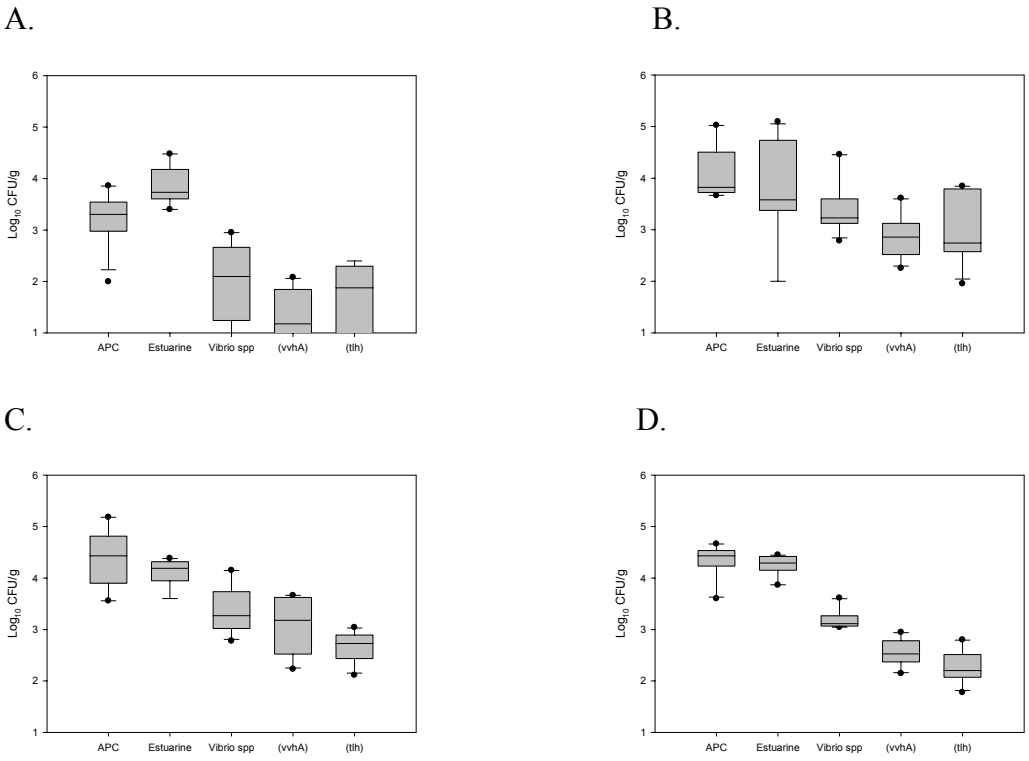
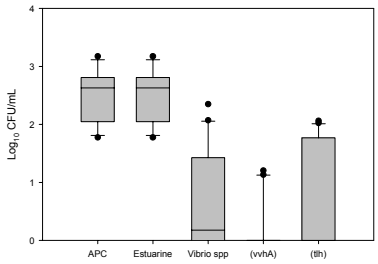


Figure 2.1. The levels of aerobic plate count (APC), total estuarine bacteria (Estuarine), total Vibrio spp., *V. vulnificus* (*vvh*A), and total *V. parahaemolyticus* (*tlh*) based on seasons (A. Winter, B. Spring, C. Summer, and D. Fall) in oysters.

A.



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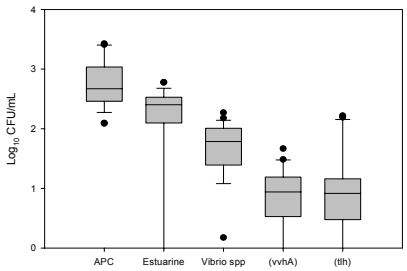

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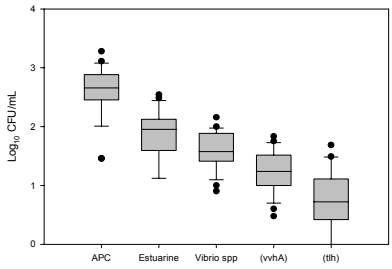

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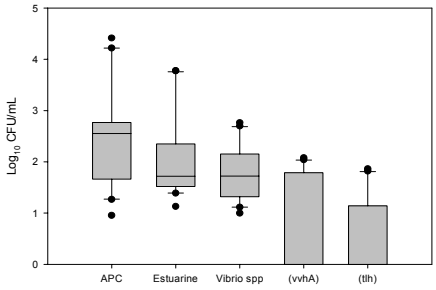
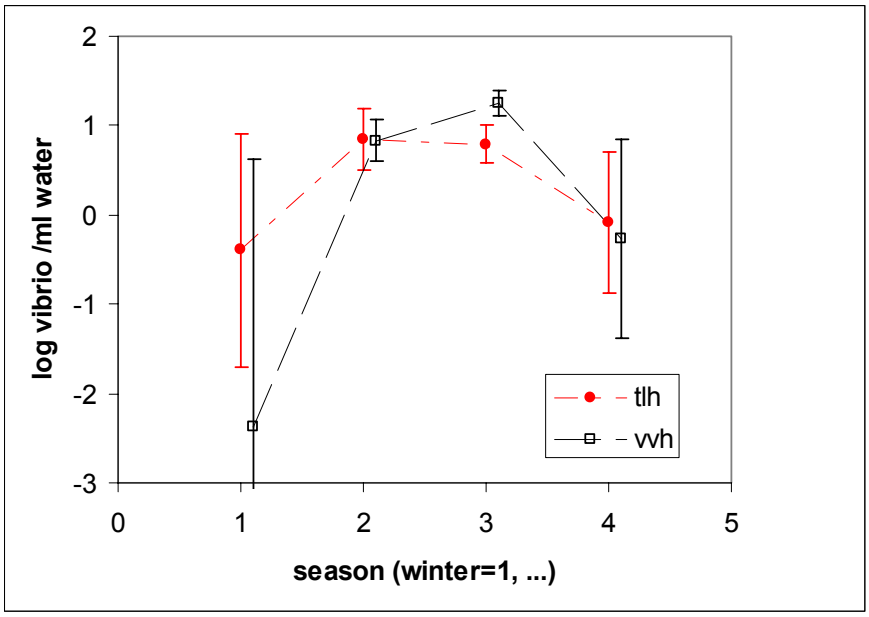


Figure 2.2. The levels of aerobic plate count (APC), total estuarine bacteria (Estuarine), total Vibrio spp., *V. vulnificus* (*vvh*A), and total *V. parahaemolyticus* (*tlh*) based on seasons (A. Winter, B. Spring, C. Summer, and D. Fall) in water.

A.



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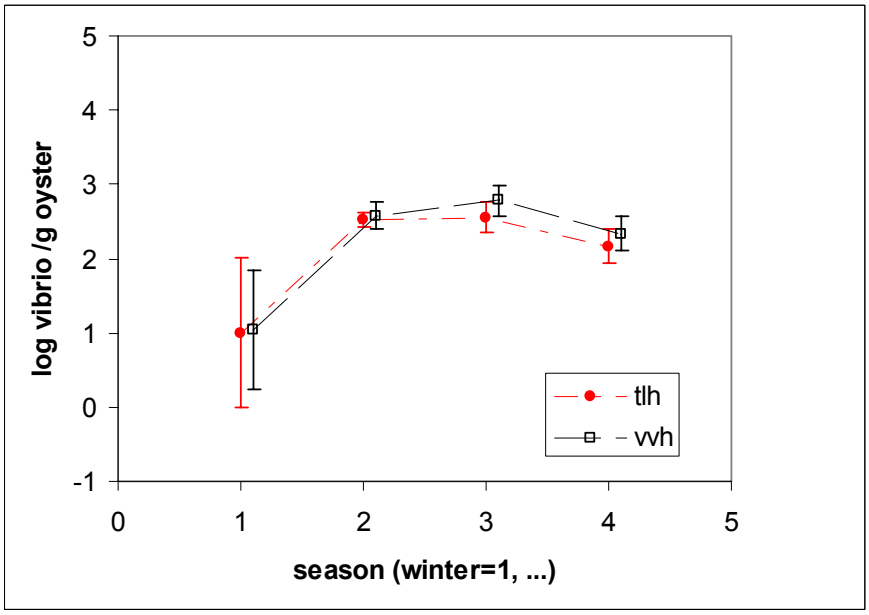

Figure 2.3. Levels of total *V. parahaemolyticus* (*tlh*) and total *V. vulnificus* (*vvh*A) for each season (A. water and B. oysters).

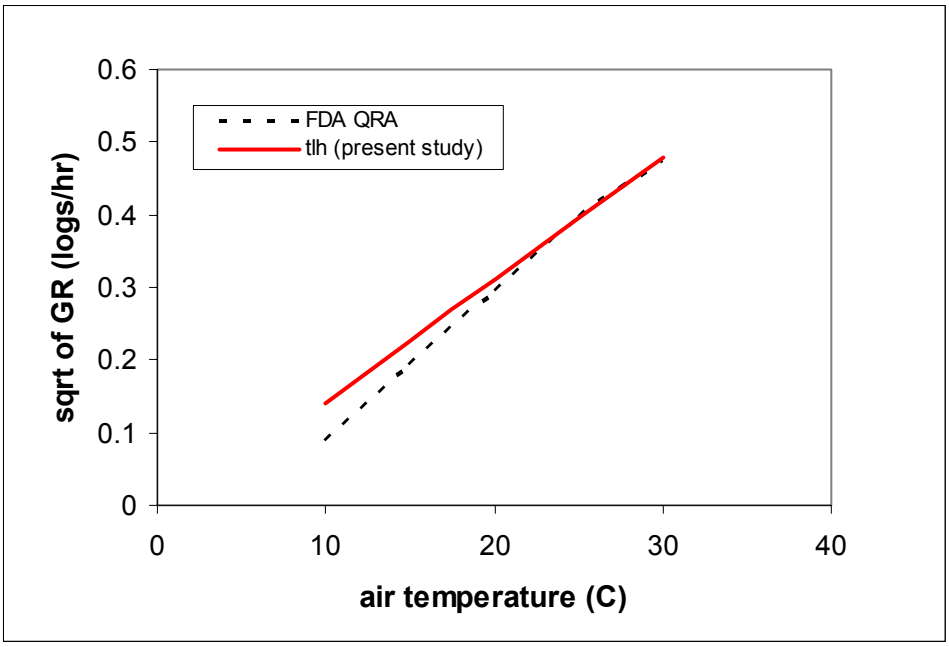

Figure 2.4. Best estimated growth rate (square root model) of *V. parahaemolyticus* (*tlh*) versus ambient air temperature based on data obtained in this study versus estimates obtained in the VPRA.

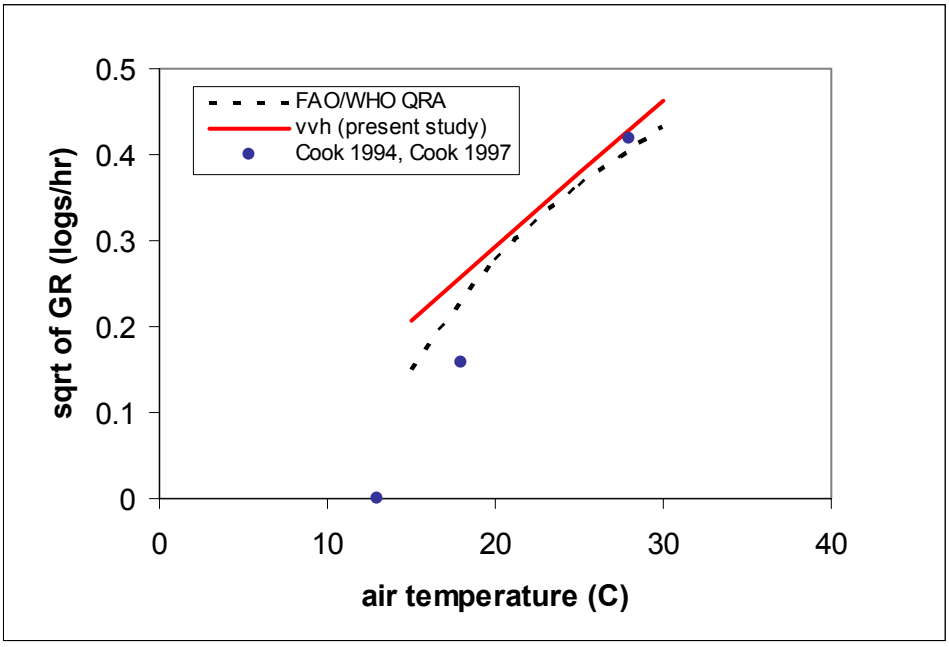

Figure 2.5. Best estimated growth rate (square root model) of *V. vulnificus (vvhA)* versus ambient air temperature based on data obtained in this study versus estimates obtained from the VVRA and Cook et al. (1994 and 1997) data.

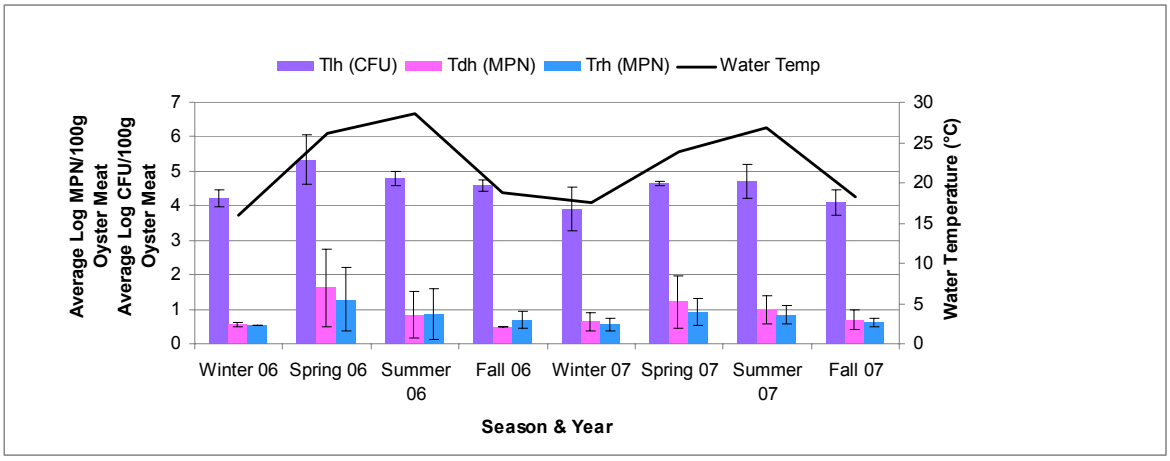

Figure 2.6. At harvest levels of pathogenic *V. parahaemolyticus* (*tdh* and *trh*) in oysters based on MPN-PCR enumeration versus non-pathogenic *V. parahaemolyticus* (*tlh*) using direct plate colony lift DNA hybridization.

Table 2.1. Environmental parameters that were measured during commercial harvesting of oysters for each season over the 2 years.

