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# Depuration as a method to reduce Vibrio vulnificus populations in live Crassostrea virginica oysters

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# DEPURATION AS A METHOD TO REDUCE *VIBRIO VULNIFICUS* POPULATIONS IN LIVE *CRASSOSTREA VIRGINICA* OYSTERS

By

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*Vibrio vulnificus* is a foodborne bacterial pathogen associated with raw oyster consumption. Shellfish depuration for 48 hours is a dynamic process where coliform bacteria are purged; however, this process is ineffective against *V. vulnificus*. The current study investigated the use of prolonged two-week depuration on *V. vulnificus* populations in Gulf Coast oysters. The study evaluated the impact of prolonged depuration on *V. vulnificus* fatty acid profile change and the ability to survive in simulated gastric fluid.

Oyster depuration in seawater (10 or 22°C, 14 days) reduced *V. vulnificus* counts, but not to non-detectable level, indicating close ecological relationship between the pathogen and mollusk. Greatest *V. vulnificus* count reductions were seen in 12 ppt 10°C seawater (2.7 $\log_{10}$ CFU/g) and in 20 ppt 22°C seawater (2.8

logs). Mesophilic vibrios dominated the overall microflora of freshly harvested oysters, while refrigeration selected for psychrotrophic bacteria. Depuration at 22°C retained dominance of mesophilic vibrios, including pathogenic species. Although aerobic plate counts were lower in 22°C depurated oysters (5.0 logs vs 6.0 logs), depuration at 10°C had little to no advantage over 22°C in terms of vibrio population reduction. Use of prolonged depuration remains economically questionable since this method failed to completely eliminate *V. vulnificus*.

Starved *V. vulnificus* behavior in artificial seawater showed that low temperature (4°C) and high seawater salinity (35 ppt) contributed to pathogen population reduction. Starved *V. vulnificus* did not adjust membrane fluidity to storage temperature within the investigated time frame. However, a significant fatty acid switch from C18:1w7c to C18:1w6c by double bond relocation was observed. The relocation was faster at ambient temperatures compared to refrigerated temperatures.

The majority of *V. vulnificus* foodborne infections occur during warm summer months. *Vibrio vulnificus* ATCC 27562 was significantly less resistant (3.7 min D-value) to simulated gastric fluid (pH 4.0) after 7-day storage at 4°C compared to the control (7.8 min D-value). Therefore, greater gastric fluid sensitivity of the pathogen may occur in winter-harvested oysters and may partially explain the low number of winter outbreaks.

Key words: oysters, *Vibrio vulnificus*, depuration, fatty acid profile, gastric fluid

# DEDICATION

I would like to dedicate this dissertation to my Mom. Without her unconditional love and support it would be impossible for me to move through all these years. And to my passed away Dad, who always dreamed about me getting my PhD. Rest in peace, and sorry.

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# CHAPTER I

# INTRODUCTION

The Eastern oyster (*Crassostrea virginica)* is an important commercially harvested oyster species in the U.S. According to the National Marine Fisheries Service (2004), this species provided 77.8% of all oyster landings in 2002 with Louisiana, Texas, Mississippi, and the west coast of Florida contributing up to 88% of the harvested eastern oysters.

*Vibrio vulnificus* emerged as a foodborne pathogen in the late 1980's, primarily associated with seawater and raw oyster consumption. The main difference between *V. vulnificus* and other foodborne pathogens was the fact that *V. vulnificus* was not associated with water pollution, but instead was indigenous to the normal microflora of warm estuarine environment of the Gulf of Mexico. The illness caused by *V. vulnificus* can be severe in susceptible individuals, often associated with mortality rates of up to 50%. According to O'Neill et al. (1992), 80% of 115 cases of shellfish-associated *V. vulnificus* infections in the U.S. (1975-1988) either occurred in southern Gulf Coast states or were associated with shellfish from these areas.

The task of reducing or eliminating *V. vulnificus* from raw oysters has been of keen interest for numerous researchers, who have investigated depuration,

relaying, refrigeration, freezing, antimicrobial applications, high pressure treatment, and irradiation methods among others to combat this problem. Depuration has been proven successful for complete coliform elimination, though it was believed that *V. vulnificus* could not be purged because of its close ecological interactions with the Eastern oyster. Because of the short duration of previous depuration studies, it is unknown whether long-term depuration exposure will be active against *V. vulnificus.* In the present work, it is hypothesized that prolonged cold-temperature depuration (10$^{o}$C; 14 days) of summer-harvested oysters harboring high numbers of *V. vulnificus* will cause population size reductions of this pathogen in live oyster tissues. In addition to analyzing *V. vulnificus* counts in non-depurated and depurated oysters, aerobic plate counts were determined and total microflora of oysters was identified using gas chromatography of fatty acid methyl esters (GC-FAME) (MIDI, Inc).

Many investigators believe that *V. vulnificus* may enter a Viable but Non-Culturable (VBNC) state at low temperatures, where the pathogen is still alive and capable of causing disease, but is not culturable on conventional microbiological media (Oliver et al., 1991; Nilsson et al., 1991). Others such as Bogosian et al. (2000) showed that possible explanation for non-cultivability might be formation of traces of hydrogen peroxide toxic to *V. vulnificus* cells.

To address the concern with *V. vulnificus* and mimic depuration tank conditions, the influence of seawater salinity (12, 20, and 35 ppt) and temperature (4, 10, and 25$^{o}$C) on the ability of pre-starved (2 hours at 25$^{o}$C) *V.*

*vulnificus* to retain viability and culturability over a fourteen day depuration period was investigated. In order to explain physiological changes occurring in starved *V. vulnificus* at different salinities and temperatures, cellular fatty acid profiles were analyzed to explore cell membrane fluidity changes over starvation time. Additionally, the ability of starved *V. vulnificus* cells to survive human simulated gastric fluid was assessed in order to determine the affect of starvation on maintaining virulence against humans.

CHAPTER II

LITERATURE REVIEW

## Microbiology of Gulf Coast oysters

### Microflora and its changes during storage of raw oysters

Eastern oyster (*Crassostrea virginica)*, also known as the American oyster, is native to the eastern seashore of North America. This important commercial species can be found in the Gulf of St. Lawrence through the Gulf of Mexico, as well as along the coasts of Argentina and Brazil (Carriker and Gaffney, 1996). Eastern oyster is the dominant oyster species harvested from natural reefs and aquaculture operations in the U.S., providing 77.8% of all oyster landings in 2002 (NMFS, 2004). Four states, namely, Louisiana, Texas, Mississippi, and the west coast of Florida, accounted for 88% of the harvested eastern oyster in the United States. Eastern oysters are harvested using dredges, tongs, or by hands, with the first method being most popular for mass production.

In biological terms, oysters are marine bivalve mollusks that filter large quantities of water to obtain necessary food and oxygen for survival (Kural and Chen, 2008). Microflora of raw oyster (*C. virginica*) is similar to the environment

the oysters inhabit and strongly correlates with water temperature and salinity (Cruz-Romero et al., 2008). According to Jay (2000), oysters are filter feeders and accumulate bacteria present in seawater; therefore, they generally contain psychrotrophic or psychrophilic Gram-negative rod-shaped bacteria belonging to the genera *Pseudomonas*, *Vibrio*, *Shewanella*, *Moraxella*, *Acinetobacter*, *Aeromonas*, *Psychrobacter*, *Serratia*, *Proteus*, as well as some Gram-positive genera such as *Clostridium* and *Bacillus*. Early studies by Colwell and Liston (1960) revealed that live Pacific oyster (*Crassostrea gigas*) microflora consisted mostly of *Pseudomonas/Vibrios* (52% of isolates), *Flavobacterium* (17.1%), *Micrococcus* (10.5%), *Achromobacter* (5.3%), *Bacillus* (4.3%), *Corynebacterium* (3.3%), and *Enterococci* (1.3%). Similarly, Kueh and Chan (1985) found whole Pacific oysters from Hong Kong contained *Pseudomonas* (49% of isolates), *Vibrio* (18%), *Acinetobacter* (9%), coliforms (3%), *Flavobacterium* (9%), *Coryneforms* (6%), *Alcaligenes* (4%), and *Micrococcus* (3%).

The microflora of aerobically stored refrigerated oysters noticeably changes over time, favoring psychrotrophic spoilage-inducing bacteria such as *Pseudomonas* spp. and *Shewanella putrefaciens*, followed by appearance of enterococci, lactobacilli, and yeasts at later stages of spoilage (Jay, 2000). The following genera have been recovered from spoiled refrigerated oysters: *Pseudomonas, Shewanella, Enterobacter, Lactobacillus, Serratia*, and *Flavobacterium* (Cruz-Romero et al., 2008). These researchers found no difference between the microflora of fresh oysters and 14-day aerobically stored

oysters at 2°C. Specifically, *Aeromonas/Vibrios* dominated in both cases (45% and 42% incidence, respectively), followed by pseudomonads (40% and 42%, respectively), while the remaining genera remained at <3% (*Moraxella/Acinetobacter, Micrococcus,* Coryneforms*, Lactobacillus, Leuconostoc, Bacillus,* Enterobacteriaceae).

## Bacterial pathogens and factors affecting their presence

Pathogenic bacteria associated with shellfish include *Vibrio*, *Salmonella*, *Clostridium*, *Listeria*, and *Escherichia coli* (Gillespie et al., 2001). Although some of these pathogenic genera are related to fecal pollution (*Salmonella* and *E. coli*), vibrios are ubiquitously present in seawater, which is their natural habitat. According to Jones et al. (1991), pathogenic vibrios are abundant in the warmer waters of the Gulf of Mexico and the southern coastal U.S.; however, they are also routinely isolated from Chesapeake Bay, Long Island Sound, and Boston Harbor on the east coast, as well as British Columbia to Southern California on the west coast. Pathogenic vibrios are occasionally detected in colder waters of northern New England and only during the four warmest months of the year (Jones et al., 1991).

The reason for such geographic distribution of pathogenic vibrios is their sensitivity to environmental conditions, such as temperature and salinity (Jones et al., 1991). For example, a seasonal difference was found for *V. vulnificus* presence in Gulf Coast eastern oysters (Birkenhauer and Oliver, 2003). They

showed that *V. vulnificus* load of Gulf Coast harvested oysters seasonally varied from non-detectable levels (<26 cfu/g) in March and February to low numbers in April and October (<3 $\log_{10}$ cfu/g), with peak numbers occurring in August (>4 $\log_{10}$ cfu/g). DePaola et al. (1994) suggested that *V. vulnificus* can overwinter in Sheepshead fish (*Archosargus probatocepahlus*) in the Gulf Coast based on the observation that winter *V. vulnificus* populations in the intestines of these fish were greater than populations in seawater and sediments. On the other hand, Motes et al. (1998) suggested that failure to detect *V. vulnificus* in Gulf Coast oysters harvested during winter months was reversed by increasing sample size eightfold to 25 grams. The authors concluded that *V. vulnificus* persists in Eastern oysters during winter months and multiplies to summer population levels when water temperatures increase.

DePaola et al. (1994) found that *V. vulnificus* is found in inshore Gulf Coast sites where salinities are lower (5 to 28 ppt), but is not found in offshore Gulf waters where salinities are higher (32 to 35 ppt). According to Motes and DePaola (1996), *V. vulnificus* was rarely recovered from Atlantic Coast oysters harvested from >30 ppt salinity seawater. O'Neill et al. (1992) found that *V. vulnificus* can be isolated from waters with temperatures of 13 to 31$^{o}$C and salinities of 0.8 to 34 ppt; however, high populations of the pathogen were more frequently observed in water with temperatures of 17 to 31$^{o}$C and salinities of 15 to 25 ppt. A notable incidence of low *V. vulnificus* numbers in raw Gulf Coast oysters occurred in June, 2001 due to tropical storm Allison, which dropped 20

inches of rainfall over the harvesting areas. This event dramatically decreased seawater salinity, which decreased *V. vulnificus* populations to what normally would be seen during winter months (Dr. Linda Andrews, personal communication). Therefore, a combination of optimal temperature and salinity is important for robust *V. vulnificus* populations in oysters.

## *Vibrio* species

### General characteristics of *Vibrio vulnificus*

Vibrios are Gram-negative, rod-shaped, non-sporoforming, halophilic, and motile bacteria (straight or curved) that produce oxidase and catalase and ferment glucose without producing gas. They are facultatively anaerobic, prefer alkaline conditions for their growth, and can grow in the presence of high concentrations of bile salts. Three *Vibrio* species, namely, *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*, are well-documented human pathogens. *Vibrio mimicus* is a newly recognized opportunistic pathogen and other species within this genus could be opportunistic human pathogens (DePaola and Kaysner, 2004). All *Vibrio* spp. are obligate halophiles with the notable exception of *V.cholera* and *V. mimicus*, which can grow in the absence of sodium chloride. Vibrios are natural inhabitants of estuarines and are associated with coastal seawater, but are not generally associated with the human gastrointestinal tract. Therefore, unlike other human bacterial pathogens, pathogenic vibrio presence in

seawater is not usually correlated with fecal pollution with the exception of epidemic *V. cholerae* O1/O139 (Montville and Matthews, 2008).

*V. vulnificus* has an optimal growth temperature of 37°C and in live oysters growth does not occur below 13°C. The pathogen is able to grow both aerobically and anaerobically in 0.5 to 5.0% NaCl. It is generally believed that the bacterium can survive low temperatures for prolonged periods of time by entering a Viable But Non-Culturable (VBNC) state, although studies have demonstrated that non-culturability might be overcome by hydrogen peroxide elimination from media (Bogosian et al., 1998).

## Isolation of *Vibrio vulnificus*

Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar is the most commonly used medium for *Vibrio* spp. isolation from seafoods. TCBS contains bile salts to select for most vibrios and inhibit non-vibrios. However, Harwood et al. (2004) found that only 51% of bacterial isolates from this medium were members of *Vibrio* genus, and its selectivity for *V. vulnificus* and *V. cholerae* was inadequate. Fortunately, a more selective medium was developed by Massad and Oliver (1987) for *V. vulnificus* and *V. cholerae*, known as Cellobiose–Polymyxin B–Colistin (CPC) agar. Its current modification (mCPC) is currently suggested for use by the Food and Drug Administration (FDA) (DePaola and Kaysner, 2004). Only *V. vulnificus* and *V. cholerae*, differentiated by cellobiose fermentation, are able to grow on mCPC among 136 strains representing 19 *Vibrio* species and

other marine isolates of the genera *Pseudomonas*, *Flavobacterium*, and *Photobacterium* (Massad and Oliver, 1987).

Classical enumeration of *V. vulnificus* in raw oysters uses the Most Probable Number (MPN) technique, which takes 4 to 7 days to complete (DePaola and Kaysner, 2004). In this method, serial dilutions of oyster meat are prepared in Phosphate-Buffered Saline and one-ml aliquots are enriched in Alkaline Peptone Water at pH 8.5. Samples from Alkaline Peptone Water tubes (24 hours, 35$^{o}$C) are streaked on mCPC agar (incubation at 39 to 40$^{o}$C overnight). Suspicious colonies from mCPC are re-streaked to Trypticase Soy Agar (TSA; 18 to 24 hours at 35$^{o}$C) agar with 2% salt and pure cultures subsequently confirmed either biochemically (for example, with API 20E bioMérieux kit), genetically (DNA probes or PCR), or by using cellular fatty acid profile analysis. Numbers are reported as MPN/g of oyster tissue. A capacitance method using this medium also has been reported (Marshall et al., 1999).
For a more detailed review of media and genetic techniques available for *V. vulnificus* isolation and confirmation from oysters and environmental sources, a recent review is available (Harwood et al., 2004).

## **Pathogenicity characteristics of *Vibrio vulnificus***

In humans, *V. vulnificus* is highly invasive, capable of causing primary septicemia with mortality rates of approximately 60%. The majority (~95%) of all foodborne *V. vulnificus* infections occur in individuals who are

immunocompromised, have diabetes, or have high serum iron levels due to chronic liver damage, such as cirrhosis or hepatitis (Rosche et al., 2006). Moreover, it appears that *V. vulnificus* infection is more likely to develop in males, presumably because females might be protected from *V. vulnificus* endotoxin by estrogen (Merkel et al., 2001). The symptoms of primary septicemia, which follows ingestion of live bacterial cells, include fever (94% of cases), chills (86%), nausea (60%), and hypotension (43%) (Linkous and Oliver, 1999). Birkenhauer and Oliver (2003) mentioned that when ingested, *V. vulnificus* is capable of rapidly crossing through gut mucosa and developing primary septicemia within 24 hours. *Vibrio vulnificus* might be the most lethal foodborne pathogen known for those with predisposing conditions. Another route of *V. vulnificus* infection includes upper or lower extremity wound contact with contaminated seafoods or seawater. Symptoms of this type of infection usually include localized pain, edema, erythema, and ultimately limb necrosis. Limb amputation is a common therapeutic treatment for necrotic cases. Mortalities following wound infection are approximately 25% (Linkous and Oliver, 1999).

One of the major virulence factors for all Gram-negative pathogenic bacteria including *V. vulnificus* is lipopolysaccharide endotoxin, a causative agent of septicemia as well as inflammatory response in wound infections. Bacterial endotoxin is released in the bloodstream after cell lysis, causing overproduction of Tumor Necrosis Factor, followed by nitric oxide production, and ending in endotoxic shock and death (Linkous and Oliver, 1999).