	Air Temp. (°C)	Water Temp. from Surface (°C)	Water Temp. from Bottom (°C)	DO from Surface (mg/L)	DO from Bottom (mg/L)	Salinity from Surface (ppt)	Salinity from Bottom (ppt)	Conductivity from Surface (mS)	Conductivity from Bottom (mS)	pH from Surface	pH from Bottom
Winter 2006	14.4 ± 2.2	16.2 ± 2.4	16.2 ± 2.4	5.9 ± 4.3	5.9 ± 4.3	16.2 ± 4.7	17.2 ± 4.3	NA	NA	8.3 ± 0.1	8.2 ± 0.1
Spring 2006	22.8 ± 3.2	26.0 ± 0.9	26.3 ± 0.5	5.7 ± 0.4	6.1 ± 0.4	18.2 ± 4.9	18.1 ± 4.9	31.2 ± 6.8	30.7 ± 6.4	8.2 ± 0.2	8.2 ± 0.2
Summer 2006	25.8 ± 1.3	28.6 ± 1.3	28.5 ± 0.8	6.0 ± 0.6	6.1 ± 0.6	15.3 ± 1.1	16.6 ± 0.9	27.2 ± 2.1	28.3 ± 1.2	8.1 ± 0.1	8.0 ± 0.1
Fall 2006	18.3 ± 2.4	18.6 ± 0.8	18.7 ± 0.7	7.5 ± 0.5	7.6 ± 0.4	17.5 ± 2.5	18.7 ± 2.1	24.8 ± 2.9	25.9 ± 3.1	7.7 ± 0.3	7.9 ± 0.2
Winter 2007	16.8 ± 0.5	15.0 ± 0.4	15.8 ± 0.2	8.4 ± 0.4	8.7 ± 0.3	10.1 ± 0.4	10.1 ± 0.4	15.5 ± 1.6	15.4 ± 1.7	8.0 ± 0.2	8.1 ± 0.1
Spring 2007	20.8 ± 2.3	24.2 ± 2.3	23.5 ± 2.1	7.4 ± 1.5	7.9 ± 1.5	14.9 ± 4.9	15.2 ± 4.9	23.5 ± 6.5	22.3 ± 5.2	8.1 ± 0.1	8.0 ± 0.1
Summer 2007	26.3 ± 2.0	26.8 ± 1.1	26.8 ± 1.2	5.6 ± 0.6	5.7 ± 0.4	16.2 ± 0.6	16.5 ± 0.7	26.5 ± 1.0	26.9 ± 0.9	7.9 ± 0.3	8.0 ± 0.1
Fall 2007	21.0 ± 1.6	19.9 ± 1.0	19.8 ± 0.8	6.8 ± 0.6	6.6 ± 0.4	18.9 ± 1.0	19.7 ± 1.1	29.2 ± 2.1	30.9 ± 2.8	7.9 ± 0.1	7.9 ± 0.4

DO = dissolved oxygen
NA = not applicable

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CHAPTER 3

CHANGES IN THE LEVELS OF *V. VULNIFICUS* DURING COMMERCIAL HARVESTING OF GULF COAST OYSTERS

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3.1 ABSTRACT

Molluscan shellfish harvesting guidelines stipulate refrigeration within a defined time based on harvest season. Nonetheless, even the more stringent summer guidelines allow for extended storage of oysters at ambient temperatures, providing an opportunity for bacterial proliferation. The purpose of this study was to evaluate the impact of on-deck (ambient) storage time on the levels and types of *Vibrio vulnificus* in commercially harvested oysters as a function of season. Oysters were harvested seasonally from multiple U.S. Louisiana Gulf Coast sites over 2 years. Samples were taken at the beginning of harvest and 2.5, 5.0, 7.5, and 10 h after holding at ambient conditions on the boat deck. Oyster samples were chilled after above ambient holding periods and were processed for enumeration of *V. vulnificus* (*vvhA* gene) using colony lift hybridization. Representative *V. vulnificus* isolates were subjected to genotyping for proposed virulence markers using two different methods (16S rRNA sequencing and virulence correlated gene). Statistical relationships were established by ANOVA. Ambient air temperatures ranged from 10-15^{o}C, 16-21^{o}C, and 26-29^{o}C for winter, spring/fall, and summer, respectively. During summer, the mean increase in *V. vulnificus* were 1.4 $\pm$ 0.26 $\log_{10}$ (CFU/g) after 10 h storage; for spring/fall oysters, a 1.0 $\log_{10}$ increase was observed; and in winter, increases were <0.5 $\log_{10}$. Statistically significant ($p \leq 0.05$) correlations between ambient air temperature and increases in *V. vulnificus* counts were noted. For most seasons, the increase in levels of *V. vulnificus* during on-deck storage exceeded those predicted in the recent *V. vulnificus* risk assessment (VVRA). Summer *V.*

vulnificus isolates contained a higher percentage of rRNA sequence genotype B (52%) and the *vcg*C (52%) strains, which appear to be more often associated with clinical illnesses, while spring/fall isolates were more often of genotype A (80%) or E (86%). These seasonal differences in strain type were statistically significant ($p < 0.05$). The study provides information about the impact of commercial harvesting conditions on the levels and strain types of *V. vulnificus* in Gulf Coast oysters and should be useful in future risk assessment iterations.

3.2 INTRODUCTION

Members for the *Vibrio* genus are ubiquitous in estuarine environments and can frequently be isolated in high numbers from molluscan shellfish (Motes et al., 1998). Three species are associated with human disease: *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The latter of these are associated with rare disease syndrome (30-40 documented cases per year) with a high mortality rate (>50%) and are almost always associated with molluscan shellfish consumption by individuals with underlying chronic diseases (Strom and Paranjpye, 2000; Levine and Griffin, 1993). *Vibrio vulnificus* is responsible for 95% of all seafood related deaths (Mead et al., 1999).

Vibrio vulnificus has been isolated from seawaters surrounding the U.S. and as far north as the Great Bay of Maine (O'Neil et al., 1992), as well as from a wide range of environmental sources including bivalves, crustaceans, finfish, sediment, and plankton (Kelly

1982: Oliver et al., 1982; Tamplin et al., 1982; O'Neil et al., 1992; DePaola et al., 1994). However, temperature and salinity play important and interrelated roles in the levels of *Vibrio* spp. in U.S. Gulf Coast oysters, and those harvested in the summer are of particular concern (Motes et al., 1998). In both estuarine water and shellfish, the highest concentration of *V. vulnificus* occurs at water temperatures between 20 and 30^{o}C and intermediate salinities of 5 to 25 ppt (Kasper and Tamplin, 1993; Motes et al., 1998). Nonetheless, it is documented that the organism persists year round (DePaola et al., 1994; Oliver et al., 1995).

Not all strains of *V. vulnificus* appear to be associated with disease (Stelma et al., 1992). Although putative virulence factors have been reported, none are definitively confirmed (Strom and Paranjpye, 2000; Chiang and Chuang, 2003; Gulig et al., 2005). There does, however, appear to be an anecdotal relationship between 16S rRNA sequence and the likelihood of association with disease in humans. This has served as a means by which to genotype *V. vulnificus* strains. Specifically, Nilsson et al. (2003) demonstrated two different 16S rRNA genotypes (designated *rrs* types A and B) based on differential restriction enzyme patterns in a 492 bp-amplified region of the gene. In general, the B sequence is more highly associated with strains of clinical origin while the A sequence is associated with strains of environmental origin. Studies in natural environments have found proportionally more genotype A isolates than genotype B isolates (Nilsson et al., 2003; Vickery et al., 2007), however there appears to be a seasonal shift (Line and Schwarz, 2003). More recently, a gene of unknown function termed the virulence correlated gene (*vcg*), originally identified by

randomly amplified polymorphic DNA (RAPD) analysis, has reportedly been associated with strain virulence. The nucleotide sequence of this gene exhibits several polymorphic regions, allowing for strain grouping into those of primarily clinical origin ("C," having the *vcgC* sequence) and those of environmental origin ("E," having the *vcgE* sequence). Rosche et al. (2005) developed a conventional PCR assay to discriminate C and E *V. vulnificus* strains and Chatzidaki-Livanis et al. (2006) later reported a 100% correspondence between *rrs* type B and *vcg* type C isolates, and *rrs* type A and *vcg*E isolates. In most instances, the *vcg*E isolates predominate (Warner and Oliver, 2008).

Recently, the World Health Organization (WHO) and Food and Agriculture Organization (FAO) (2005) conducted a quantitative risk assessment for *V. vulnificus* in raw oysters originating from the U.S. Gulf Coast (VVRA). Water temperature was considered the major factor affecting *V. vulnificus* density at harvest and different models were constructed to predict the levels of the organism as a function of season. Air temperature was considered secondary to water temperature. A very important consideration in the modeling was the fact that *V. vulnificus* continues to multiply after harvest unless shellstock temperatures are brought down to $<13^{o}$ C very rapidly. To illustrate the purported effect of temperature, the VVRA estimated that the highest levels of *V. vulnificus* at harvest in the U.S. Gulf Coast was 3.3 $\log_{10}$ CFU/g during the summer, and the risk assessment model predicted that *V. vulnificus* levels increased by approximately 0.7 $\log_{10}$ CFU/g during 5 hr of exposure to ambient air prior to first refrigeration. The increase did impact disease risk estimates.

Currently, molluscan shellfish harvesting and handling guidelines stipulate that oysters must be refrigerated within a defined time frame which changes seasonally. Until recently, harvesters were allowed extended time during the summer months (up to 10 h before first refrigeration), during which the oysters are usually sitting on the boat deck at ambient air temperatures. Despite the recent risk assessment estimates, there are actually limited data about how *V. vulnificus* levels change during the time from harvest (exit from the water) to first refrigeration, especially under common commercial harvesting practices. The purpose of this study was to characterize how extended ambient on-deck storage of shellstock oysters, which frequently occurs during commercial harvesting, affects the levels and types of *V. vulnificus* in Gulf Coast oysters as a function of season. Therefore, the objective study was to determine the changes in the total levels of *V. vulnificus* and the more virulent *V. vulnificus* populations as a function of season and ambient storage time prior to 1st refrigeration.

3.3 MATERIALS AND METHODS

3.3.1 Bacteriological media

All bacteriological media were obtained from Becton, Dickinson and Co. (Sparks, MD) unless otherwise stated and were prepared according to manufacturer's recommendations, or altered accordingly based on requirements of the experimental design.

3.3.2 Sampling and sample collection

Oysters were collected by dredge on commercial oyster harvesting vessels from multiple Louisiana coastal locations, which included a total of 7 different sites sampled over the course of the study. For most seasons, 3 locations were sampled per season at approximately 3-month intervals over a total of two years (January 2006 through December 2007). In a few cases (winter 2006 and fall 2006), only two locations could be accessed. For each sampling event, surface and bottom water temperature and salinity were measured using a YSI model 85 salinometer (Yellow Springs, OH) and ambient air temperature was recorded using an ACR Smartbutton temperature logger (ACR Systems Inc., Surrey, B.C., Canada). The first dredge of oysters was placed on the boat dock and oyster samples (15 specimens for each collection point) were taken immediately after harvest (time 0) and then at 2.5, 5.0, 7.5, and 10 h intervals after being held on the boat deck. Immediately after collection, the oysters were placed in burlap bags and placed in an ice chest with ice. Upon boat docking, the ice chests containing the oyster samples were shipped to North Carolina State University by overnight courier, where internal shellfish temperature ($< 10^{o}C$) was verified upon receipt. Microbiological analyses were begun immediately upon receipt. In most instances, this was <24 h after first sample collection, but the time before sample processing never exceeded 30 h.

3.3.3 Sample preparation

The oysters from each collection time point were washed with brushing under cold running tap water, drained, shucked, and pooled (150 to 200 g meat and shell liquor). In slight contrast to the APHA method (1998), each pool was separated into 6 oyster aliquots (representing 2 replicates per collection time point). This was done to eliminate the effect of a single "hot" oyster, e.g., one containing levels $\geq$10-fold higher than the mean for all oysters harvested within a 1 m^2 proximity (Kaufman and others, 2003). For each aliquot, an equal amount of sterile alkaline peptone water (APW) was added to each sample followed by blending for 2 min. Additional replications were created by doing duplicate samples of each of the 6 oyster subsamples for each collection time point.

3.3.4 Isolation of *V. vulnificus* from oysters

Isolation of *V. vulnificus* was done using the colony lift hybridization method of the U.S. Food and Drug Administration Gulf Coast Seafood Laboratory (FDA/CFSAN, 2004). All media and buffers were prepared in accordance with the FDA Bacteriological Analytical Manual (BAM) (DePaola, 2004). In brief, 0.2 $\pm$ 0.01 g of 1:1 homogenate and 100 µl of the 1:10 dilution were spread plated in duplicate onto the surface of *Vibrio vulnificus* agar (VVA) plates. The plates were incubated at 37^{o}C overnight followed by colony lift using Whatman #541 filters (VWR International, Bristol, CT). Replicate filters were treated for cell lysis by microwave. The filters were then neutralized using ammonium acetate buffer,

treated with 1X SSC supplemented with 20 µl stock proteinase K (Sigma Chemical Co., St. Louis, MO) at 42°C for 30 min with shaking, and washed three times at room temperature in 1X SSC. Pre-hybridization was done at 55°C for 30 min with shaking, followed by hybridization in 10 ml buffer supplemented with 5 pM of the *vvh*A probe [5'-XGA GCT GTC ACG GCA GTT GGA ACC A-3'] (DNA Technologies A/S, Demark) for 1 hr at 55°C with shaking. Filters were removed and rinsed 2 times in 1X SSC for 10 min at 55°C. Development was achieved by the addition of 20 ml of NBT/BCIP solution (Roche Applied Science, Indianapolis, IN) followed by incubation at 35°C with shaking in the dark. Filters were air dried and counted; colonies were visible as dark brown spots. Control strips were used in each run, consisting of *V. vulnificus* (9075-96) and a *V. parahaemolyticus* (TX2103) strain as positive and negative controls, respectively. Presumptively positive strains were identified by aligning the hybridized filters to the plates, and 5 - 10 typical colonies were streaked for isolation on modified cellobiose-polymyxin B-colistin (mCPC) agar. After overnight incubation at 42°C, all presumptive isolates were reconfirmed by DNA hybridization with the *vvh*A gene probe. Isolates were stored indefinitely in trypticase soy agar supplemented with 2% NaCl ($TSAN_2$) with mineral oil overlay at room temperature.

3.3.5 Genotypic tests

Oligonucleotides used in this study were obtained from Integrated DNA Technologies (Coralville, IA; primers) and Sigma-Aldrich (St. Louis, MO; probes) Primers and probes

used to classify strains by 16S rRNA genotype have been previously reported (Vickery et al., 2007). For C and E genotyping, sequence data for the virulence correlated gene (*vcg)* of *V. vulnificus* (Genebank accessions AY626575 – AY626584, AE016795, BA000037) was aligned using AlleleID (Premier Biosoft, Palo Alto, CA); primers and dual-labeled fluorogenic probes were designed using proprietary algorithms to discriminate the two variants from one another, with each oligonucleotide spanning a variable region in the alignment (see Chapter 4 for details). Traditional PCR performed according to Rosche et al. (2005) was used to verify the real-time assay.

Multiplexed, real-time PCR was used for both genotyping protocols. The method for 16S rRNA genotyping have been detailed elsewhere (Vickery, 2007; Chapter 4). For discrimination of the *vcg* C or E variants, the assay used the newly developed primers and probes. A 25 µl reaction volume was used with final concentrations of 1 x PCR buffer (20mM Tris-HCl, 50 mM KCl) (Invitrogen, Carlsbad, CA), 5 mM $MgCl_2$, 300 nM each dNTP (Applied Biosystems, Foster City, CA), 200 nM each primer, 75 nM each probe, 1.25 U Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) and 2 µl template DNA. Templates for all PCR reactions were prepared by boiling 0.5 ml pure culture for 10 min. Thermocycling was done using a Smart-Cycler II system (Sunnyvale, CA) with the following cycling parameters: 95˚C for 60 sec, followed by 40 cycles of 95˚C for 10 sec, 62˚C for 40 sec; fluorescence was measured during annealing/extension at 62˚ with the FTTC dye set.

Results were analyzed using the default settings, with the exception of the Manual Threshold Fluorescent Units setting, which was changed from 30 to 15 units above background.

3.3.6 Statistical analysis

The main effect tested in this study was that of amount of time that oysters remained on the boat deck prior to first refrigeration. The effect of time on *V. vulnificus* levels was evaluated by analysis of variance (ANOVA) with Fisher's least significant difference for means separation (Statistical Analysis Software, Version 8, SAS Institute, Cary, North Carolina).

3.4 RESULTS

With the exception of the winter season, there were no statistically significant differences between year 1 and year 2 data and hence the two years of data were averaged on a seasonal basis (Figure 3.1). During the spring season, an increase in *V. vulnificus* levels of 0.3 to 1.1 $\log_{10}$ CFU/g was noted after 10 h of on-deck non-refrigerated storage (Figure 3.1b). Ambient air temperature for this sampling season ranged from 18-24^{o} C. This increase in *V. vulnificus* levels was statistically significant when comparing the 0 and 10 h sampling time points. For the summer season, there was a 1.2 to 1.9 $\log_{10}$ CFU/g increase in *V. vulnificus* levels after 10 h (Figure 3.1c), with water temperatures of 28.5 $\pm$ 1.1 ^{o}C and ambient air temperature of 26.5 $\pm$ 1.5 ^{o}C (Figure 3.1c). However, there was no shift in the ratio of

genotype A (and C) compared to B (and E) based on the 16S rRNA and *vcg* when comparing storage temperature.. Again, the increase in *V. vulnificus* levels was statistically significant over extended storage at ambient air temperatures. In the fall season, a 0.4 to 1.1 $\log_{10}$ increase in *V. vulnificus* levels after 10 h of on-deck storage (Figure 3.1d) was noted, which was also statistically significant. In this case, the ambient air temperatures ranged from 16-20^{o}C.