Another definite virulence factor is acidic polysaccharide capsule, which is present on the surface of some *V. vulnificus* strains and is associated with opaque, not translucent, colony phenotype (Simpson et al., 1987). Yoshida et al. (1985) showed that *V. vulnificus* opaque phenotype had greater tissue invasiveness, serum resistance, antiphagocytic activity, and mouse lethality, compared to translucent phenotype, which is generally avirulent in mouse models. The only disadvantage of the presence of capsular polysaccharide is its ability to inhibit *V. vulnificus* biofilm formation (Joseph and Wright, 2004). It is widely accepted that the colony phenotype of the same *V. vulnificus* strain can be spontaneously switched, with frequencies of $10^{-5}$ in rich medium and up to 60% in chemically-defined medium for opaque-to-avirulent translucent state transition, while the reverse switch occurs less frequently (Yoshida et al., 1985; Simpson et al., 1987; Grau et al., 2005). Other less common phenotypes of *V. vulnificus* are rugose positive, which was more resistant to serum killing and produced copious amounts of biofilm (Grau et al., 2005), and intermediate phenotype, which had intermediate capsular polysaccharide expression on its surface (Rosche et al., 2006).

Various extracellular enzymes produced by *V. vulnificus* (mucinase, lipase, DNase, cytolysin-hemolysin, elastase, collagenase, and metalloprotease) are well known and might play some role in virulence (Oliver et al., 1986; Moreno and Landgraf, 1998; Kawase et al., 2004). Cytolysin-hemolysin is present in both environmental strains and clinical strains (Johnson and Calia, 1981; Oliver et al.,

1986), but is not sufficient to account for virulence, since its gene knockout did not eliminate lethality towards the mouse (Wright and Morris, 1991). Kim et al. (2007) showed that metalloprotease was essential for *V. vulnificus* swarming and destroying IgA and lactoferrin in intestinal mucosa. It also has been shown that *V. vulnificus* produces bradykinin, which increases vascular permeability and induces pain, smooth muscle contraction, and vasodilation (Maruo et al., 1998).

The ability of *V. vulnificus* to utilize iron has been suggested as a pathogenic trait (Wright et al., 1981), with infection progression possible only if elevated serum iron level is present in infected host. It has been shown that *V. vulnificus* produces siderophores, which are capable of acquiring iron from saturated iron-protein complexes (transferrin, ferritin, and lactoferrin) after protein cleavage by protease and iron release (Morris et al., 1987; Litwin et al., 1996). In addition, Litwin et al. (1996) showed that *V. vulnificus* mutant for phenolate siderophore production lost its ability to obtain iron from transferrin, and had reduced virulence in mouse compared to the parent strain.

Although it is believed that the two most important factors required to initiate *V. vulnificus* infection are the presence of capsular polysaccharide and endotoxin; however, based on epidemiological data some strains possessing these attributes, especially environmental isolates, might not be able to cause disease based on epidemiological data (Linkous and Oliver, 1999). It has been shown that *V. vulnificus* isolates display a wide range of virulence in the injected, iron-overload mouse model, and there is no correlation with clinical or

environmental origin (Stelma et al., 1992; DePaola et al., 2003). According to Jackson et al. (1997), high host specificity might be the reason for the low number of *V. vulnificus* infections occurring each year in the U.S. They noted the occurrence of only 5 to10 cases per year in Florida among an at-risk, oyster-eating population of approximately 70,000. The infectious dose of *V. vulnificus* is unknown, but cell densities as high as $10^4$ to $10^5$ MPN/g occur in raw oyster tissues during summer months. Therefore, Rosche et al. (2005a) agreed that higher rates of *V. vulnificus* septicemia should be expected in the summer. Jackson et al. (1997) hypothesized that only certain strains of *V. vulnificus* can cause disease, while other strains are not virulent even if consumed in high numbers.

Rosche et al. (2005a) used Polymerase Chain Reaction (PCR) of a specific *V. vulnificus* DNA repeatable region to differentiate between clinical and environmental isolates. They found two PCR products groups, one called Clinical type (C-type) and second Environmental type (E-type). Ninety percent of C-type strains were clinical isolates, while 93% of environmental isolates were classified as E-type. They noted that C-type strains were rarely found in the environment. In contrast, 7 E-type strains were found among the investigated 25 clinical isolates, which indicate that these strains are capable of causing human disease. Another interesting observation was noted by Hilton et al. (2006) about opaque phenotype C-type strains having greater resistance to translucent state switch, compared to opaque E-type strains. Strain differentiation might show differences

between clinical and environmental isolates, but it still does not exclude the possibility of environmental isolates causing disease, as has been shown in iron-overload mouse models (DePaola et al., 2003).

## Viable but non-culturable state of *Vibrio vulnificus* cells

Roszak and Colwell (1987) discussed survival strategies of bacteria in the natural environment and suggested that real numbers of marine bacteria might be underestimated due to the difficulties in finding an "ultimate" growth medium and growth conditions suitable for all diverse bacteria present. Moreover, some bacteria may enter stressed, injured, or "Viable But Nonculturable" (VBNC) state. VBNC cells metabolize but do not divide, and are naturally adapted for low-nutrient environment without ability to grow in rich laboratory media within a short period of time to form visible growth. In the case of stressed or injured cells, resuscitation is needed.

It's been long believed that *V. vulnificus* cells can enter VBNC state upon immediate refrigeration of cells in broth or artificial seawater to temperatures below 10$^{o}$C (Oliver et al., 1991; Nilsson et al., 1991). Oliver et al. (1991) wrote that VBNC entry was highly temperature dependent, with entry occurring at 5$^{o}$C but not 10$^{o}$C. Oliver (1991) showed that *V. vulnificus* culture with contaminating nutrients removed by centrifugation underwent division while incubated at room temperature under starvation in artificial seawater, with culturable cell densities reaching a one log increase after 24 hours. Such starved population maintained

culturable cell densities of 4 to 5 $\log_{10}$ CFU/ml even after 90 days of incubation at room temperature.

Different studies showed various time frames were needed for *V. vulnificus* to enter a non-culturable state. For example, Oliver (1991) showed a 5-$\log_{10}$ reduction (from 6 to 1 $\log_{10}$ cfu/ml) within fourteen days at 5$^{o}$C in artificial seawater, while acridine orange direct count and viability (respiration activity) direct counts showed less than a one log reduction. Several factors were suggested to influence entry into VBNC state. Cell growth phase analysis showed that logarithmic cells were more susceptible to entry compared to stationary phase cells (Oliver, 1991; Biosca et al., 1996). Pre-starvation in artificial seawater at room temperature for 1 to 2 hours caused division, but didn't prevent entry into VBNC state after temperature downshift to 5$^{o}$C. However, if cells were starved at room temperature for longer than 4 hours before temperature downshift, they didn't enter VBNC state and maintained culturable cell density of at least 5 $\log_{10}$ cfu/ml within 14 days (Oliver, 1991).

At the time, Oliver et al. (1991) challenged the conventional definition of "dead" bacteria as those that failed to multiply in a favorable environment under optimal conditions. This definition was supported by microbiologists who were primarily concerned with populations rather than individual bacterial cells. Oliver argued that cells may lose their culturability, but still be physiologically active. Much earlier, Hoppe (1976) suggested that non-culturable cells are primarily responsible for organic turnover in the open ocean.

Loss of *V. vulnificus* culturability in the estuarine environment during winter was attributed to entry into VNBC state. Nilsson et al. (1991) wrote that non-culturable cells can be resuscitated back to culturable state by simply warming of 5$^{o}$C VBNC state culture in seawater without any nutrient addition. Oliver et al. (1995) showed that culturable *V. vulnificus* cells placed into estuarine waters entered into VBNC state in January and February, when water temperatures were low (average <15$^{o}$C; median 10$^{o}$C), while cells in VBNC state placed in the same waters in the warmer months of August through November (average water temperature 21$^{o}$C) underwent rapid resuscitation to the fully culturable state. Whitesides and Oliver (1997) further hypothesized that in order to resuscitate cells in VBNC state culture (5$^{o}$C), warm-up should be performed under starvation condition without added nutrients because nutrient constituents may be toxic to starved VBNC cells.

An alternative VNBC hypothesis was proposed by Bogosian et al. (1998) who suggested that VBNC cells are actually “dead”. According to this hypothesis, the previously observed “resuscitation” occurs only as a re-growth of residual culturable cells, which use nutrients provided by cells that are dead and lysed. Bogsian et al. (1998) argued that VNBC state was merely an experimental artifact caused by count methodology deficiencies. For example, total acridine microscopic count can be used to count all bacterial cells (Oliver et al., 1995), while direct viable microscopic count with nalidixic acid and yeast extract can be used to count viable cells (Whitesides and Oliver, 1997). Direct viable cell count

by cell respiration detection (Oliver et al., 1991; Oliver and Bockian, 1995) and agar plating can be used to count viable cells capable of reproducing by cell division. These methods can give different results, which might lead to the conclusion that cells might be non culturable but still alive and not dead.

Recent studies provide additional insight on the VBNC cell state (Bogosian et al., 2000; Kong et al., 2004; Abe et al., 2007; Bang et al., 2007). Early explanations of VBNC state in *V. vulnificus* were summarized by Kong et al. (2004) and included the possibility that nutrient-rich media may be toxic for *V. vulnificus* incubated at low temperature, such that cells would not grow on nutrient-rich agar. They speculated that exposure of growth-arrested cells to high nutrient concentration would cause metabolism imbalance and production of superoxides and other toxic radicals. However, Bogosian et al. (2000) showed that refrigerated *V. vulnificus* culture contained two subpopulations consisting of hydrogen peroxide-resistant cells and hydrogen-peroxide sensitive cells, with populations of the later increasing over time. Most nutrient-rich media after autoclaving contain trace quantities of hydrogen peroxide, which is toxic to sensitive cells. Therefore, VBNC cells might be non-culturable due to the presence of hydrogen peroxide in the media (Kong et al., 2004).

Kong et al. (2004) constructed a *V. vulnificus oxyR* mutant that lacked catalase activity to degrade toxic hydrogen peroxide. The generated mutant was non-culturable on solid media even at ambient temperature due to hydrogen peroxide presence. It has been shown that *V. vulnificus* cells entering VBNC

state also lacked catalase activity, making them unable to grow in media containing hydrogen peroxide. Going further, Abe et al. (2006) isolated a *V. vulnificus* VBNC-suppression mutant. Cell recoveries of the discovered mutant did not decrease by more than one log after storage at 5$^{o}$C in artificial seawater for thirty days. The mutant showed 10x greater catalase activity than the parent strain, which was not recovered on agar following fourteen days storage at 5$^{o}$C in artificial seawater. The authors concluded that oxidative stress contributed a significant role in *V. vulnificus* inability to grow on media after exposure to refrigeration.

## Methods to control *Vibrio vulnificus* in raw oysters

### Control and monitoring of oyster harvesting areas

The U.S. Food and Drug Administration (FDA) regulates *V. vulnificus* in shellfish through its National Shellfish Sanitation Program (NSSP) (FDA-CFSAN, 2005). Control is achieved through patrol of growing areas, required licensing of commercially harvested shellstock, and mandatory tagging of each container of harvested shellfish. According to NSSP, all growing areas are classified into five major groups: approved, conditionally approved, restricted, conditionally restricted, and prohibited. Approved growing areas should have median or geometric mean of fecal coliforms of less than 14 MPN/100 ml of seawater. Restricted growing areas should have median or geometric mean of fecal

coliforms of less than 88 MPN/100 ml of seawater, and shellstock harvested from these areas could enter commerce only after appropriate depuration or relaying. Regardless of growing area classification, other factors, such as presence of biotoxins in seawater will also determine open or closed status of growing waters (less frequently - reopened, inactive, etc). Because *V. vulnificus* can multiply in harvested oysters at ambient temperatures, mandatory refrigeration is required to prevent pathogen proliferation after harvesting. If harvesting areas have not been implicated in illnesses associated with *Vibrio* spp., harvested shellstock should be cooled to 10$^{o}$C within 24 hours after harvesting if seawater temperature range is 19 to 27$^{o}$C, and within 20 hours if temperature exceeds 27$^{o}$C. If harvested areas have been implicated in two or more *V. vulnificus* outbreaks, harvested shellstock should be cooled to 10$^{o}$C within 14 hours if seawater temperature range is 18 to 23$^{o}$C, within 12 hours if temperature range is 23 to 28$^{o}$C, and within 10 hours if temperature is above 28$^{o}$C (FDA-CFSAN, 2005).

To control *V. vulnificus* infections, the Interstate Shellfish Sanitation Conference (ISSC) proposed implementation of post-harvest treatment of shellfish resulting in a 5-log reduction in pathogen level with an end-point population density of non-detectable (<3 MPN/g) (Kural and Chen, 2008). According to Birkenhauer and Oliver (2003), the ISSC goal was to reduce shellfish-related *V. vulnificus* illness by 60% by the year 2007. If the number of mentioned illnesses is not reduced, then control measures such as closing of

shellfish growing waters from May through September or post-harvest treatments will be mandated.

## Antimicrobial interventions

Several efforts to reduce *V. vulnificus* presence and growth in raw oysters by using antimicrobials have been attempted. For example, Ren and Su (2006) investigated the effect of electrolyzed oxidizing water treatment of raw Pacific oysters on *V. vulnificus* numbers. Although electrolyzed water was bactericidal to *V. vulnificus* in pure culture (>6.6 log reduction), 4 to 6 hours of exposure yielded pathogen population reduction of only 1.05 MPN/g in oysters. Extended exposure (>12 hours) of oysters to electrolyzed water resulted in oyster fatality. Chhabra et al. (2006) attempted to reduce *V. vulnificus* populations in live oysters using chitosan. They noted no difference in *V. vulnificus* counts between chitosan-treated and negative control samples. Pelon et al. (2005) used a pool of *V. vulnificus* specific bacteriophages and extract of eastern oyster to reduce *V. vulnificus* counts in oysters. They found both interventions to be successful, with combined effect noted.

As previously found by Oliver (1981), Pelon et al. (2005) confirmed the presence of an anti-*Vibrio vulnificus* factor in raw oyster extract by showing a 5 log *V. vulnificus* reduction in oyster extract compared to only a 0.5 log reduction in estuarine water within 66 hours storage time at 4$^{o}$C. Greer (2005) noted that

bacteriophage might be both strain and species specific, and it is unknown if a selected pool of *V. vulnificus* phages could reliably reduce natural contamination.

Sun and Oliver (1994) investigated FDA Generally Recognized as Safe (GRAS) compounds against *V. vulnificus* in raw oysters. They found diacetyl to be most promising, with lactic acid and butylated hydroxyanisole being somewhat effective as well. Follow-up studies by Birkenhauer and Oliver (2003) confirmed the efficiency of diacetyl (0.05 to 0.1%) in shucked oysters, but not in live depurated oysters, since in the later case many oysters ceased pumping after diacetyl was added to the depuration tank. In shucked oysters, treatment with 0.26% diacetyl reduced *V. vulnificus* numbers by less than 1 log during 5, 9, and 25 hour storage at 5$^{o}$C.

## Novel and required processing techniques

Extra processing steps, such as immediate on-board oyster refrigeration or ice immersion, freezing, gamma irradiation, and high-pressure treatments have been explored. Several studies focused on survival and growth of *V. vulnificus* in harvested oysters (Cook and Ruple, 1989; Kaysner et al., 1989; Cook and Ruple, 1992; Cook, 1994; Cook, 1997). Cook (1994) found that *V. vulnificus* failed to multiply in shellstock oysters kept at 13$^{o}$C or below for up to 30 hours; however, pathogen numbers significantly increased at 18$^{o}$C within the same time frame. More detailed studies by Cook (1997) revealed 0.75, 1.30, 1.74, and 1.94 log increases of *V. vulnificus* in shellstock oysters harvested and

held without refrigeration (23.6 to 31°C) for 3.5, 7, 10.5, and 14 hours, respectively. Therefore, it is generally believed that if shellstock are chilled immediately after harvest to <13°C, *V. vulnificus* counts do not increase within 48 hours and remain the same as counts at the time of harvest (Motes et al., 1998). Immediate icing of harvested oysters caused slight reduction in *V. vulnificus* numbers (Quevedo et al., 2005). In their study, ice immersion resulted in increased fecal coliform counts and total bacterial contamination of shellstock. Similar resulted were obtained by Melody et al. (2008), who concluded that on-board and dockside icing before refrigeration did not reduce levels of *V. vulnificus*, but negatively affected oyster survival during shelf life at refrigerated temperatures.

Parker et al. (1994) investigated the effects of frozen storage and vacuum packaging on survival of *V. vulnificus* in Eastern oysters. They found that freezing (-20°C) reduced naturally present and inoculated *V. vulnificus* loads in shucked oysters from 5.5 and 4 $\log_{10}$ cfu/g to 3 and <1 $\log_{10}$ after 14 days of storage, respectively. Vacuum packaging significantly enhanced the lethal effect of freezing. Similarly, Cook and Ruple (1992) found that natural *V. vulnificus* numbers (5.2 $\log_{10}$ MPN/g) decreased in individually quick frozen (-30°C) and blast (-23°C) frozen shucked oysters to 0.88 and 1.97 $\log_{10}$ MPN/g after fourteen days of storage at -20°C.