Harvest location was shown to have a significant effect on *V. vulnificus* counts ($p < 0.05$), but due to a low number of replicate sampling from the same site(s), this was only observed for a few locations and during the spring and summer seasons. In the spring, oysters harvested from two different sites [B (year 1) and E (year 2)] exhibited the highest numbers of *V. vulnificus*. In the summer, (site B, years 1 and 2) had the highest levels of *V. vulnificus* as well. There was also variability in site location for one season (fall, year 2). In this case, two sampling sites averaged a 0.6 $\log_{10}$ increase in *V. vulnificus* levels after 10 h, which was not considered a statistically significant increase. However, in the third site, the organism was barely detectable and no increase was seen during on-deck storage (data not shown). Sites B and E were harvested in early November and the ambient air temperature was 20^{o}C for these two sites; on the other hand, site C was harvested in early December with an ambient air temperature of 14^{o}C. This significant difference was probably associated with substantial differences in water (20 ^{o}C vs 15 ^{o}C) and ambient air (20 ^{o}C vs 14.8 ^{o}C) temperatures for November and December, respectively.

A total of 281 naturally-occurring strains of *V. vulnificus* in oysters were obtained over the course of this study. All of these isolates were obtained from oysters collected in spring, summer and fall seasons; no winter isolates were obtained for either sampling year. Figures 3.2 and 3.3 detail the proportion of strain types as a function of season and year (Figures 3.2a, 3.2b, 3.3a, and 3.3b). When comparing years, the proportion of genogroups A and E relative to B and C, were quite different for the summer season. For example, in the summer, isolates were classified as genotype A (24% and 53% for years 1 and 2, respectively); genotype B (71% and 32% for years 1 and 2, respectively); and genotype A/B (5% and 15% for years 1 and 2, respectively). Using the *vcg* method, results were similar, with 28% and 65% of strains classified as genotype E (for years 1 and 2, respectively), while 74% and 33% being genotype C (for years 1 and 2, respectively). When combining the seasonal data from both years (Figures 3.2c and 3.3c), it appears that there were proportionally more genotype B (and C) strains relative to A (and E) strains during the summer season, although a high degree of variability between the years is noted. Unfortunately, we were unable to analyze if the proportions of A and B genotypes changed as a function of time of on-board (ambient) storage because of small number of isolates obtained for any one sampling location or time point.

3.5 DISCUSSION

The purpose of this study was to characterize how extended boat deck storage of shellstock, which frequently occurs during commercial harvesting, affects the levels and

types of *V. vulnificus* in Gulf Coast oysters as a function of season. As expected, we observed the greatest increase in *V. vulnificus* levels in the summer season (range of 1.2- 1.9 $\log_{10}$ CFU/g) after 10 h storage. Spring and fall increases in *V. vulnificus* levels were similar (0.4 to 0.9 $\log_{10}$). In the winter season we observed minimal if any *V. vulnificus* proliferation during extended deck storage at ambient air temperatures. *Vibrio vulnificus* isolates obtained during the summer months had a proportionally higher percentage of genotype B strains (52%), while spring/fall isolates were more often genotype A (80%).

According to the VVRA, the highest estimated levels of *V. vulnificus* immediately after oyster harvest in the Gulf of Mexico would be 1.6, 3.4, 3.7, and 2.7 $\log_{10}$ CFU/g during the winter, spring, summer, and fall seasons, respectively (WHO-FAO, 2005). It is important to note that in Louisiana the harvesting areas are very remote. It takes several h (2-3 h) to reach these harvesting sites and get back to the boat docks for unloading; therefore, there is the issue of longer times for oysters to go unrefrigerated in this state. Overall, the initial levels of *V. vulnificus* that we found in this study were similar to those predicted by the VVRA.

However, it is clear that extended storage of oysters at ambient air temperature has a substantial impact on the levels of *V. vulnificus* levels achieved at the end of the commercial harvest. Specifically, there was a statistically significant increase in *V. vulnificus* levels during extended boat deck storage for most seasons. This is well documented in the literature (Cook, 1994; Cook, 1997). In an early study, Cook (1999) monitored the levels of *V.*

vulnificus in commercially harvested oysters held at ambient temperatures in a laboratory setting at a storage temperature of 24-32^{o}C. After 3.5, 7, 10.5, and 14 h, the investigator reported increases of 0.75, 1.30, 1.74, and 1.94 log units, respectively. Our data are more or less consistent with those of Cook (1997). Furthermore, the risk assessment model which uses data from Cook (1994 and 1997) predicted that *V. vulnificus* levels increased substantially during post-harvest storage, with predicted increases of 0.1, 0.5, 0.7, and 0.2 $\log_{10}$ CFU/g in the winter, spring, summer, and fall seasons, respectively, assuming a 5 h lapse between harvest and first refrigeration. For the winter months, we observed that *V. vulnificus* levels increased by an average of 0.4 ± 0.3 $\log_{10}$ CFU/g over 5 h, which is similar to prediction of the VVRA. In the spring season, we observed a mean increase of 0.2 ± 0.1 $\log_{10}$ CFU/g *V. vulnificus* over 5 h, a value which is lower than the VVRA prediction of 0.5 $\log_{10}$ CFU/g *V. vulnificus*. However, in the summer, there was a mean 1.0 ± 0.2 a $\log_{10}$ increase in *V. vulnificus* levels after 5 h of extended boat storage at ambient air temperature, which was higher than the VVRA predication of 0.7 a $\log_{10}$ increase. In the fall season, we observed *V. vulnificus* counts increased by of 0.4 ± 0.1 $\log_{10}$ CFU/g over 5 h, which was higher than the VVRA prediction of a 0.2 $\log_{10}$ CFU/g increase.

The increase in *V. vulnficus* levels after 10 h of extended boated deck storage was notable. Until recently, Louisiana oyster harvesters were able to stay out for a total of 10 h prior to requiring refrigeration of their product in the summer season. To illustrate how this impacts *V. vulnificus* for the winter months, we observed levels of *V. vulnificus* to range from

a low of non-detectable (limit of detection 10 CFU/g) to a high of 2.9 $\log_{10}$ CFU/g after 10 h of on-deck storage a ambient temperatures, an increase ranging from 0.3 to 1.0 $\log_{10}$ CFU/g, compared to the VVRA prediction of a 0.1 $\log_{10}$ increase during the winter season. In the spring season of year 1, the lowest *V. vulnificus* levels were 2.8 $\log_{10}$ CFU/g, while the highest were 3.6 $\log_{10}$ CFU/g, but this only constituted a maximum increase of 1.6 $\log_{10}$ from levels immediately after harvest in *V. vulnificus* levels, also higher than the VVRA prediction of a 0.5 $\log_{10}$ increase in *V. vulnificus* levels at the time of consumption. In the summer, on-deck increases resulted in total *V. vulnificus* counts ranging from a low of 3.5 $\log_{10}$ CFU/g to a high of 4.5 $\log_{10}$ CFU/g after 10 h of on-deck storage at ambient conditions, which was higher (1.9 $\log_{10}$) than predictions in the VVRA (a 0.7 $\log_{10}$ CFU/g increase). In the fall season, *V. vulnificus* counts ranged from a low of 2.0 $\log_{10}$ CFU/g to a high of 3.6 $\log_{10}$ CFU/g after 10 h of on-deck storage, with on-deck storage increases ranging from 0.5 to 1.3 $\log_{10}$ CFU/g, again higher than the VVRA prediction of a 0.2 $\log_{10}$ CFU/g increase. Overall, our data demonstrated higher *V. vulnificus* counts occurring as a consequence of on-board storage of oysters at ambient temperature for all seasons, when compared to VVRA predicted values. However, it should be noted that the VVRA assumed that oysters were only unrefrigerated on average for 5 h. Furthermore, the VVRA took into account potential reductions in *V. vulnificus* populations that might occur during extended cold storage over the entire 2 week shelf-life of shellstock oysters. Our study was not designed to evaluate further decreases in populations which might occur during such extended post-harvest storage.

We isolated proportionally more strains of environmental origin (genotypes A or E) than strains associated with clinical disease (genotypes B or C) with the exception of the summer season. This is certainly consistent with previous reports (Nilsson et al., 2003; Rosche et al., 2005). Further, previous studies have reported proportionally more type A strains to be isolated during early summer (June and July), while more genotype B strains were isolated in late summer (September) from oysters harvested in the Galveston, TX (Lin and Schwarz, 2003). Our year 1 summer data (September) also showed a higher percentage (71%) of genotype B strains compared to those of genotype A, but this trend was not replicated during year 2 of sampling. Interestingly, Warner and Oliver (2007) have shown that enrichment followed by plating to cellobiose-polymyxin B-colistin (CPC) agar allows for a significant ($p < 0.05$) selective advantage of genotype B or C strains over those of genotype E or A, meaning that lower proportions of genotype B strains in the summer of year 2 may be an artifact of the microbiological recovery method. Unfortunately, the Warner and Oliver (2007) data were not yet publicly available when we initiated our study.

If the seasonal shift from genotype *rrs* A or *vcg* E to genotype *rrs* B or *vcg* C in the warmer months of the years is in fact true, this may be an important consideration in future iterations of the VVRA. In its present form, the VVRA assumes that all strains of *V. vulnificus* are equally virulent. However, if there are a higher proportion of virulent strains in summertime oysters, this, along with the higher total *V. vulnificus* counts which occur in warmer weather, could substantially impact the dose-response relationship. However, it is

possible that environmental isolates of *V. vulnificus* (*rrs* A and/or *vcg* E) play a role in illness since some research has shown there to be no strain-specific differences in disease symptoms using the mouse model (Starks et al, 2000; DePaola et al., 2003). Further research on strain-specific virulence, and its impact on the dose-response relationship, is needed to clarify the importance of changes in strain proportions as they may influence disease risk.

There have been several recent changes in oyster harvesting regulations in the Gulf Coast of the U.S. In June of 2008, the length of harvesting time during the summer months (June, July, August and September) was reduced from 10 h to 5 h, although this was instituted to control *V. parahemolyticus*, not *V. vulnificus*. The ISSC established a goal of 3 *V. vulnifiucs* cases per year (60% illness reduction) by mid 2008; if these goals were not met, the oyster producers in the Gulf of Mexico region would be required to move to mitigations such as post-harvest processing of all oysters intended for raw consumption; shucking and labeling all oysters "for cooking purposes only;" or closing oyster harvesting areas. As this 60% reduction was not achieved, a *V. vulnificus* management plan must be submitted by September 2, 2008 that includes specific actions to achieve the 60% illness reduction goal. These controls will become effective on May 1, 2010 (ISSC, 2008).

Recently, a "risk calculator" was developed to aid in prediction of the degree of risk for *V. vulnificus* disease associated with the consumption of Gulf Coast oysters. The calculations in this model are based on certain critical parameters (personal communication, Angelo DePaola) (Table 3.1). In particular, the risk calculator contains baseline monthly

values for maximum time unrefrigerated (h), maximum time to cooldown (h) once under refrigeration, and the number of (raw) servings consumed by at risk consumers nationwide. To determine how changes in harvesting impact risk, the model also allows the user to enter input variables such as mean monthly air temperature (^{o}F), water temperature (^{o}F), maximum time unrefrigerated (h), and cooldown time (h). The risk calculator then predicts mean $\log_{10}$ CFU *V. vulnificus*/g, the risk of illness per 100,000 servings, the expected number of cases, and juxtaposes this to the number of expected cases based on calculations using baseline values. It also provides a quantification of the difference in disease rates relative to the baseline.

The risk calculator is a useful tool for the shellfish industry, and helps illustrate how *V. vulnificus* levels can be controlled by length of harvest time and implementation of more stringent cooling requirements. For instance, in September the current baseline values for maximum time unrefrigerated are based on a 12 h unrefrigerated harvest time and a maximum time to cool down of 10 h, which gives a predicted mean $\log_{10}$ 4.2 CFU *V. vulnificus*/g, the risk of illness 4.2 per 100,000 servings, the expected number of cases of 4.2 total cases. When the maximum time unrefrigerated is changed to 5 h and the maximum time to cool down remains at 10 h, the calculator gives a predicted mean $\log_{10}$ CFU *V. vulnificus*/g of 3.7, a risk of illness per 100,000 servings of 3.3, and an estimated number of cases at 3.2; this equates to a per-month disease reduction from baseline of 23%. If one were to use the maximum September water and air temperatures that we observed in the risk calculator, with

a 10 h harvest time (unrefrigerated) and holding the maximum time to cool down at 10 h, the calculator predicts a mean $\log_{10}$ CFU *V. vulnificus*/g of 3.4, a risk of illness per 100,000 servings of 2.9, and an expected number of cases of 2.8; together, this represents a reduction from baseline of 13%. This can be directly compared to the mean 3.4 $\log_{10}$ CFU *V. vulnificus*/g observed in our study after 10 h of on deck storage, which is lower than the predicted mean $\log_{10}$ CFU *V. vulnificus*/g of 3.7 associated with 5 h of non-refrigerated on deck storage, apparently due to the fact that our water temperature was approximately 10^{o}C cooler then the baseline water temperature. On the other hand, using the minimum September water and air temperature observed in our study, with a maximum time unrefrigerated of 5 h and a maximum time to cool down of 10 h, the calculator predicted a 3.1 mean $\log_{10}$ CFU *V. vulnificus*/g, a risk of illness per 100,000 servings of 1.9, and an expected number of cases of 1.9, which reflects an expected 42% reduction of disease risk compared to baseline values. This risk calculator helps illustrate how harvesting time and cooldown time (although we did not look at different cooling methods in this paper) will affect *V. vulnificus* levels which in turn will effect the risk per 100,000 servings.

In conclusion, the results presented here demonstrate that significant proliferation of *V. vulnificus* occurs during extended boat deck exposure at ambient temperatures during commercial oyster harvesting in the Gulf of Mexico. For most cases, the levels of *V. vulnificus* measured after harvest but before first refrigeration exceeded those predicted by the VVRA, suggesting the potential for under-estimation of risk. This effect was most

pronounced for oysters harvested during the winter months, although higher water temperatures (13.1^{o}C) were observed in our study as compared to the VVRA predictions. Although genotype B was more prevalent (71%) in the summer season of year 1, this was not the case for year 2 samples. It is possible that factors other than temperature and salinity may influence the distribution of genotypes in oysters and their overlay waters. Overall, there is a clear seasonal pattern of higher occurrence of pathogenic strains of *V. vulnificus* during the summer season; however for the other seasons there are no clear patterns of occurrence of pathogenic strains of *V. vulnificus* meaning that additional data is necessary in order to establish a definitive relationship between strain virulence and disease risk for these seasons.

3.6 ACKNOWLEDGEMENTS

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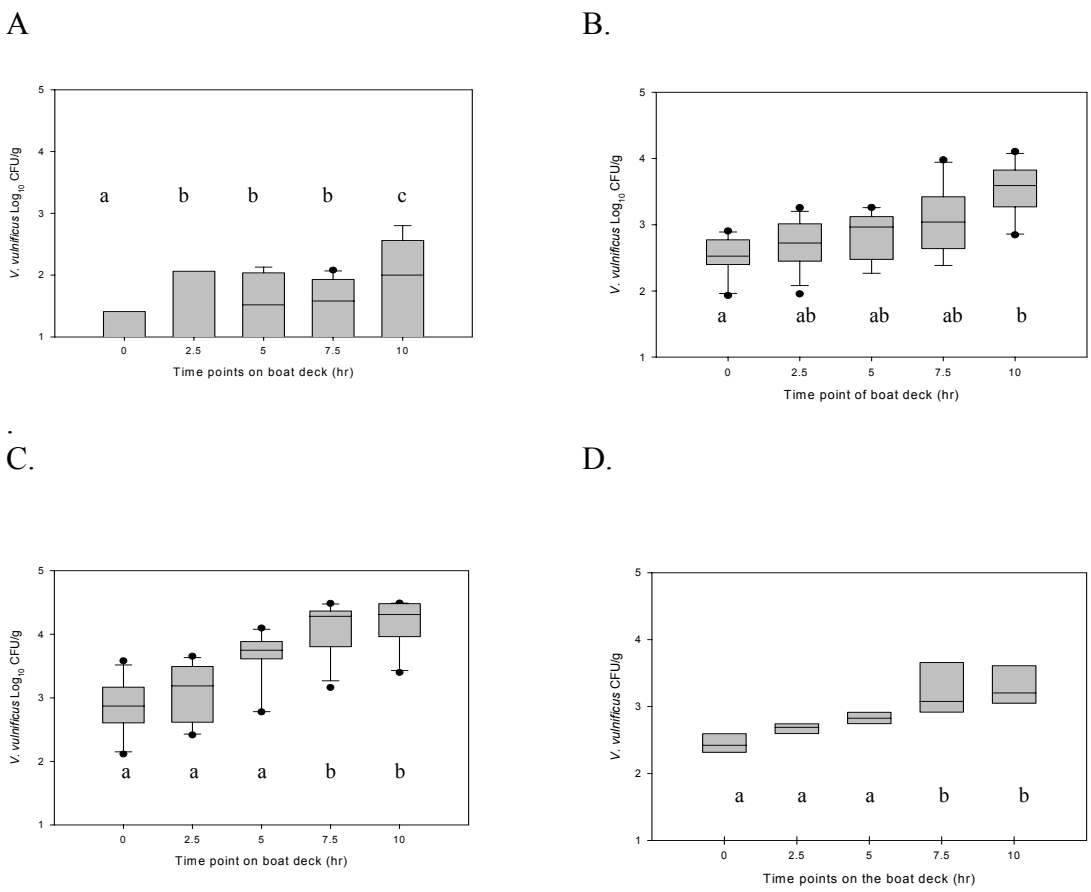

Figure 3.1. The influence of extended boat deck storage at ambient air temperatures for each season (A. winter, B. spring, C. Summer, and D. Fall). Locations are averaged together. Letters indicate statistically significant difference ($p < 0.05$).

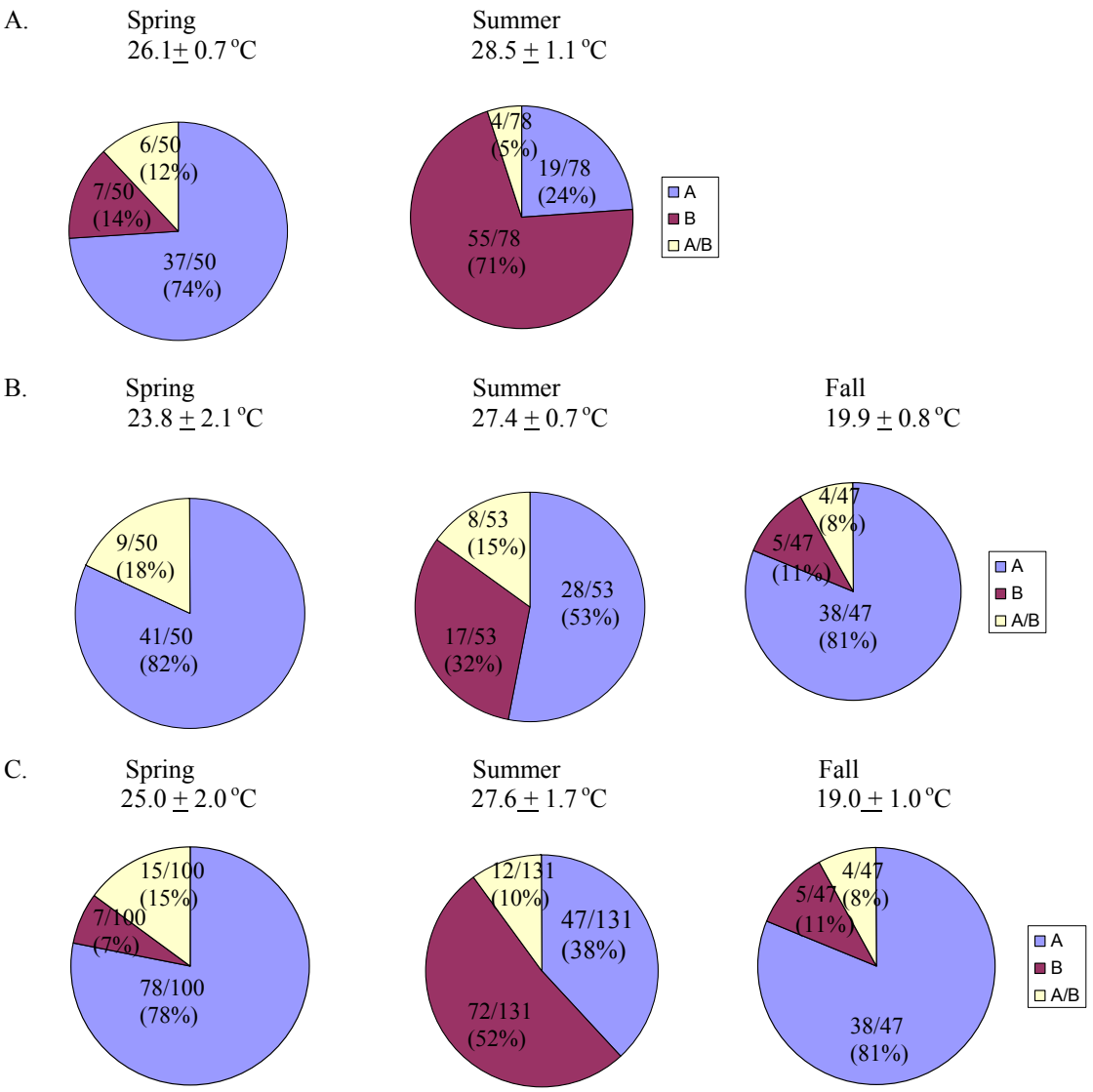

Figure 3.2. Genotypes of *V. vulnificus* isolates from oysters for each season based on the 16S rRNA gene (A. year 1, B. year 2, and C. combined). Number of isolates for each genotype are indicated. Percentages are indicated by parentheses.

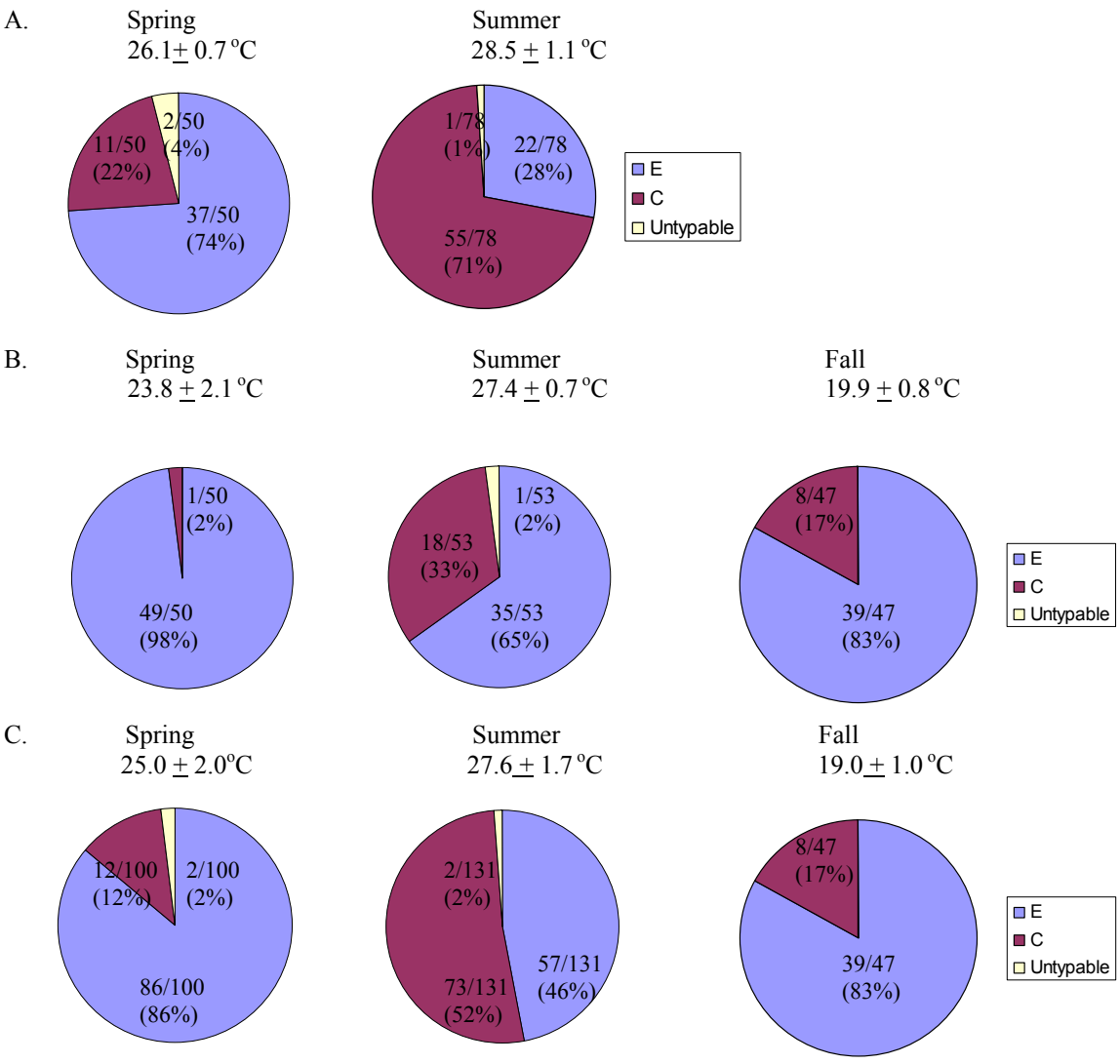

Figure 3.3. Genotypes of *V. vulnificus* isolates from oysters for each season based on the virulence correlated gene (A. year 1, B. year 2, and C. combined). Number of isolates for each genotype are indicated. Percentages are indicated by parentheses.

Table 3.1. Baseline of the *V. vulnificus* risk calculator

month	Water temperature (F)	air temperature (F)	Baseline: maximum time unrefrigerated (hr)	Baseline: maximum time to cooldown (hrs)	# of servings	mean log10 Vv/g at retail	risk (per 100,000 servings)	Expected number of cases
Jan	57	56	36	10	128,000	0.3	0.0	0.0
Feb	55	53	36	10	132,000	-0.1	0.0	0.0
Mar	67	64	36	10	151,000	2.2	0.7	1.0
Apr	71	68	14	10	131,000	2.9	1.6	2.21
May	77	75	12	10	110,000	3.8	3.5	3.8
Jun	84	82	12	10	105,000	4.5	4.8	5.0
July	85	82	10	10	97,000	4.4	4.5	4.4
Aug	84	80	10	10	88,000	4.3	4.3	3.8
Sep	81	78	12	10	99,000	4.2	4.2	4.2
Oct	79	77	12	10	127,000	4.0	4.0	5.1
Nov	70	67	14	10	146,000	2.7	1.3	2.0
Dec	55	52	36	10	149,000	-0.1	0.0	0.0
					1,463,000			31.3

Assumption that it takes 10 hr for oysters to cooldown once under refrigeration

3.7 REFERENCE

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CHAPTER 4

COMPARISON OF PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF *VIBRIO VULNIFICUS* STRAINS ISOLATED FROM OYSTERS AND WATERS IN THE GULF OF MEXICO

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Running title: Phenotypic and Genotypic Characteristics of *V. vulnificus*

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4.1 ABSTRACT

Vibrio vulnificus strains (n = 469) isolated from Gulf of Mexico oysters and waters were subjected to phenotypic and genotypic characterization. A high degree of concordance was observed between 16S rRNA type and virulence correlated gene (*vcg*) type (A to E = 92% for environmental genotype; B to C = 94% for clinical genotype) and between each genotyping method and D-mannitol fermentation (*vcg* = 84% and 16S rRNA = 91%). D-mannitol fermentation should be considered as a simple and less expensive alternative to screen *V. vulnificus* isolates for virulence potential, particularly when analyzing large strain banks.

4.2 INTRODUCTION

Vibrio vulnificus is ubiquitous in estuarine waters and can be pathogenic to humans (Strom and Paranjpye, 2000). Historically, *V. vulnificus* strains have been classified by biotyping, which combines different phenotypic, serologic, and host range characteristics. Three biotypes are currently recognized. Biotype 1 strains cause disease in humans only, have different immunologically distinct lipopolysaccharide (LPS) types, and are indole-positive (Biosca et al., 1996). Biotype 2 strains can cause disease in both humans and eels and express a common LPS type; the majority of biotype 2 strains are indole-negative (Biosca et al., 1996). Biotype 3 consists of a rare group of closely

related strains that have so far only been associated with human wound infections from handling tilapia in Israel (Bisharat et al., 1999).

Genotyping based on 16S rRNA sequence is commonly used for *V. vulnificus* strain discrimination. Nilsson et al. (2003) demonstrated two different 16S rRNA types based on differential restriction enzyme patterns in a 492 bp-amplified region of the gene. The 16S rRNA type A sequence was associated with environmental isolates and the16S rRNA type B sequence was highly correlated with clinical strains. More recently, a gene of unknown function termed the virulence correlated gene (*vcg*), originally identified by randomly amplified polymorphic DNA (RAPD) analysis was reported to be associated with strain virulence (Roshe et al., 2005). This gene exhibits several polymorphic regions and can be used to group strains into two categories, i.e., those of primarily clinical origin ("C" strains, having the *vcgC* sequence variation) and those of environmental origin ("E" strains, having the *vcgE* sequence variation). This finding has been used to design of a PCR-based assay to classify biotype 1 strains of *V. vulnificus* into these two distinct genotypes. In this study, we explored the relationships between genotype and phenotype by analyzing a large *V. vulnificus* strain bank.

4.3 MATERIALS AND METHODS

4.3.1 Sampling

Strains were isolated from oysters and water samples collected from three Louisiana coastal locations four times a year for two years (January 2006 through December 2007). Oyster and water samples were shipped overnight on ice to North Carolina State University where internal shellfish temperature (< 10^{o}C) was verified upon receipt. All microbiological analyses were begun within one day of sample collection.

4.3.4 Isolation of *V. vulnificus* from oysters

Samples were processed for isolation of *V. vulnificus* using the colony lift hybridization method of the U.S. Food and Drug Administration (FDA) (DePaola, 2004) with the *vvh*A probe [5'-XGA GCT GTC ACG GCA GTT GGA ACC A-3'] (DNA Technologies A/S, Demark). *Vibrio vulnificus* (9075-96) and *V. parahaemolyticus* (TX2103) strains served as positive and negative controls, respectively, and were included in each run. Five to ten presumptively positive colonies were chosen per plate, streaked for isolation on modified cellobiose-polymyxin B-colistin (mCPC) agar [made according to DePaola (2004)] and confirmed by DNA hybridization with the *vvh*A gene probe. A total of 469 strains of *V. vulnificus* from oysters (281) and water (188) were obtained and analyzed.

4.3.5 Phenotypic tests

All isolates were screened for ornithine decarboxylase and *o*-nitrophenyl-β-D-galactoside (ONPG) using tubed media as previously described (DePaola, 2004). A select group of isolates were screened for indole production using API 20E strips (BioMerieux, Inc., Durham, NC). Isolates were also streaked onto D-sorbitol and D-mannitol agar plates to observe fermentation. All *V. vulnificus* isolates were negative ONPG, and ornithine decarboxylase, consistent with biotype 1 (Bisharat et al., 1999) (data not shown). No biotype 3 strains were identified. Approximately 60% of the *V. vulnificus* isolates were D-mannitol negative and 40% were positive for D-mannitol fermentation.