Andrews et al. (2003) reported that low-dose gamma irradiation (0.75 to 1.0 kGy) reduced *V. vulnificus* load of naturally contaminated or artificially

contaminated raw eastern oysters from 6 $\log_{10}$ MPN/g to <3 MPN/g. Triangle difference sensory evaluation data showed no influence of irradiation on product quality .

High-pressure processing is a non-thermal technology involving high pressure to reduce bacterial loads while keeping foods fresh with only minor changes in sensory and nutritional characteristics. High pressure treatment can reduce *V. vulnificus* counts in raw oysters (Koo et al., 2006; Kural and Chen, 2008; Cruz-Romero et al., 2008). Koo et al. (2006) showed a 5.4-log reduction of *V. vulnificus* populations in oysters after treatment with 345 MPa pressure and 6 min pressure come-up time. Similarly, Kural and Chen (2008) showed more than a 5 log reduction in *V. vulnificus* numbers in raw oysters after >250 MPa treatment at -2 or 1$^{o}$C. High-pressure treatment of oysters can improve the ease of shucking and increase yield without loss of fresh-like sensory and nutritional characteristics (Kural and Chen, 2008; Cruz-Romero et al., 2008). However, color and texture of treated oysters can be negatively affected (Cruz-Romero et al., 2008).

To summarize, a few processing methods, such as gamma irradiation and high pressure treatment, proved to successfully eliminate *V. vulnificus* from raw oysters, with others having lesser (freezing) or little effect (immediate chilling). Because ionizing irradiation is expensive, lacks processing facilities, and faces resistance from consumers, high pressure treatment could be more promising if color and texture changes are not objectionable. Freezing could be effective,

although questions about economic feasibility and “freshness” labeling claims remain. Older techniques, such as depuration and relaying, might need re-visiting to investigate new factors that might influence effectiveness.

## Depuration

According to Richards (1988), depuration is a dynamic process where shellfish are allowed to purge contaminants, either chemical or microbial, in a natural setting or inland-based controlled facilities. Inland-based processes, also known as controlled purification or depuration, takes place when shellfish are cleansed in human-made tanks with circulated seawater that is continuously decontaminated. Depuration systems most often utilize circulated water, which must be disinfected to prevent microbial build up. Water disinfection can be accomplished with chlorine, ozone, or UV light treatment (the one used exclusively in the U.S) (Richards, 1988). Regulations for bivalve mollusk depuration facilities and criteria for purification effectiveness regarding coliform counts is available (FDA-CFSAN, 1995).

According to Richards (1988), depurated shellfish is considered a premium product because of improved quality and safety attributes. These attributes include less grittiness due to sand purge, improved flavor because shellfish harvested from low salinity water and depurated in higher salinity water have more saltiness, and greater safety due to reduction in microbial loads and chemical contaminant concentrations. Perkins et al. (1980) demonstrated that

48-hour depuration of live oysters (*C. virginica*) reduced fecal coliform contamination from a median of 2,200 MPN/100 g to a median of <18 MPN/100 g. Similar results were found by Eyles and Davey (1984) for Sydney Rock oyster (*Crassostrea commercialis*) depurated in a commercial recirculation depuration plant employing UV light for water sterilization. They found that aerobic counts of purified oysters were generally lower ($4.8 \times 10^2$ cfu/g) than those of unpurified oysters ($1.2 \times 10^3$ cfu/g). In addition, coliforms and *Escherichia coli* were detected considerably less frequently and at a lower level in purified oysters compared to unpurified oysters.

It is generally believed that indigenous naturally present marine bacteria, such as *Vibrio* spp., do not depurate well and might multiply at elevated temperatures in depurating shellfish. Jones et al. (1991) eliminated total coliforms, but not total vibrios or pathogenic vibrios from Eastern oysters in a commercial depuration facility in Maine. Their short duration of depuration (48 hours) may be insufficient to reduce populations of pathogenic vibrios. Greenberg et al. (1982) artificially inoculated hardshell clams with *Vibrio parahaemolyticus, Vibrio harveyi*, and *Escherichia coli* and conducted depuration. They found that *E. coli* was easily removed, while vibrios showed persistent presence during depuration for 72 hours. They observed an initial slight decrease in both vibrios counts, then an increase followed by a steep decrease, with initial numbers of approximately 3 $\log_{10}$ eventually falling to 1 to 2 $\log_{10}$ cfu/g. The short duration of depuration limits the value of this study to see

what actually might be happening during low-temperature depuration. Son and Fleet (1980) used a 3 day depuration period at 18 to 22$^{o}$C with UV-decontaminated natural seawater circulating at a rate of 60 liters/hour to treat oysters (*Crassostrea commercialis*) artificially contaminated with *V. Parahaemolyticus.* This process reduced counts from 9 x $10^{5}$ cfu/g to 8 cfu/g.

Kelly and Dinuzzo (1985) showed that depuration was effective in reducing *V. vulnificus* numbers from 25 cfu/g to non-detectable levels in artificially inoculated live oysters stored in 14 ppt seawater for 16 days at room temperature. Others, however, have failed to replicate these results. For example, Tamplin and Capers (1992) showed that UV-light depuration tanks were ineffective to control *V. vulnificus* in live oysters at temperatures above 23$^{o}$C because of rapid pathogen growth in oyster tissues and release in the water. They concluded that *V. vulnificus* is persistent in oyster tissues and a complex ecological relationship between the bacterium and the oyster might be present. Similarly, Eyles and Davey (1984) suggested that *V. parahaemolyticus*, which became associated with oysters under natural conditions, might be more resistant to depuration compared to the bacterium artificially introduced into oysters in the laboratory. Eyles and Davey (1984) showed that *V. parahaemolyticus* incidence and numbers were not affected by oyster depuration, although in their study naturally present pathogen numbers were low (48 MPN/g). Chae et al. (2009) showed 1.2, 1.7, 2.9, and 2.0 log MPN/g reduction of *V. vulnificus* in laboratory-contaminated Eastern oysters during

depuration at 5, 10, 15, and 22$^{o}$C during a 48 hours depuration period in 30 ppt seawater. Froelich et al. (2010) showed that clinically- associated (C-type) *V. vulnificus* strains were able to colonize oyster tissues better than environmental (E-type) strains; however, both types were equally depurated from live oysters. In the previous studies, depuration was not evaluated on naturally-contaminated oysters collected during the spring and summer, when greater numbers of pathogenic vibrios are expected. To summarize, short-term depuration efficiently eliminates coliforms from oyster tissue but cannot give a true picture of *Vibrio* spp. behavior, especially at lower temperatures. More research is needed to evaluate long-term depuration and the influence of other factors, such as depuration time and water temperature and salinity.

## Relaying

Relaying involves moving shellfish harvested from contaminated areas to clean open seawater areas (Richards, 1988). Motes and DePaola (1996) demonstrated the effectiveness of offshore relaying of harvested oysters to areas of greater salinities (from 15 to 25 ppt in harvest waters to 32 to 35 ppt in relay waters) for 17 days to reduce *V. vulnificus* numbers in summer months. They found that natural *V. vulnificus* numbers decreased from 3 to 4 $\log_{10}$ MPN/g to <10 MPN/g in five of six studies. They further discovered that oyster acclimatization to higher salinities before relaying was critical to improve oyster

survival rates. Unfortunately, the extra cost to shellfish harvesters prohibits widespread adoption of the method.

## Purpose of the study

Depuration studies in the past have focused on remediation of coliform bacteria so that oysters harvested from restricted sites might be utilized in the half-shell market. This has proven to be successful and specific guidelines for the process are outlined in the NSSP (FDA-CFSAN, 1995). Early oyster depuration studies by Kelly and Dinuzzo (1985) showed effectiveness in reducing *V. vulnificus* numbers from 25 cfu/g to non-detectable level in artificially inoculated live oysters stored in 14 ppt seawater after 16 days; however, later studies showed inefficiency of this technique. For example, Tamplin and Capers (1992) showed that UV-light depuration tanks were ineffective to control *V. vulnificus* in live oysters at temperatures above 23$^{o}$C because of rapid pathogen growth in oyster tissues and its release in the water. Longer duration depuration studies (1 to 2 weeks) at refrigerated temperatures (4 to 10$^{o}$C) have not been performed since the time needed to purge coliforms from oyster tissues is usually less than 48 hours. Low-temperature depuration under high salinity (25 to 35 ppt) has to date only been used for flavor enhancement of oysters on the half shell (Porter, 2003).

The major hypothesis of the present work is that prolonged cold-temperature depuration (10$^{o}$C; 14 days) of summer-harvested oysters harboring

large numbers of *V. vulnificus* will cause reduction in populations of this pathogen in live oyster tissues. Beyond *V. vulnificus* counts, it is also hypothesized that such depuration treatment will change the overall microbial profile of oysters. To measure such effect, Aerobic Plate Counts (APC) were performed and overall microflora of oysters were analyzed by picking random colonies from countable plates and identifying them using GC-FAME microbial identification system (MIDI, Inc).

Many believe that *V. vulnificus* cells can enter a viable but not culturable state upon immediate refrigeration (<10$^{o}$C) of cells in broth or artificial seawater (Oliver et al., 1991; Nilsson et al., 1991). Oliver et al. (1991) revealed that VBNC entry was highly temperature dependent, with entry occurring at 5$^{o}$C but not 10$^{o}$C. They showed a 5-log *V. vulnificus* reduction (from 6 to 1 $\log_{10}$ cfu/ml) within fourteen days at 5$^{o}$C in artificial seawater. Several factors were suggested to influence entry into VBNC state. Cell growth phase analysis showed that logarithmic cells are more susceptible to entry versus stationary phase cells (Oliver et al., 1991). Pre-starvation in artificial seawater at room temperature for 1 to 2 hours caused cell division, but didn't prevent entry into VBNC state after temperature downshift to 5$^{o}$C. However, if cells were starved at room temperature for 4 hours or longer before temperature downshift, they didn't enter VBNC state, and maintained culturable cell density of at least 5 $\log_{10}$ cfu/ml throughout the 14-day study.

Recent studies have offered explanations of VBNC cell state (Bogosian et al., 2000; Kong et al., 2004; Abe et al., 2007). Bogosian et al. (2000) showed that *V. vulnificus* culture under conditions of refrigeration is split into two subpopulations of hydrogen peroxide-resistant cells and hydrogen-peroxide sensitive cells, with populations of the later increasing during storage. Most nutrient-rich media after autoclaving contain trace quantities of hydrogen peroxide, which is toxic to sensitive cells. As a result, VBNC cells might be, at least in part, non-culturable due to the hydrogen peroxide presence in the media (Kong et al., 2004).

The second objective of the present investigation was to evaluate the influence of seawater salinity (12, 20, and 35 ppt) and temperature (4, 10, and 25$^{o}$C) on the ability of pre-starved (2 hours at room temperature) *V. vulnificus* to maintain viability and culturabilty over a fourteen day storage period. Bacterial cell counts were monitored by plating on solid medium (TSA-2% NaCl), by turbidity measurement (O.D. 600), and by viability staining using LIVE/DEAD BacLight fluorescent dyes.

The ability of bacteria to multiply or survive under suboptimal conditions requires membrane fluidity modification, a phenomenon known as homeoviscous adaptation. Membrane fluidity can be altered through membrane fatty acid profile modification. Fatty acid profile changes are usually caused by temperature shifts, toxic antimicrobials, and other related stressors. Except for a single study by Linder and Oliver (1989), no one has described the effect of survival in seawater

at different salinities and temperatures on changes in cellular fatty acid profile *of V. vulnificus*. Therefore, the influence of those effects mimicking depuration conditions on changes in cellular fatty acid profile of *V. vulnificus* was the third objective of this study.

According to O'Neill et al. (1992), among 115 cases of shellfish-associated *V. vulnificus* infections in the U.S. (1975 to1988), over 80% either occurred in southern Gulf Coast states or were associated with shellfish harvested from these areas. To further prove abundance of virulent *V. vulnificus* during summer months in Gulf Coast oysters, Shapiro et al. (1998) investigated the role of Eastern oysters harvested in warmer months on *V. vulnificus* infections in the United States during 1988 to 1996. They found that among seventy *V. vulnificus* traced cases caused by raw oyster consumption, 89% of cases were tracked back to seawater harvesting temperatures of above 22$^{o}$C. The ability of starved, cold-stored cultures of *V. vulnificus* to cause disease has not been sufficiently studied. Furthermore, most *V. vulnificus* infection studies have used intravenous injection of the pathogen into mouse models. For example, Oliver and Bockian (1995) showed that *V. vulnificus* VBNC cells retained their virulence against mice after injection of inoculated refrigerated (5$^{o}$C, 7 days storage) artificial seawater containing <0.04 CFU of culturable cells but >$10^5$ VBNC cells. Culturable *V. vulnificus* cells were recovered from blood and peritoneal cavities of dead mice. It is notable that no virulence studies have been conducted on gastric infectivity of cold-starved *V. vulnificus.* Therefore, the third objective of the present study was

to investigate survival of starved *V. vulnificus* at different temperatures in simulated gastric fluid.

# CHAPTER III

# DEPURATION OF RAW OYSTERS TO REDUCE *VIBRIO VULNIFICUS* COUNTS

## Introduction

The influence of refrigerated (10°C) and ambient (22°C) water depuration at different salinities on *Vibrio vulnificus* counts, Aerobic Plate Counts, and microbiological profile of raw oysters was the objective of this study. Manual of Operations of National Shellfish Sanitation Program (FDA-CFSAN, 1995) suggests a number of rules for oyster depuration tank design:

1. UV light treatment of re-circulating water
2. Dissolved oxygen in depuration tank no less than 50% of saturation
3. Minimum temperature of seawater of 10°C and maximum temperature of 25°C for oyster depuration, if no adaptation to higher or lower temperature exists
4. Minimum flow rate in each depuration tank of process water to be no less than 107 liters per minute per cubic meter of shellfish, or one gallon per minute per standard U.S. bushel of shellfish.
5. The minimum volume of process water to be 6400 liters per cubic meter of shellfish based upon effective empty tank volume

6. The maximum depth of shellfish in containers to be 7.6 centimeters for oysters
7. Shellfish should be depurated for at least 48 hours to ensure coliform purge.

The National Shellfish Sanitation Program (CFSAN, 1995) requires that all depuration lots must be treated at least 48 hours and turbidity of the water in the depuration tank before UV disinfection to be less than 20 nephelometric turbidity units (NTUs). It also establishes verification of depuration plant performance, based on fecal coliform count (number of cells/100 grams).

## Materials and methods

### Depuration tank design

The depuration tank design and oyster depuration procedure was in compliance with recommendations made by the Manual of Operations of National Shellfish Sanitation Program (FDA-CFSAN, 1995). The depuration tank was 1.2 x 2.4 meters, equipped with a varying flow (284 to 568 liters/hour) circulating water system with a cooling unit through which the water passes (Figure 1). The filtration system consisted of a multilayer rock and sand gravity filter to remove large particles followed by a charcoal filter equipped with an ultra violet radiation source to minimize bacterial buildup. Water filtration units occurred prior to the water passing through the chiller. Water from the chiller

was then dispensed into the tank via pressurized air holes in PVC pipe to provide sufficient aeration for the livelihood of the oysters. Oysters were fed initially with 5 milliliters of Marine Snow® Plankton Diet (Two Little Fishies, Inc., Miami Gardens, FL) purchased at a local aquarium store. This amount, together with algae already present in the oyster gastrointestinal gut was sufficient to sustain animals for 2 weeks depuration time.


Figure 1. Depuration tank design (Pascagoula, MS)

Immediately after harvest (Table 1), oysters were transported to the Mississippi State University Experimental Seafood Processing Laboratory in Pascagoula, Mississippi in insulated coolers and placed into a specially designed tank within 12 hours of their harvest. A representative sample

Table 1. Harvest date, harvesting area, and depuration water salinity of oysters

| Delivery date (2006) | Harvesting area | Seawater salinity for depuration |
|---|---|---|
| April, 10 | LA area #5 | 12 ppt |
| May, 10 | LA area #6 | 16 ppt |
| May, 23 | LA area #6 | 20 ppt |
| July, 18 – set I | LA area #4 | 20 ppt |
| July, 18 – set II | LA area #4 | 20 ppt |
| August, 8 | LA area A La Hatch | for fresh refrigeration only |
| September, 8 – set I | LA area #4 | 12 ppt |
| September, 8 – set II | LA area #4 | 12 ppt |
| September, 27 – set I | LA area #6 | 16 ppt |
| September, 27 – set II | LA area #6 | 16 ppt |

of the same set of freshly harvested oysters was transported to the Mississippi State University Department of Food Science, Nutrition, and Health Promotion, Mississippi State, MS. The tank circulated and filtered water enriched with Sea Salt (CrystalSea MarineMix, Bioassay Laboratory formula, Marine Enterprise International, Baltimore, MD) at a selected salinity level (12, 16, or 20 ppt). A cooling system connected to the filtration system in the tank allowed temperature manipulation. The oysters were placed in two depuration tanks at ambient temperature (22$^{o}$C) and then the temperature was lowered in one of the tanks to reach a target temperature of 10$^{o}$C within 12 hours for a slow acclimatization period. The control tank maintained the water at the ambient temperature (22$^{o}$C). The lower temperature tank simulated winter water temperatures. Four bins of 50 oysters were placed in each of the depuration tanks. Oysters within the bins were no deeper than 7.6 cm. Oysters were fed once by adding 5 ml of

MarineSnow® Plankton Diet (Two Little Fishies, Inc., Miami Gardens, FL) to each tank immediately after oyster insertion.