4.3.5 Genotypic tests

Strains were also characterized by 16S rRNA genotyping using a multiplexed real-time PCR as previously reported (Vickery et al., 2007). For *vcg* variant genotyping, sequence data for the two variants (Genebank accessions AY626575 – AY626584, AE016795, BA000037) were aligned using AlleleID (Premier Biosoft, Palo Alto, CA). Primers and dual-labeled fluorogenic probes were designed to discriminate the two variants from one another, with each oligonucleotide probe spanning a variable region. All oligonucleotide sequences are provided in Table 1. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA; primers) and Sigma-Aldrich (St. Louis, MO; probes). For real-time PCR *vcg* variant typing, a 25 µl reaction volume was

used with final concentration of 1 x PCR buffer (20mM Tris-HCl, 50 mM KCl) (Invitrogen, Carlsbad, CA), 5 mM $MgCl_2$, 300 nM each dNTP (Applied Biosystems, Foster City, CA), 200 nM each primer, 75 nM each probe, 1.25 U Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) and 2 µl template DNA. Templates for all PCR reactions were prepared by boiling 0.5 ml pure culture for 10 min. Thermocycling was done using a Smart-Cycler II system (Sunnyvale, CA) with the following cycling parameters: 95˚C for 60 sec, followed by 40 cycles of 95˚C for 10 sec, 62˚C for 40 sec; fluorescence was measured during annealing/extension at 62˚ with the FTTC dye set. Results were analyzed using the default settings, with the exception of the Manual Threshold Fluorescent Units setting which was changed from 30 to 15 units above background. Conventional PCR performed according to Rosche et al. (2005) and compared to the real-time PCR method verified 100% concordance between the two assays (data not shown).

4.4 RESULTS AND DISCUSSION

Of the 469 isolates, all were typeable using 16S rRNA genotyping; four strains were not typeable by *vcg* real-time PCR and were designated "non-typeable." The typeable strains were classified as genotype A 272/465 (58%), genotype B 135/469 (29%), and genotype A/B 62/469 (13%) (Table 2). Of the 465 strains that could be typed using the *vcg* method, 303/465 (65%) were classified as genotype E while 162/465 (35%)

were genotype C. Consistent with the work of others (Nilsson et al., 2003; Warner and Oliver, 2008), the majority of isolates were identified as 16S rRNA type A or *vcg* type E. Also similar to other reports (Gordon et al., 2008), we identified 62/469 (13%) 16S rRNA A/B genotypes but no strains had both the *vcgC* and *vcgE* genes. Of these 62 16S rRNA A/B isolates, 46/62 (74%) were *vcg* type E and 16/62 (26%) were type *vcg* type C.

Confidence intervals (95%) were calculated for the difference between proportions obtained by 16s rRNA and real-time PCR (*vcg*) genotyping and D-mannitol fermentation to assess the relative concordance of the three assays (Newcombe, 1998). Test sensitivity, specificity and predictive value (Altman, 2000; Newcombe, 1998), as well as corresponding 95% confidence intervals for detecting strains with the "clinical" designation, were also calculated to compare the diagnostic value of the *vcg* real-time PCR and D-mannitol fermentation with 16S rRNA genotyping. Such calculations are dependent upon comparison of one assay to the so-called "gold standard" (Altman, 2000). Since 16S rRNA genotyping is routinely done to identify clinically relevant strains, we considered this assay to be the "gold standard" for comparing the two genotyping methods. Both genotyping assays, because they definitively classify the strains as clinical or environmental (non-clinical) were considered "gold standards" for comparison with D-mannitol fermentation. The clinical relevance of strains identified as 16S rRNA type A/B is not clear so these strains were not included in the comparative analyses. While sensitivity and specificity are inherent properties of the test, predictive

value, which varies with the prevalence of the variable being detected in the population, is considered a better indicator of test performance.

Of the 135 *V. vulnificus* isolates with the 16S rRNA B genotype, 128 (94%) were identified as genotype *vcg*C (Table 2). Conversely, of the 272 *V. vulnificus* isolates with the 16S rRNA A genotype, 250 (92%) were identified as genotype *vcg*E. Minimal variation was apparent in the 95% confidence intervals implying little difference in the ability of all three assays to correctly identify strains as falling into the "clinical" designation (Table 3). The positive and negative predictive values of the *vcg* typing method, when compared to the "gold standard" 16S rRNA method, were 0.88 (95% CI, 0.81-0.92) and 0.97 (95%CI, 0.94-0.99) respectively, supporting a high degree of concordance between the two genotyping approaches (Table 4). This finding is consistent with that of Chatzidaki-Livanis et al. (2006a, 2006b) who were the first to demonstrate a strong correlation between the 16S rRNA strain type and *vcg* variant genotyping designations.

D-mannitol fermentation was also highly predictive of genotype. Of the 135 *V. vulnificus* 16S rRNA genotype B isolates, 123 (91%) were positive for fermentation of D-mannitol, while D-mannitol fermentation was negative for 228/272 (84%) of the 16S rRNA genotype A strains and 44/62 (71%) of the 16S rRNA genotype A/B strains (Table 2). Positive and negative predictive values for the detection of strains falling into the "clinical" designation were 0.74 (95% CI, 0.66-0.80) and 0.95 (95% CI, 0.91-0.97),

respectively (Table 5). Similarly, of the 162 *V. vulnificus* isolates classified as *vcg*C genotype, 138 (85%) were positive for fermentation of D-mannitol; 258/304 (85%) genotype *vcg*E strains were negative for D-mannitol fermentation. In this case, the positive predictive value for detection of a "clinically" relevant strain was 0.75 (95% CI, 0.68-0.81) and the negative predictive value was 0.91 (95% CI, 0.87-0.94) (Table 6). Others have also reported that a high proportion of biotype 1 *V. vulnificus* strains are positive for D-mannitol fermentation (Biosca et al., 1996; Bisharat et al., 1999), but a predictive relationship between genotyping and D-mannitol fermentation has never been firmly established.

It should be clear that fermentation of D-mannitol agar is highly predictive of strain designations identified by both 16S rRNA and *vcg* gene variant genotyping as applied to *V. vulnificus*. However, the concordance between genotyping methods and D-mannitol fermentation is not absolute, perhaps due to a high degree of phenotypic and genotypic variation for this species. For example, Oliver (2005) noted that phenotypic variation is so great for *V. vulnificus* that rapid identification systems are of little value. Genotypic variability has also been demonstrated using pulsed field gel electrophoresis (PFGE), ribotyping and RAPD (Lin et al., 2003; Tamplin et al,1996; Vickery et al., 2000). Nonetheless, since D-mannitol fermentation is so much simpler than genotyping, we suggest that this may be a good preliminary screening method to discriminate the

potential virulence of *V. vulnificus* isolates, particularly when analyzing large strain banks.

4.5 ACKNOWLEDGEMENTS

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Table 4.1. Oligonucleotide primers and flourogenic probes used for this work

	Target	Sequence (5'→ 3')	Position	Reference
Primers				
Vvu16S51-F	16S rRNA	CAAGTCGAGCGGCAGCA	51-67[a, b]	Vickery et al 2007
Vvu16S221-R	16S rRNA	TCCTGACGCGAGAGGCC	221-205[a, b]	Vickery et al 2007
P1	*Vcg*	AGCTGCCGATAGCGATCT	166-173[c]	Rosche et al 2005
P2	*Vcg*	CTCAATTGACAATGATCT	157-174[d]	Rosche et al 2005
P3	*Vcg*	CGCTTAGGATGATCGGTG	433-416[c, d]	Rosche et al 2005
VcgC-F	*Vcg*	CTGATGGGCGCAGTTCAAAC	122-141[c]	This work
VcgC-R	*Vcg*	TAGCCTGTTCAGATGACACATTAG	220-197[c]	This work
VcgE-F	*Vcg*	CTTGGTCTCAGAAAGGCTCAATTG	141-164[d]	This work
VcgE-R	*Vcg*	GGTGCTTTCGTTACTGCTCAATG	283-261[d]	This work
Probes				
Vvu16SA-P	16S rRNA	6FAM-TGATAGCTTCGGCTCAA – MGBNFQ	173-189[a]	Vickery et al 2007
Vvu16SB-P	16S rRNA	TET-CCCGTAGGCATCATGC – MGBNFQ	185-170[b]	Vickery et al 2007
VcgC-P	*Vcg*	TET-ACGAGATCGCTATCGGCAGCTCCT-BHQ1	153-176[c]	This work
VcgE-P	*Vcg*	6FAM-AGTGATCCCACTCCGCCGACCGC-BHQ1	218-240[d]	This work

[a] Genbank Accession X76333
[b] Genbank Accession X76334
[c] Genbank Accession AY626575
[d]Genbank Accession AY626582

Table 4.2. Key phenotypic and genotypic characteristics of 469 naturally-occurring isolates of *V. vulnificus*.

16S rRNA gene	**Number of Strains (%)**	***Vcg* genotype**	**Number of Strains (%)**	**D-Mannitol**	**Number of Strains (%)**
A	272/469 (58%)	E	303/469 (65%)	Neg.	284/469 (61%)
B	135/469 (29%)	C	162/469 (34%)	Pos.	185/469 (39%)
A/B	62/469 (13%)	Untypable	4/469 (1%)		

Table 4.3. Confidence intervals for the difference between the proportions of *V. vulnificus* isolates identified as clinical isolates.

Assay	95% CI
16S rRNA vs. PCR (vcg)	0.02-0.04
16S rRNA vs. D-mannitol	0.01-0.06
PCR (vcg) vs. D- mannitol	0.02-0.05

Table 4.4. Sensitivity, specificity and predictive value of real-time PCR (*vcg* gene) genotyping compared with 16S rRNA strain characterization as applied to "clinical" isolates

	Estimated value	**95 % CI**	
Prevalence of 16S "clinical" isolates	0.33	0.29	0.38
Sensitivity of PCR (*vcg*) assay	0.95	0.89	0.98
Specificity of PCR (*vcg*) assay	0.93	0.89	0.96
Positive predictive value PCR (*vcg*) assay	0.88	0.81	0.92
Negative predictive value PCR (*vcg*) assay	0.97	0.94	0.99

Table 4.5. Sensitivity, specificity and predictive value of D-mannitol fermentation when compared with 16S SRNA genotyping strain characterization as applied to "clinical" isolates.

	Estimated value	**95 % CI**	
Prevalence of 16S "clinical" isolates	0.33	0.29	0.38
Sensitivity of D-mannitol assay	0.91	0.84	0.95
Specificity of D-mannitol assay	0.84	0.79	0.88
Positive predictive value of D-mannitol assay	0.74	0.66	0.80
Negative predictive value of D-mannitol assay	0.95	0.91	0.97

Table 4.6. Sensitivity, specificity and predictive value of D-mannitol plating/fermentation when compared with *vcg* genotyping as applied to "clinical" isolates.

	Estimated value	**95 % CI**	
Prevalence of 16S "clinical" isolates	0.35	0.31	0.39
Sensitivity of D-mannitol assay	0.85	0.79	0.90
Specificity of D-mannitol assay	0.85	0.80	0.90
Positive predictive value of D-mannitol assay	0.75	0.68	0.81
Negative predictive value of D-mannitol assay	0.91	0.87	0.94

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CHAPTER 5

STRAIN-TO-STRAIN DIFFERENCES IN RECOVERY OF *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* EXPOSED TO STARVATION AND COLD STRESS CONDITIONS

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Running title: Survival of *V. parahaemolyticus* and *V. vulnificus* based on genotypes

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5.1 ABSTRACT

The pathogenic *Vibrio* spp., specifically *Vibrio parahaemolyticus* and *V. vulnificus*, display strain-to-strain differences in their degree of pathogenicity. Among the total population of these organisms in estuarine environments, the so-called "pathogenic" strains (particularly those of *V. parahaemolyticus*) are greatly under-represented, although the reason(s) for this is unknown. This study was designed to explore if there were strain-to-strain differences in the survival of these organisms when subjected to environmental stresses. Four strains of *V. parahaemolyticus* with different genotypes (combinations of the *tlh, tdh,* and *trh* genes) and three strains of *V. vulnificus* with different genotypes (A, B, A/B) were subjected to starvation and cold stress over extended storage. Surviving cells were enumerated using four different culture conditions (i.e., a nutrient medium with and without sodium pyruvate supplementation, incubated under anaerobic or aerobic conditions). For *V. vulnificus*, there were no significant genotype-specific differences in recovery under either starvation or cold stress. Although there were no statistically significant differences in the recovery of *V. parahaemolyticus* by strain type over extended cold temperature storage, there were statistically significant differences in D-values when comparing different *V. parahaemolyticus* strains subjected to starvation. For example, the average D-values were 23.8 $\pm$ 1.9, 7.9 $\pm$ 0.1, 16.3 $\pm$ 1.6, and 12.9 $\pm$ 1.6 d for strains of *V. parahaemolyticus* having no virulence factors, *tdh+/trh-, tdh-/trh+, tdh+/trh+* virulence gene patterns, respectively. In some instances, culture media and conditions impacted *V. parahaemolyticus* (starvation) and

V. vulnificus (cold) recovery, extending D-values by 1-3 d when media was supplemented with sodium pyruvate and/or incubated under aerobic conditions. This study provides preliminary evidence of differential survival and recovery of *V. parahaemolyticus* on a strain-to-strain basis, which may explain in part the difficulty in recovering the more pathogenic strains from estuarine environments and molluscan shellfish.

5.2 INTRODUCTION

The *Vibrionaceae* are environmentally ubiquitous to estuarine waters, with their numbers typically peaking during the warm summer months. Two species in particular, *V. vulnificus* and *V. parahaemolyticus*, are important human pathogens that are commonly transmitted by the consumption of contaminated molluscan shellfish. Strains of both of these species can be further subdivided based on the presence or absence of putative virulence markers (*V. vulnificus*) or virulence genes (*V. parahaemolyticus*). Specifically, Nilsson et al. (2003) demonstrated two different 16S rRNA types of *V. vulnificus* (designated A and B) based on differential restriction enzyme patterns in a 492 bp -amplified region of the gene. The B sequence was more highly associated with clinical strains and the A sequence was more often associated with environmental isolates. All strains of *V. parahaemolyticus* harbor the thermolabile hemolysin (*tlh*) gene, which is used for species identification. However, it appears that the clinical and hence more "virulent" strains of *V. parahaemolyticus*is also

harbor one or both of the virulence genes designated thermostable direct hemolysin *(tdh*) and TDH-related hemolysin (*trh*).

Both *V. vulnificus* and *V. parahaemolyticus* are able to enter the so-called viable-but-nonculturable (VBNC) state upon exposure to sublethal stress, including starvation, cold temperature, and suboptimal pH (Gauthier, 2000). An early theory to explain the VBNC phenomenon was increased sensitivity to hydrogen peroxide (Whiteside and Oliver, 1997). It is well documented that injured cells frequently demonstrate increased sensitivity to the toxic effects of hydrogen peroxide, which has been ameliorated for some organisms by media supplementation with sodium pyruvate or catalase (Baird-Parker and Davenport, 1965; Rayman et al., 1978; Bogosian et al., 2000; Drake et al., 2006; Bang and Drake, 2007; Bogosian et al., 2000; Drake et al., 2006). Anaerobic storage of media does not allow accumulation of toxic hydrogen peroxide during metabolism (Carlsson et al., 1978; F. Breidt, personal communication). For example, Grimes et al. (1988) demonstrated that *V. cholerae, V. parahaemolyticus* and *V. vulnificus* strains were capable of surviving and carrying out active metabolism and growth under prolonged anaerobic incubation. Nonetheless the use of anaerobic incubation to promote the recovery of sublethally injured cells of pathogenic *Vibrio* spp. has not been investigated.

Seasonal variation in the prevalence and levels of total *V. parahaemolyticus* and *V. vulnificus* is well established, with the levels of both organisms peaking during the warm summer months, followed by a gradual reduction in the colder months of the year (Motes and

et al., 1998). Less is known about the relative proportions of pathogenic to non-pathogenic strains of these organisms as a function of temperature, season, or other mitigating circumstances. One of the reasons for this is lies in the difficulty in isolating the more pathogenic strains amongst a high background level of relatively non-pathogenic strains. In a field study in our laboratory, the counts of nonpathogenic *V. parahaemolyticus* ranged from 4.0, 5.2, 5.5, and 4.9 $\log_{10}$ CFU/100 g of oyster meat for the winter, spring, summer, and fall, respectively. On the other hand, the counts of strains with virulence markers were much lower, at <0.5, 2.5, 1.5, and 1.4 log MPN/100 g of oyster meat for the winter, spring, summer, and fall, respectively (Chapter 2). The reason(s) for this discrepancy in counts is unknown, but could be associated with strain-specific differences in survival or growth. Based on these findings, the purpose of this study was to investigate if there were virulence-factor specific differences in the survival of various strains of *V. parahaemolyticus* and *V. vulnificus* subjected to stress conditions. In so doing, we also compared various culture conditions (anaerobic/aerobic, presence or absence of sodium pyruvate in plating media) to determine if these made a difference in the recovery of sublethally injured cells.

5.3 MATERIALS AND METHOD

5.3.1 Media

All bacteriological media were obtained from Becton, Dickinson and Co. (Sparks, MD) unless otherwise stated and were prepared according to manufacturer's

recommendations, or altered accordingly based on requirements of the experimental design. All incubations were done at 37^{o}C. Alkaline pepton water (APW) was used as a diluent, trypticase soy broth supplemented with 2% NaCl ($TSBN_2$) was used for culture propagation, and trypticase soy agar supplemented with 2% NaCl ($TSAN_2$) was the base medium used for enumeration. In recovery experiments, cells were enumerated using four different media-incubation combinations, i.e., $TSAN_2$ alone with subsequent incubation under aerobic conditions; $TSAN_2$ incubated under anaerobic conditions; $TSAN_2$ supplemented with sodium pyruvate (Sigma Chemical Co., St. Louis, M.O.) at a concentration 80 mg/ml ($TSAN_2$-P) and incubated under aerobic conditions; and $TSAN_2$-P incubated under anaerobic conditions. Anaerobic conditions were achieved using GasPak Systems (Remel Inc., Lenexa, KS).

5.3.2 Strains

Three strains of *V. vulnificus* [98-641 (genogroup A), 99-738 (genogroup A/B), and 9070-96 (genogroup B)] and four strains of *V. parahaeamolyticus* [FIHES98 (*tdh-/trh-*), TX2103 (*tdh+/trh-*), AQ4037 (*tdh-/trh+*), and F11-3A (*tdh +/trh +*)] were evaluated in this study. Genotype A and A/B isolates of *V. vulnificus* were environmental in origin, while genotype B was a clinical isolate. The FIHES98 *V. parahaemolyticus* (*tdh -/trh -*) strain was clinical in origin, isolated in 1998 from a Japanese patient. *V. parahaemolyticus* TX2103 (*tdh+/trh-*) was a clinical isolate from the 1998 Texas outbreak, while *V. parahaemolyticus* AQ4037 (*tdh-/trh+*) was a clinical isolate obtained from a Japanese traveler in 1985. *V.*

parahaemolyticus F11-3A (*tdh+/trh+*) was an environmental isolate obtained from a contaminated clam originating from Washington state in 1988. All strains were provided by Jessica Jones (Gulf Coast Seafood Laboratory, FDA/CSFAN, Dauphin Island, AL) and were selected as they are frequently used control strains in real-time multiplex PCR assays. The TX2103 isolate is a pandemic *V. parahaemolyticus* O3:K6 strain.

Stock strains of *V. vulnificus* and *V. parahaeamolyticus* were maintained at room temperature on $TSAN_2$ slants with sterilized mineral oil overlays (Sigma Chemical Co., St. Louis, MO). The stock strains were transferred monthly to maintain viability. Prior to experiments, strains were grown overnight at 37°C in 10 mL $TSBN_2$, centrifuged at 10,000 x g and resuspended in 1 mL of $TSBN_2$.

5.3.3 Cold and Starvation Stress Study

To evaluate the long-term effect of cold storage, the resuspended overnight cultures of each *V. vulnificus* and *V. parahaemolyticus* strain were separately transferred to 99 ml of sterile $TSBN_2$ to reach an initial population density of approximately 10^6 CFU/ml. For cold storage studies, $TSBN_2$ was pre-chilled to 5°C before the addition of the inoculum and was kept at 5°C for the duration of the study. A similar design was used to evaluate the combined effect of starvation and cold temperature, but in this case the resuspended overnight stock cultures were inoculated into 99 mL of pre-chilled sterile artificial seawater prepared

according to Drawbridge (2003) to achieve an initial concentration of 10^6 CFU/ml, with subsequent storage at 5 ^oC in the dark.

For both treatments, subsamples were obtained every three days and plated for enumeration on $TSAN_2$ with and without sodium pyruvate supplementation and incubated at 37 ^{o} C under aerobic or anaerobic conditions. Plating for recovery continued until the counts were non-detectable (limit of detection 1 CFU/mL) or until only 10 mL of sample volume was left, whichever came first. In an effort to determine if any viable cells remained, when only 10 mL of the sample remained, the entire 10 mL volume was concentrated by centrifugation at 10,000 x g, re-suspended in 4 mL of fresh media (ASW or $TSBN_2$ for starvation or cold stress, respectively), and plated for enumeration under all four media-incubation conditions.

5.3.4 Statistical Analysis

Three replications were performed for each strain and each treatment using all four media-incubation recovery conditions and were averaged together and analyzed. All statistical analyses were done using Statistical Analysis Software (SAS) version 8.0 (SAS Institute, Cary, NC). For log linear data, D-values, defined as the time (in days) to achieve a one $\log_{10}$ reduction of the population were calculated using regression analysis (PROC REG). Statistical comparison of D-values was done by ANOVA (PROC MIXED), and the least-squares' method was used to determine significant differences ($p<0.05$). Data which

were not log linear were analyzed with (PROC NLIN) using the log transformed equation: $\log S = \log 2 - \log \{1 + e(\beta t)\}$, where S is the proportion of survivors relative to time 0 population levels. In this case, D-values were determined by the calculation $D = 2.94/\beta$ (Taormina and Beuchat 2001, Pruitt and Kamau 1993). Comparison of D-values was done by ANOVA using PROC GLM with least-squares means evaluated to determine statistically significant differences ($p<0.05$).

5.4 RESULTS

During extended refrigerated storage, no statistically significant differences in D-values were observed when comparing the counts obtained for different strains of *V. vulunificus* within any one media category (Table 5.1 and Figure 5.1). However, for all three genotypes, there were statistically significant differences in D-values when comparing recovery on aerobic media with sodium pyruvate supplementation versus the other three media, i.e., aerobic media with pyruvate supplementation provided the highest counts (and hence lengthier D-values). Recoveries were more or less equivalent using the other three media formulations and incubation conditions (aerobic, anaerobic, and anaerobic with sodium pyruvate supplementation).

Under the same cold stress conditions, there were no statistically significant differences in D-values (and hence recovery) of *V. parahaemolyticus* strains when comparing

different culture media or conditions, regardless of virulence factors (Table 5.2); D-values approximated 5 d in all cases. All *V. parahaemolyticus* strains remained detectable for 25 d (Figure 5.2).

In the starvation study, no statistically significant strain-to-strain differences in D-values was observed for *V. vulnificus*, nor were there differences for different media and culture conditions (Table 5.3). Nonetheless, when visualizing the inactivation curves (Figure 5.3), it did appear that aerobic conditions hindered recovery of starved *V. vulnificus* cells, while pyruvate supplementation along with aerobic incubation conditions favored recovery of cells.

The most dramatic results were seen for *V. parahaemolyticus* strains subjected to starvation, where statistically significant differences in D-values were observed when comparing different culture media and conditions (Table 5.4). In this case, better recovery of cells (longer D-values) was consistently achieved under aerobic conditions with sodium pyruvate media supplementation, as observed for the *tdh-/trh-, tdh-/trh+*, and *tdh+/trh+* strains. For instance, D-values for these strains were 30.8 $\pm$ 1.5, 20.0 $\pm$ 1.2, and 16.8 $\pm$ 1.6 d (for *tdh-/trh-, tdh-/trh+*, and *tdh+/trh+* strains, respectively) under aerobic conditions with sodium pyruvate supplementation, as compared to D-values of 23.8 $\pm$ 1.9, 16.3 $\pm$ 1.6, and 12.1 $\pm$ 1.9 d (for *tdh-/trh-, tdh -/trh+*, and *tdh+/trh+* strains, respectively) for cells recovered aerobically without pyruvate supplementation. Recoveries were approximately the same using the other three media formulations and incubation conditions. The D-value for the

tdh+/trh- V. parahaemolyticus strain was the shortest at approximately 8-9 d for all media and incubation conditions. This finding was very interesting since this strain was a pandemic O3:K6 strain which usually are the most stress resistant when it comes to other stresses such as heat (Andrews et al., 2003) and high pressure (Cook, 2003).

When comparing D-values of *V. parahaemolyticus* strains with different virulence markers, a number of significant differences were noted (Table 5.4). For example, *V. parahaemolyticus* cells recovered on non-supplemented media under aerobic conditions showed D-values of 23.8 $\pm$ 1.9, 7.9 $\pm$ 0.1, 16.3 $\pm$ 1.6, and 12.1 $\pm$ 1.9 d for *tdh-/trh -, tdh+/trh -, tdh -/trh+*, and *tdh+/trh +* strains, respectively. The *tdh-/trh- V. parahaemolyticus* strain (for which virulence factors were absent) remained culturable after 65 d of starvation, while the *tdh+/trh-* strain was the least persistent under starvation conditions (Figure 5.4). This strain remained culturable for only about 30 d, which was consistent across all media and incubation conditions.

We observed log linear kinetics for loss of culturability for all *V. parahaemolyticus* strains held under cold stress conditions, some strains of *V. parahaemolyticus* (*tdh+/trh-* and *tdh+/trh+*) under starvation conditions, and all strains of *V. vulnificus* held under starvation and cold stress conditions. Inactivation kinetics were not log linear for *V. parahaemolyticus* strains (*tdh-/trh-, tdh-/trh+,* and *tdh+/trh+*) exposed to starvation conditions.

5.5 DISCUSSION

Our hypothesis was that strains of *V. parahaemolyticus* and *V. vulnificus* having genes specific for virulence would have different survival characteristics under stress conditions (such as starvation and cold) relative to strains in which such virulence determinants were absent. Therefore, our purpose was to investigate if there were genotype-specific differences in the survival of *V. parahaemolyticus* and *V. vulnificus* as a function of both physiological stress and culture conditions. Few differences were noted for *V. vulnificus* except that culture conditions which included media supplementation with sodium pyruvate and aerobic incubation increased (improved) recovery of cells, as reflected in higher counts. Although few differences were seen for *V. parahaemolyticus* subjected to cold stress, there were statistically significant differences in D-values under starvation conditions, with *tdh-/trh-* strains (without virulence markers) surviving better than any of the strains with virulence genes. Interestingly, strains having the *tdh* gene were the least persistent under stress conditions.

This latter finding is perhaps the most interesting. Despite many efforts to isolate pathogenic *V. parahaemolyticus* strains (containing *tdh* and/or *trh* genes) from their natural environment (Gulf of Mexico), our group has found this to be difficult. We were only able to isolate 55 pathogenic *V. parahaemolyticus* (*tdh*+ and/or *trh*+) strains compared to the 640 generic (*tlh*+ only) *V. parahaemolyticus* isolates obtained. This is supported by the work of others. For instance, DePaola et al. (2003) reported that the levels of pathogenic strains

(*tdh*+) ranged from 10 to 20 CFU/g in 40% of the oysters harvested during early summer, for which the total *V. parahaemolyticus* counts ranged from 100 to 1,000 CFU/g from June through August. Using these numbers, this would mean that approximately 0.1 to 1% of the summer strains were actually positive for the *tdh* virulence gene. The decreased survivability of *V. parhaemolyticus* strains containing virulence markers (*tdh* and/or *trh* genes) which we observed may explain why these organisms are so difficult to isolate. In addition, DePaola et al. (2003) reported that the pathogenic (*tdh*+) strains of *V. parahaemolyticus* constituted a higher percentage of the total *V. parahaemolyticus* population when water temperatures and total *V. parahaemolyticus* levels were lower (water temperature < 15^{o}C and total *V. parahaemolyticus* were 10 CFU/g). It is also possible that pathogenic strains are relatively poor competitors compared to their non-pathogenic counterparts, although this was not specifically evaluated in our study. Recently, Mudoh et al. (2008) used Baranyi D and linear models to estimate the maximum growth rate (GR) of total *V. parahaemolyticus* (*tlh*) and pathogenic *V. parahaemolyticus* (*tdh*+ and/or *trh*+) at various storage temperatures. GR estimates for *tlh*+ *V. parahaemolyticus* at 5, 10, 15, 25, and 30^{o}C were 0.0005, 0.015, 0.061, 0.12, and 0.17 $\log_{10}$ CFU/h, respectively. On the other hand, the best estimates of GR for *tdh*+ and *trh*+ positive *V. parahaemolyticus* strains at 5, 10, 15, 25, and 30^{o}C was 0.006, <0.001, 0.16, 0.27, 0.15; and 0.024, 0.006, 0.25, 0.21, 0.16 log MPN/h, respectively. This data suggests that pathogenic *V. parahaemolyticus* may multiply more rapidly at lower temperatures (10-25^{o}C) compared to nonpathogenic *V. parahaemolyticus*.

Linear $\log_{10}$ reduction kinetics were observed for the *V. parahaemolyticus* strain with the *tdh+/trh-* virulence maker under starvation conditions. This *V. parahaemolyticus* strain lost culturability fairly rapid under starvation conditions, suggesting it was not able to adapt to starvation conditions as well as the other strains showed nonlinear kinetics. This may be another possible reason for the low prevalence of *V. parahaemolyticus* (*tdh*) strains in the environment. A tailing response (nonlinear data) has been reported for other microorganism (Palumbo et al.,1987; Condon et al.,1992; Peleg and Cole, 1998), such as that observed for other *V. parahaemolyticus* strains (*tdh-/trh-, tdh-/trh+,* and *tdh+/trh+*) subjected to starvation conditions. A possible explanation for these nonlinear curves is that variation of cold resistance can occur within a population. Furthermore, non-linear kinetics may occur because the cells are adapting to the stress. Several studies have addressed stress adaptation occurring in *Vibrio* spp. (Bryan et al., 1999; Bang and Drake, 2002; Chang et al., 2004: Lin et al., 2004; Wong et al., 2008).

Consistent with the work of others (Bang et al., 2002; Drake et al., 2006; Bang et al., 2007; Vasudevan and Venkitanarayanan, 2006), the recovery of viable cells of *V. vulnificus* and *V. parahaemolyticus* was impacted (reduced) when the cells were subjected to cold and/or starvation conditions, irrespective of strain type. Although we did not evaluate whether this was due to cell injury vs. VBNC, we observed that, in some cases, cell recovery was improved by culturing in the presence of sodium pyruvate, which quenches the toxic effects of hydrogen peroxide. Others have reported similar results (Bogosian et al., 2000;

Drake et al., 2006; Bang et al., 2007), although Wong et al. (2004a) found that supplementation of media with catalase did not improve the recovery of *V. parahaemolyticus* cells, suggesting that peroxide sensitivity may be a phenomenon specific to *V. vulnificus*. In our study we did observe better recovery of stressed *V. parahaemolyticus* cells using sodium pyruvate supplementation of media, and the differences between our findings and those of Wong et al. (2004a) may be a function of slightly different experimental design.

Some have postulated that loss of culturability or entry into the VBNC state may in reality reflect loss in catalase activity undergone by stressed cells. In our study, we observed a faster loss in culturability under starvation conditions for *V. vulnificus* cells recovered on media without sodium pyruvate, and extended culturability on media with sodium pyruvate supplementation. This is consistent with the findings of Kong et al. (2004), who constructed a *V. vulnificus* deletion mutant which lacked catalase (*oxyR*) activity, resulting in loss of culturability on solid media containing 7.2 μM H_2O_2. Additionally, low temperature incubation resulted in reduced catalase activity that appeared to be the result of the inability to synthesize catalase de novo upon a return to ambient temperature. Taken together, these results suggest a role for low catalase activity in loss of culturability and an inability for resuscitation of non-culturable cells following a shift from refrigeration to ambient temperatures. These data also suggest that the loss of catalase activity may be the result of peroxide sensitivity which lead to nonculturable cells. Smith and Oliver (2006) demonstrated that the loss catalase activity was a direct results of *kat*G (hydroperoxidase I) repression. It is

possible that there are strain-specific differential rates in the decline in catalase activity, and this may be an interesting avenue for future exploration.