Within each tank, two bins were used to determine oyster mortality rate during depuration and the other two were used to determine oyster *V. vulnificus* populations, Aerobic Plate Count populations, and microflora changes before and after a 14 day depuration period and after post-depuration storage at 4°C for 14 days.

## Microbial analysis

Population levels of *V. vulnificus* in oysters were determined using a three tube alkaline peptone water MPN procedure (DePaola and Kaysner, 2004), followed by selective plating on mCPC agar, and confirmation of isolates using biochemical profile (API 20E, bioMérieux, Durham, NC) at the Experimental Seafood Processing Laboratory.

Aerobic Plate Counts and general microflora of oysters were determined at the Department of Food Science, Nutrition, and Health Promotion. Two dozen freshly harvested and two dozen depurated oysters were transported on ice without direct contact from the Experimental Seafood Processing Laboratory to the Department of Food Science, Nutrition, and Health Promotion and analyzed within eight hours. Each set of oysters was split in half immediately upon arrival. One half was analyzed immediately and second half was placed in a plastic box containing a small amount of water on the bottom to prevent dehydration and

stored at 4$^{o}$C for two weeks before analysis. Oysters were manually cleaned with an autoclaved brush under continuously flowing tap water to remove soil from the shells. Oyster viability was judged by absence of gaping and no sign of mantle shrinking (Aaraas et al., 2004).

At each sampling point, a set consisted of 10 pooled oysters. For analysis, oysters were shucked using an ethanol dipped flamed oyster knife. Oyster meats with liquor were aseptically transferred to a sterile blender jar. Weight of sample was recorded and equal amount of sterile Phosphate Buffered Saline (PBS, pH 7.4, Sigma, St. Louis, MO) was added to each blender jar (1:2 dilution) followed by homogenization for 1 min on high using a Warring blender (model 31BL92, Warring Products Division, New Hartford, CT). Serial dilutions of the homogenate were performed using 9 ml PBS blanks and dilutions were spread plated on Trypticase Soy Agar (TSA, BD Diagnostic Systems, Sparks, MD). Plates were incubated for 48 hours at 35$^{o}$C and counted.

For fresh oysters, 18 colonies (9 from each duplicate plate) were randomly selected from countable APC plates (25 to 250 cfu/plate) to account for colony diversity. Because of lack of colony diversity only eight colonies were randomly selected (four from each countable plate of the same dilution) for all other treatments (fresh stored, depurated, depurated stored). The identity of each selected colony was determined using GC-FAME (MIDI, Inc., Newark, DE) based on similarity indexes of isolate fatty acid composition as described elsewhere (Duran and Marshall, 2005). If the similarity index of an isolate was low (<0.3),

there was no corresponding species in the database. For these isolates, the closest identification was chosen. All other bacterial isolates with similarity indexes of >0.3, were represented as a single or several species. Because this method gives results as probabilities, it is possible that several species can be associated with an isolate. If the similarity index difference between first choice and consecutive choices was more than 0.1, only the first choice was reported. If the index difference was lower than 0.1, all choices within the 0.1 range from the primary choice were reported.

## Statistical analysis

The experiment was repeated three times for each of the three salinities (12, 16, and 20 ppt) using the experimental setup shown in Figure 2.

APC data for all salinities were combined since no obvious influence of salinity was noted. One-way ANOVA procedure was applied to determine treatment affect on APC. Treatment had six levels - fresh, fresh stored, ambient depurated, ambient depurated stored, cold depurated, and cold depurated stored. Means were separated using Fisher's Least Significant Difference (LSD) test if the influence of treatment factor was significant ($p<0.05$).

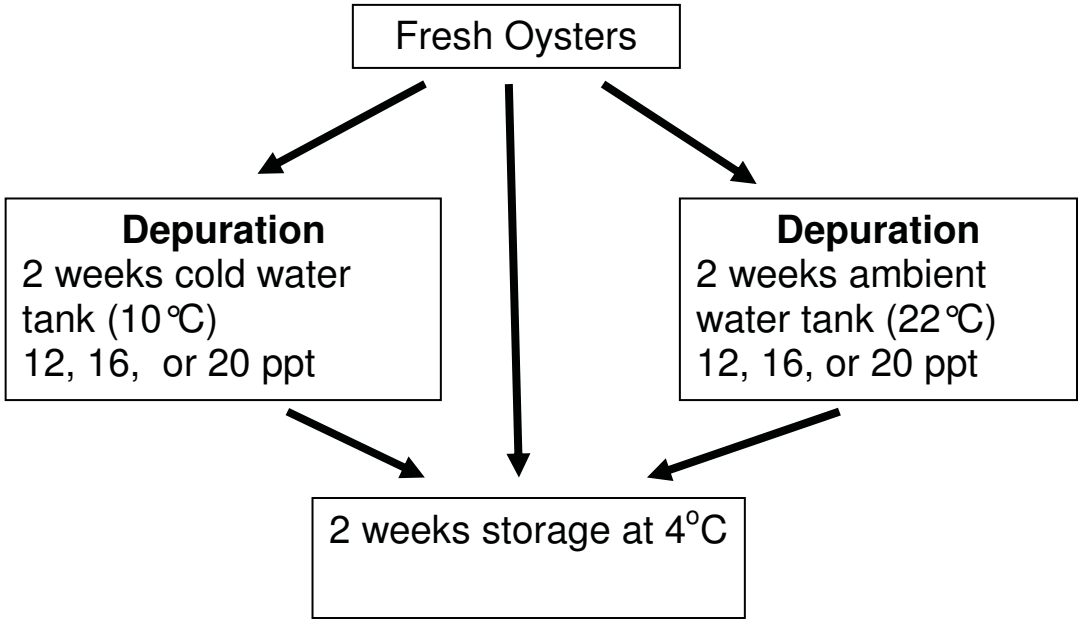


Figure 2. Oyster processing scheme

A two-way factorial design was used to test the effects of depuration method and salinity, as well as their interaction, on *V. vulnificus* counts in live oysters. Seawater salinity had three levels, 12, 16, and 20 ppt. Depuration method was either ambient water depuration (22°C) or cold water depuration (10°C). If the influence of factors was significant ($p<0.05$), means were separated using Fisher's Least Significant Difference (LSD) test. Statistical analysis was done using SAS ver. 9.2 (SAS Institute Inc., Cary, NC).

Identified bacterial isolates from each group of analyzed oysters were pooled together for all salinities to determine incidence before and after treatment. A total of 180 bacterial isolates were analyzed from freshly harvested oysters. Eighty bacterial isolates were analyzed from fresh stored oysters, and

seventy two from each of ambient water depurated oysters, cold water depurated oysters, ambient depurated stored oysters, and cold depurated stored oysters. The incidence of each specific bacterial genus/species was calculated and presented as a percentage based on the total number of bacterial isolates.

## Results and discussion

There was a significant treatment influence ($p<0.05$) on Aerobic Plate Counts of live oysters. APC data for all salinities were combined since no obvious influence ($p>0.05$) of salinity was noted (results not shown). Figure 3 shows APC of freshly harvested oysters, depurated oysters (2 weeks at 10 or 22°C), refrigerated fresh oysters (2 weeks at 4°C), and refrigerated 10 or 22°C depurated oysters (2 weeks at 4°C). The average APC for fresh harvested oysters was 5.6 $\log_{10}$ cfu/g. Fresh bivalve mollusks are generally considered good quality when APC is less than 5.7 $\log_{10}$ cfu/g (ICMSF, 1980). Cook and Ruple (1989) found that APC were greater in oysters harvested in the summer months compared to colder months, but levels rarely exceeded 5.7 $\log_{10}$ cfu/g.

On average, ambient temperature (22°C) depuration significantly ($p<0.05$) decreased APC by 0.6 log compared to counts of fresh oysters (Figure 3). Cold temperature (10°C) depuration had no affect ($p>0.05$) on APC compared to counts of freshly harvested oysters. Fresh oysters stored at 4°C for 2 weeks had significantly ($p<0.05$) greater counts (6.7 $\log_{10}$ cfu/g) compared to their freshly-harvested counterparts (5.6 $\log_{10}$ cfu/g) (Figure 3). Likewise, oysters depurated

at 22℃ had 1 log greater ($p<0.05$) counts after storage at 4℃ for 2 weeks than depurated oysters that were not stored. Storage at 4℃ for 2 weeks had no effect ($p>0.05$) on APC of oysters depurated 10℃.