Anaerobic storage of media does not allow accumulation of toxic hydrogen peroxide during metabolism (Carlsson et al., 1978; F. Breidt, personal communication). We therefore investigated whether anaerobic incubation of recovery media would result in improved recovery of stressed cells. This is also relevant because the *Vibrio* spp. initiate disease in the gastrointestinal tract, suggesting that the organisms must survive under anaerobic conditions prior to causing disease. In our study, anaerobic conditions (with or without sodium pyruvate supplementation) did not appear to improve the recovery of stressed cells of either *V. vulnificus* or *V. parahaemolyticus* to any significant degree. This is expected as the level of oxygen radical species (i.e. H_2O_2) under anaerobic conditions is low and the action of sodium pyruvate is not needed for recovering cells.

In conclusion, recovery of viable *V. parahaemolyticus* and *V. vulnificus* cells was, on occasion, significantly improved by media supplementation with sodium pyruvate, but not impacted by incubation under anaerobic vs. aerobic conditions. The effect of media supplementation was most obvious when comparing *V. parahaemolyticus* cells with different virulence markers which were treated under starvation conditions. The presence of the virulent genotype in *V. vulnificus* did not appear to impact survival of cells subjected to sublethal stresses. The fact that *V. parahaemolyticus* strains with the *tdh+/trh-* genotype demonstrated significantly decreased survival under starvation conditions relative to less

virulent *V. parahaemolyticus* strains could explain, at least in part, their low proportions in natural estuarine environments.

5.6 ACKNOWLEDGMENTS

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Table 5.1. Comparison of *V. vulnificus* D-values under cold conditions with different plating media and incubation conditions

Strains	Aerobic	Aerobic + sodium pyruvate	Anaerobic	Anaerobic + sodium pyruvate	Type of kinetics
A type	4.4 ± 0.5b,y	6.7 ± 0.2a,x	5.3 ± 0.2 b,y	4.6 ± 0.4b,y	Log Linear
B type	4.3 ± 0.2b,y	6.5 ± 0.2a,x	4.6 ± 0.1b,y	4.1 ± 0.1b,y	Log Linear
A/B type	5.4 ± 0.9b,y	7.5 ± 0.5a,x	4.7 ± 0.1b,y	5.4 ± 1.0b ,y	Log Linear

a, b, c letters in a row indicate statistically significant difference ($p<0.05$) between different culture conditions

x, y, z letters in a column indicate statistically significant difference ($p<0.05$) between different genotypes

Table 5.2. Comparison of *V. parahaemolyticus* D-values under cold conditions with different plating media and incubation conditions

Strains	Aerobic	Aerobic + sodium pyruvate	Anaerobic	Anaerobic + sodium pyruvate	Type of kinetics
tlh	5.1 ± 0.4a,x	5.0 ± 0.2a,x	4.9 ± 0.5a,x	4.8 ± 0.3a,x	Log Linear
tdh	6.5 ± 1.0a,x	5.4 ± 0.5a,x	6.0 ± 1.1a,x	6.0 ± 1.2a,x	Log Linear
trh	4.7 ± 0.6a,x	4.6 ± 0.1a,x	4.6 ± 0.2a,x	5.0 ± 1.0a,x	Log Linear
tdh/trh	5.5 ± 0.4a,x	5.8 ± 1.1a,x	5.2 ± 0.5a,x	5.9 ± 0.8a,x	Log Linear

a, b, c letters in a row indicate statistically significant difference ($p<0.05$) between different culture conditions

x, y, z letters in a column indicate statistically significant difference ($p<0.05$) between different virulence markers

Table 5.3. Comparison of *V. vulnificus* D-values under starvation conditions with different plating media and incubation conditions

Strains	Aerobic	Aerobic + sodium pyruvate	Anaerobic	Anaerobic + sodium pyruvate	Type of kinetics
A type	10.3 ± 2.0a,x	8.3 ± 0.2a,x	9.6 ± 0.4a,x	8.5 ± 0.9a,x	Log Linear
B type	9.8 ± 0.4a,x	9.2 ± 1.5a,x	9.9 ± 1.8a,x	9.7 ± 2.1a,x	Log Linear
A/B type	10.2 ± 2.1a,x	9.1 ± 2.2a,x	9.6 ± 0.2a,x	8.9 ± 1.9a,x	Log Linear

a, b, c letters in a row indicate statistically significant difference ($p<0.05$) between different culture conditions

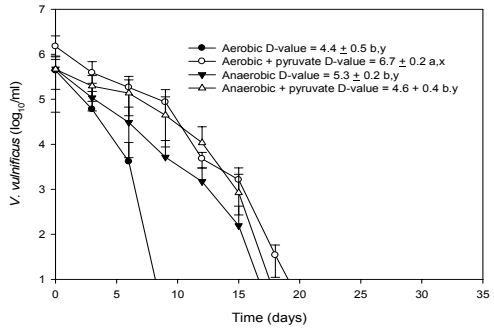
x, y, z letters in a column indicate statistically significant difference ($p<0.05$) between different genotypes

Table 5.4. Comparison of *V. parahaemolyticus* D-values under starvation conditions with different plating media and incubation conditions

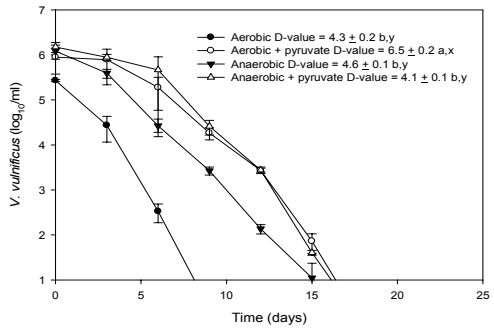
Strains	Aerobic	Aerobic + sodium pyruvate	Anaerobic	Anaerobic + sodium pyruvate	Type of kinetics
tlh	23.8 ± 1.9b,w	30.8 ± 1.5a,w	20.6 ± 1.9b,w	20.7 ± 1.9b,w	Nonlinear
tdh	7.9 ± 0.1a,z	8.2 ± 0.5a,z	8.5 ± 0.1a,y	9.8 ± 0.9a,y	Log Linear
trh	16.3 ± 1.6b,x	20.0 ± 1.2a,x	16.6 ± 0.7b,x	21.4 ± 2.8a,w	Nonlinear
tdh/trh	12.1 ± 1.9b,y	16.8 ± 2.6a,y	9.9 ± 0.5c,y	12.8 ± 1.5b,x	Nonlinear

a, b, c letters in a row indicate statistically significant difference ($p<0.05$) between different culture conditions
x, y, z letters in a column indicate statistically significant difference ($p<0.05$) between different virulence markers

A.



B.



C.

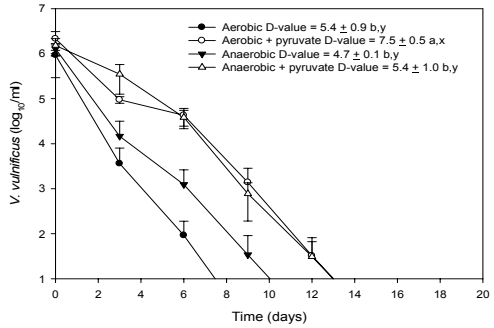


Figure 5.1. Survival of different *V. vulnificus* strains (A. genotype A, B. genotype B, and C. genotype A/B) under cold condition using different culture conditions.

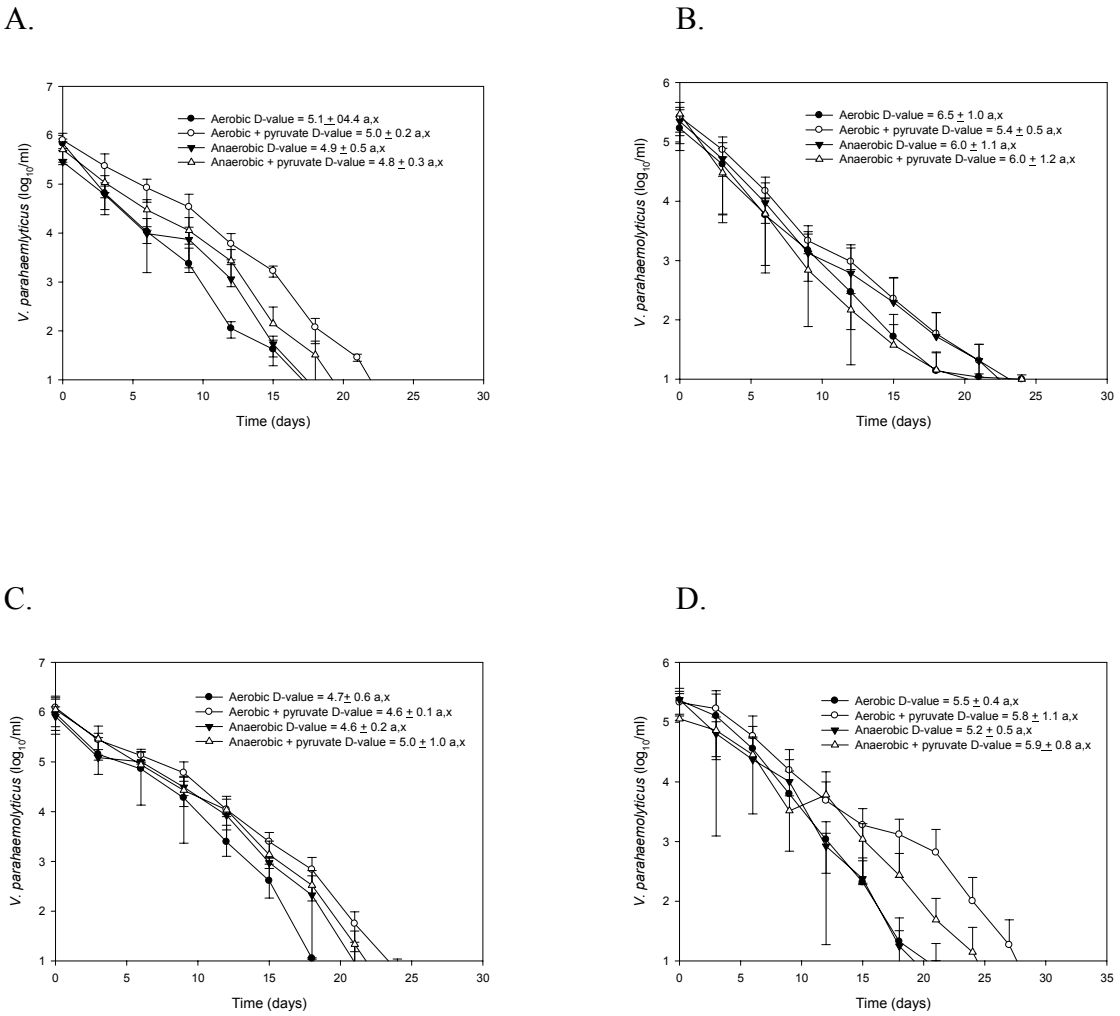
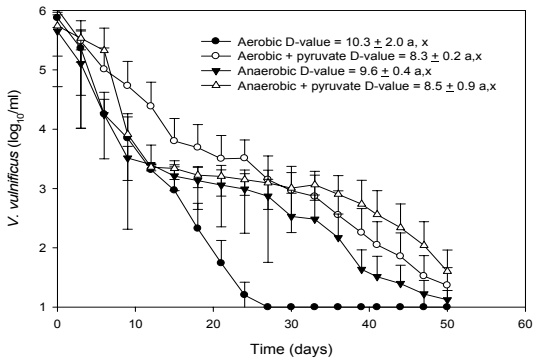
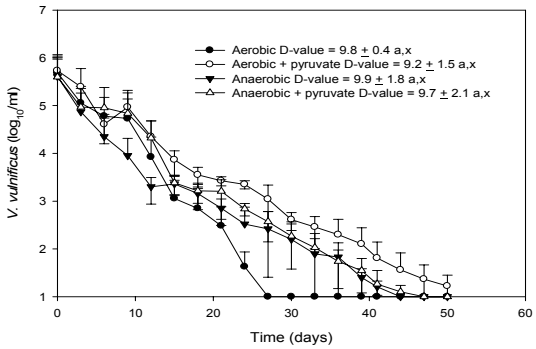


Figure 5.2. Survival of different *V. parahaemolyticus* strains (A. *tdh-/trh-*, B. *tdh+/trh-,* C. *tdh-/trh+,* and D. *tdh+/trh+*) under cold condition using different culture conditions.

A.



B.



C.

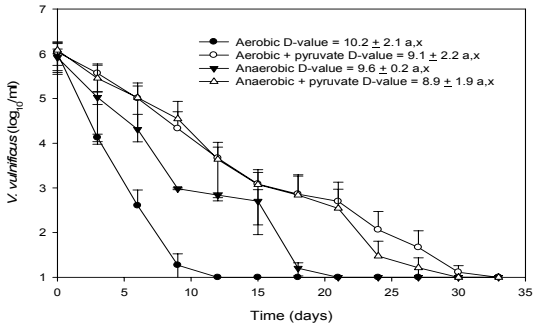


Figure 5.3. Survival of different *V. vulnificus* strains (A. genotype A, B. genotype B, and C. genotype A/B) under starvation conditions using different culture conditions.

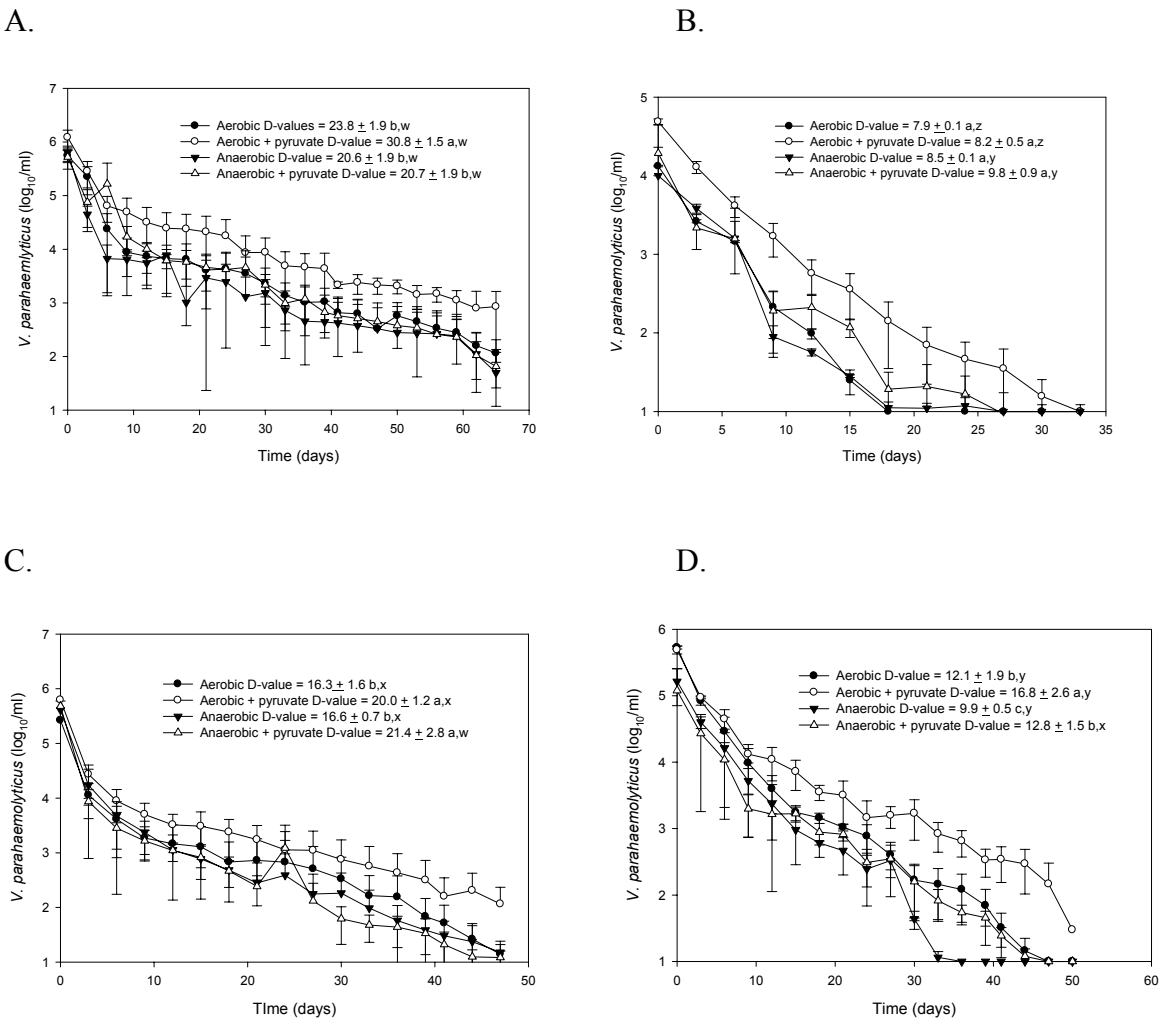


Figure 5.4. Survival of different *V. parahaemolyticus* strains (A. *tdh-/trh-*, B. *tdh+/trh-,* C. *tdh-/trh+,* and D. *tdh+/trh+*) under starvation conditions using different culture conditions.

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CHAPTER 6

A SIMPLIFIED METHOD TO MONITOR INTERNAL OYSTER MEAT TEMPERATURE ON A COMMERCIAL SCALE

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6.1 ABSTRACT

Temperature control is an important aspect of food safety and thermocouples have long been used for temperature monitoring. Nonetheless, thermocouples are not ideal for all products, particularly those of irregular size or which are subjected to multiple handling steps throughout the farm-to-fork continuum. Newer electronic time-temperature recording devices are smaller, portable, and less prone to slippage. However, their accuracy in comparison to traditional thermocouples has yet to be validated. The purpose of this study was to compare traditional thermocouples and button data loggers with respect to their ability to accurately record the temperature of shellstock oysters during normal commercial handling. Commercial burlap bags of oysters were obtained and the temperature of individual oyster specimens was monitored internally and externally using T-type thermocouples and button data loggers. Specimens with thermocouples or button data loggers were placed side by side in the commercial burlap bags at different locations (top, middle, and bottom) to achieve representative cooling profiles based on product location. No statistically significant differences in oyster cooling profiles were observed when comparing thermocouple versus button data logger data, irrespective of location in the commercial sacks (top, middle, and bottom) or temperature monitor location (internal vs. external). The results support the use of button data loggers as a practical and inexpensive alternative for monitoring the temperature of oysters and perhaps other food products as they pass through the farm-to-fork continuum.

6.2 INTRODUCTION

Temperature control is an important aspect of food safety. Thermocouples are widely used for temperature sensing because they are inexpensive, interchangeable, and can measure over a wide range of temperatures. They are, however, less suitable for applications where smaller temperature differences need to be measured with high accuracy (Anonymous, 2008). Furthermore, using thermocouples for temperature monitoring in certain circumstances, for instance in molluscan shellfish, is difficult due to concerns about portability, slippage, sensitivity to moisture. Taken together, this means that temperature data collected using thermocouples may not always be accurate, reliable, or straightforward.

Improvements in time-temperature indicators have resulted in miniaturization, waterproofing, and ease and security of data retrieval. These new devices, termed button data loggers, are also inexpensive and durable in moist environments. An appealing application for these button data loggers is the monitoring of temperatures of seafood, particularly molluscan shellfish, during harvest, handling, shipment, and storage. The purpose of this study was to compare traditional thermocouples and button data loggers with respect to their ability to accurately record the temperature of shellstock oysters during normal commercial handling.

6.3 Materials and Methods

6.3.1 Cooling Curves of Oysters

Commercial size burlap bags of oysters (approx. 120 kg/bag) were obtained three times on different days from a commercial oyster harvester immediately after docking. These commercial oyster bags were transported to the Food Microbiology Laboratory, Louisiana State University (LSU) AgCenter (Baton Rouge, Louisiana) in covered trucks. To obtain temperature data for oysters, one thermocouple was placed within the oyster meat and a second thermocouple was placed on the outside of an oyster. To measure internal oyster temperature, a 1/4 in. hole was made by drilling into the shell approximately ½ in. from the bottom center of the oyster. These holes were cleaned of any drilling debris and blotted dry of water and oyster liquor with absorbent paper, consistent with the method described by Martin et al. (2004). A T-type thermocouple (copper-constant) (TMQSS-032U-6, OMEGA Engineering Inc, Stanford, CT) was inserted approximately 1 in. into the oyster meat and then the hole was sealed with modeling clay (Crayola, PA). To measure the temperature of oysters externally, a second thermocouple was secured to the outside of the shell of a different oyster specimen using duct tape. The thermocouple data were collected using an OM-3000 portable datalogger (OMEGA Engineering Inc, Stanford, CT). As an alternative method for measuring the temperature of oysters, a SmartButton data logger (ACR Systems Inc., Surrey, B.C., Canada) was taped to the outside of a third oyster (Figure 1). These three individual

oysters to which temperature probes were attached at different locations (thermocouple inside the meat, thermocouple on the outside, and button data logger on the outside) were placed side by side in the commercial oyster bags. Placement was done in three locations per bag (top, middle, and bottom) in an effort to record temperatures representative of different product locations. In addition, a thermocouple wire was placed inside and outside of the burlap bags to monitor ambient temperature. The commercial oyster bags were allowed to equilibrate to room temperature (18 - 20^{o}C) and then placed in a walk-in cooler (5 ^{o}C). The data loggers and smart buttons were programmed to record temperatures every two minutes. The study was repeated three times for each type of temperature recorder and placement location.

6.3.2 STATISTICAL ANALYSIS

The 2005 *Vibrio parahaemolyticus* risk assessment (CFSAN/FDA, 2005) notes that growth of this organism does not occur at temperatures <10 ^{o}C. Therefore, temperature data were stratified for statistical analysis using this biologically relevant cut-off value. The Wilcoxon signed-rank test for paired data was used to compare button logger with internal probe measurements, and to compare button logger and internal probe measurements with concurrent ambient external thermocouple temperature readings (Noether, 1991). The Bonferroni correction (0.05/3, number of comparisons=3) was used to adjust the target *p* value for hypothesis testing in multiple comparisons

(Sokal and Rohlf, 1995); hence, a p value of ≤ 0.0167 was considered significant. Statistical analysis was performed using JMP, version 6.03 (SAS Institute, Cary NC).

6.4 RESULTS AND DISCUSSION

There was no practical difference observed between the two techniques and locations (thermocouples and SmartButton data loggers). More specifically, in the total data set of 553 compared values (average of three replicates), the data obtained using the SmartButton data loggers did not exceed the internal probe values by more than 1.3^{o} C, and external thermocouple recordings did not exceed the internal thermocouple recordings by more than 1.2^{o} C (Table 1). These data suggest that placement of the button logger on the outside of the oyster was as good at monitoring temperature as was the internal placement of the thermocouples. Analysis of cooling curves for different placement of the thermocouple wires also showed no statistically significant differences between oyster temperature when using thermocouple wires placed inside the oyster meat vs. on the outside of the oyster shell (representative cooling curve in Figure 2). Healthy oysters open every 2 h whether they are in water or not, so it is not surprising that the internal oyster meat temperature rapidly equilibrates to that of the surrounding ambient environment (Eble and Scro, 1996). In addition, there were no statistically significant differences between thermocouple wires and button data logger readings when each was

placed on the outside of the oyster and used to monitor oyster temperature (representative cooling curve in Figure 3).

Thermocouples are the traditional means of monitoring storage temperatures during product holding and transport (Pollock, 1991). But thermocouple placement can be awkward, and likely to be displaced during handling and shipment. Button loggers more easy to place and may provide a robust alternative for monitoring temperature in oysters and other molluscan shellfish during shipment. Their ease of use may have the added benefit of enhancing compliance with temperature monitoring guidelines.

6.5 CONCLUSIONS

Button data loggers have potential applications for monitoring temperatures in a variety of perishable foods, including other seafood products, meat and poultry products, and produce and could simplify temperature monitoring during transportation and storage. For example, button data loggers could be placed in containers in different locations of the transport truck to monitor refrigeration temperatures as a function of product location. The ease of external placement and lower expense provided by the button loggers could enhance compliance with temperature monitoring guidelines. The availability of a simplified method for real-time temperature monitoring should result in decreased risk for pathogen proliferation, as well as potential improvements in product shelf-life, both of which would help assure the quality and safety of the food supply.

6.6 ACKNOWLEDGEMENTS

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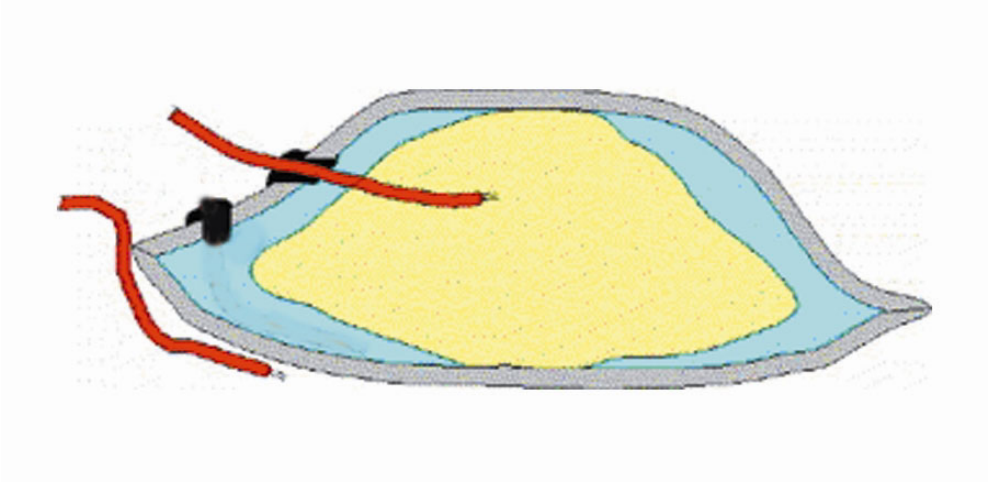

Figure 6.1. Thermocouple placement of the internal probe versus the external probe on an experimental oyster specimen.

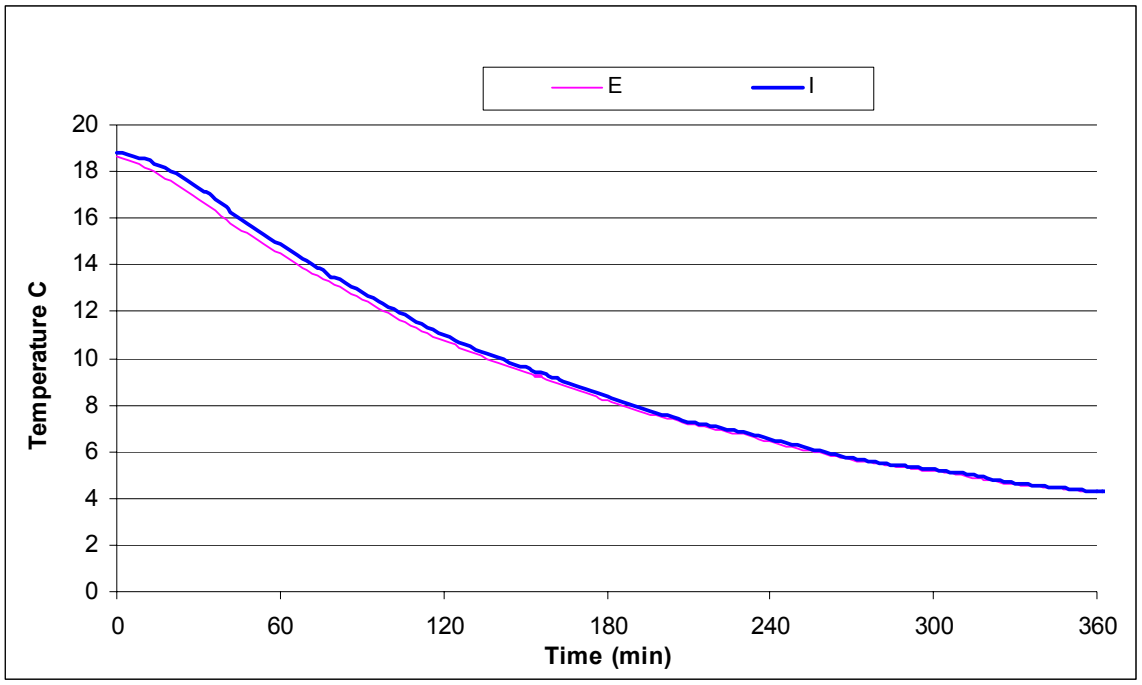

Figure 6.2. Representative cooling curves for different locations of thermocouples (internal (I) and external (E))

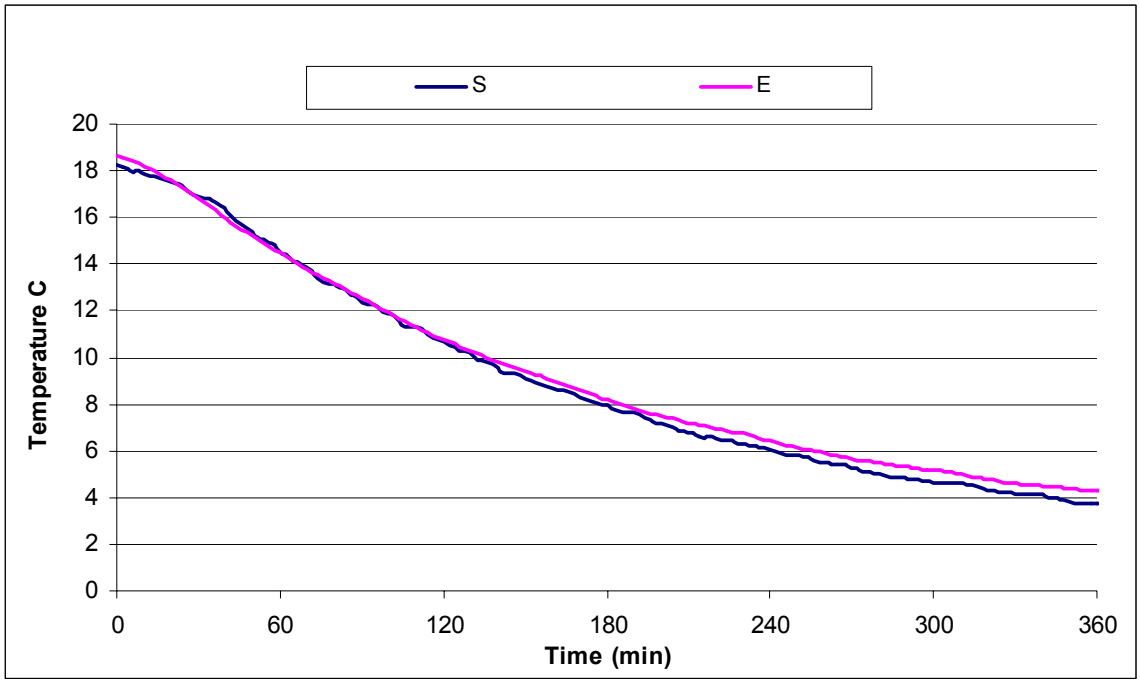

Figure 6.3. Representative cooling curves for comparison of SmartButton data logger (S) and external thermocouple (E).

Table 6.1. Summary statistics for the difference between button logger, internal and external thermocouple temperature recordings

Logger Comparison	Internal Logger < 10°C		Internal Logger ≥ 10°C	
	Median	Range in Diff. °C	Median	Range in Diff. °C
Top (n = 107)				
B vs I	0.3	-0.1,0.6	0	-1,0.6
E vs I	0.3	0.1,0.6	0.8	0.3,1.2
E vs B	0	-0.5,0.7	0.8	-0.1,2.1
Middle (n = 87)				
B vs I	1	0.8,1.2	1	0.7,1.3
E vs I	0.3	0.0,0.5	0.5	0.0,0.7
E vs B	-0.7	-0.9,0.6	-0.6	-1.1
Bottom (n = 136)				
B vs I	0.6	0.4,1.0	0.8	0.5,1.0
E vs I	0	-0.1,0.2	0.2	0.1,0.4
E vs B	-0.6	-0.9,-0.4	-0.5	-0.8,- 0.3

B = button data logger
I = internal thermocouple
E = external thermocouple

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