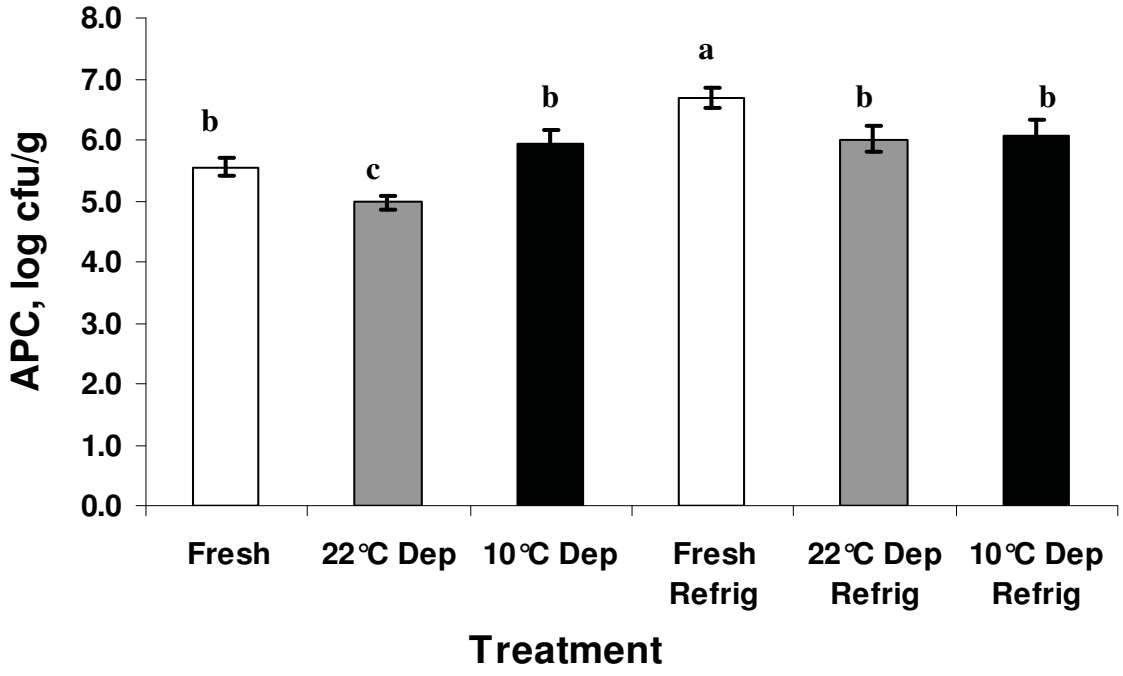


Figure 3. Aerobic Plate Counts of freshly harvested oysters, depurated oysters (2 weeks at 10 or 22℃), refrigerated fresh oysters (2 weeks at 4℃), and refrigerated 10 or 22℃ depurated oysters (2 weeks at 4℃)

NOTE: Means with the same letters are not significantly different ($P>0.05$)

Refrigerated storage of fresh and 22℃ depurated oysters resulted in increased APC likely because of proliferation of psychrotrophic and psychrophilic bacteria (Jay, 2000; Cruz-Romero et al., 2008). Similarly, Cook and Ruple (1989) found a slight APC increase of 0.2 $\log_{10}$ cfu/g in live oysters stored at 10°C for 5

days. Refrigerated storage of 10°C depurated oysters had no affect on APC possibly because of the minimal temperature change (10 to 4°C).

*V. vulnificus* counts significantly ($p<0.05$) declined in oysters depurated at 10 and 22°C (Figure 4). At 12 ppt salinity *V. vulnificus* count reduction was 1 log greater ($p<0.05$) in oysters depurated at 10°C compared with oysters depurated at 22°C. Greatest *V. vulnificus* count reductions were seen with depuration in 12 ppt, 10°C water (2.7 logs) and 20 ppt, 22°C water (2.8 logs). Depuration water salinity had no influence ($p>0.05$) on *V. vulnificus* count reductions. Kaspar and Tamplin (1993) found a 10-fold reduction in endogenous *V. vulnificus* populations in eastern oyster shellstock after 14 days at 2 to 4°C, while storage at 30°C for three days resulted in more than a 2 log *V. vulnificus* population increase. In the present study, a significant decrease in *V. vulnificus* counts was seen for both cold-water depurated (as expected) and warm-water depurated (not expected) oysters. Perhaps the 14-day depuration time used in the present study accounts for the reductions seen here compared to only 3 days in the previous report. In addition, the temperature difference (22 vs. 30°C) may also have influenced results, with a lower depuration temperature perhaps resulting in count reductions. Indeed, the optimum temperature for growth of *V. vulnificus* is 37°C (Anonymous, 2001).

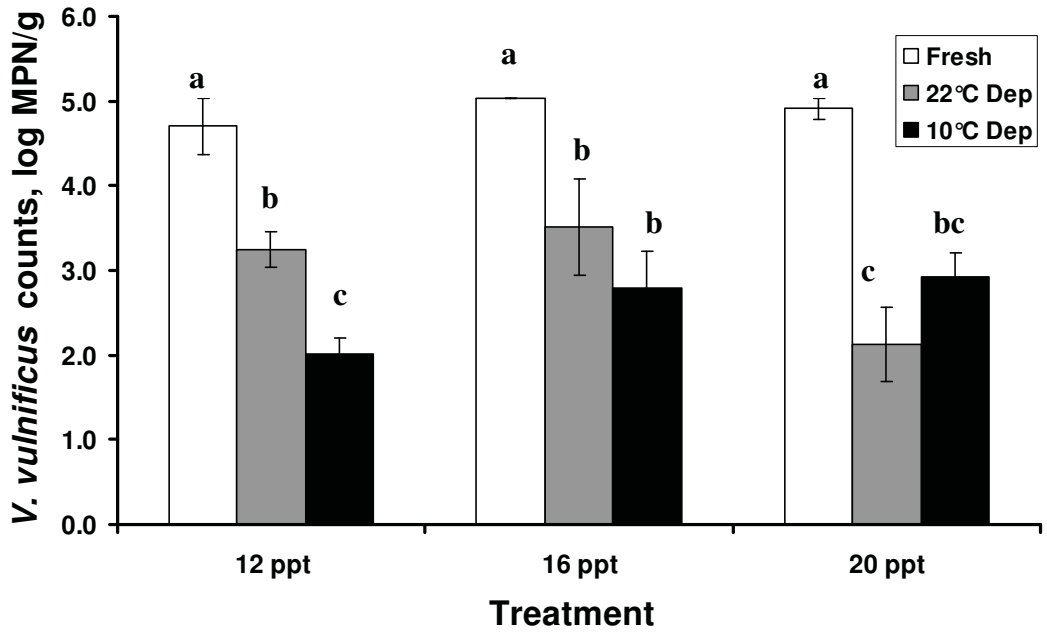


Figure 4. *V. vulnificus* counts of freshly harvested and 2 weeks depurated oysters at different salinities

NOTE: Means with the same letters are not significantly different

Early studies with oyster depuration showed its efficacy for total coliform count reduction (Perkins et al., 1980; Jones et al., 1991), but not pathogenic vibrios count reduction (Eyles and Davis, 1984). The important distinction, however, is that these studies used short-duration depuration times, whereas the present study used a longer duration. The present study showed that prolonged depuration (2 weeks) of warm-water harvested Gulf Coast oysters at either ambient (22$^{o}$C) or chill (10$^{o}$C) temperatures reduced *V. vulnificus* counts, but not to non-detectable levels. The inability of the depuration process used here to completely eliminate *V. vulnificus* is likely due to persistent localization of the

bacterium within oyster tissues, making elimination difficult (Tamplin and Capers, 1992).

Depuration water salinity (12 to 20 ppt) did not affect effect *V. vulnificus* count reductions after depuration. Using laboratory and field studies, others have found that *V. vulnificus* is only sensitive to salinities greater than 30 ppt (O'Neill et al., 1992; Motes and DePaola, 1996). Present results confirm these observations.

Overall, prolonged oyster depuration failed to reduce *V. vulnificus* numbers by 5 $\log_{10}$ as required by ISSC (Birkenhauer and Oliver, 2003), which means more aggressive depuration conditions must be considered. Future studies should investigate the effect of gradual increases of depuration seawater salinity to 30 to 35 ppt, as this salinity level has proved effective during relaying (Motes and DePaola, 1996). Refrigeration itself without depuration might not be effective, as Kaspar and Tamplin (1993) found only a 10-fold reduction in endogenous *V. vulnificus* populations in Eastern oyster shellstock after 14 days storage at 2 to 4$^{o}$C.

Figure 5 shows the predominant microflora identified in freshly harvested oysters. Of 180 bacterial isolates, the most abundant genera were *Vibrio* (99 isolates), *Shewanella* (37), *Photobacterium* (19), *Pseudomonas* (9), *Aeromonas* (8), and *Alcaligenes* (5).

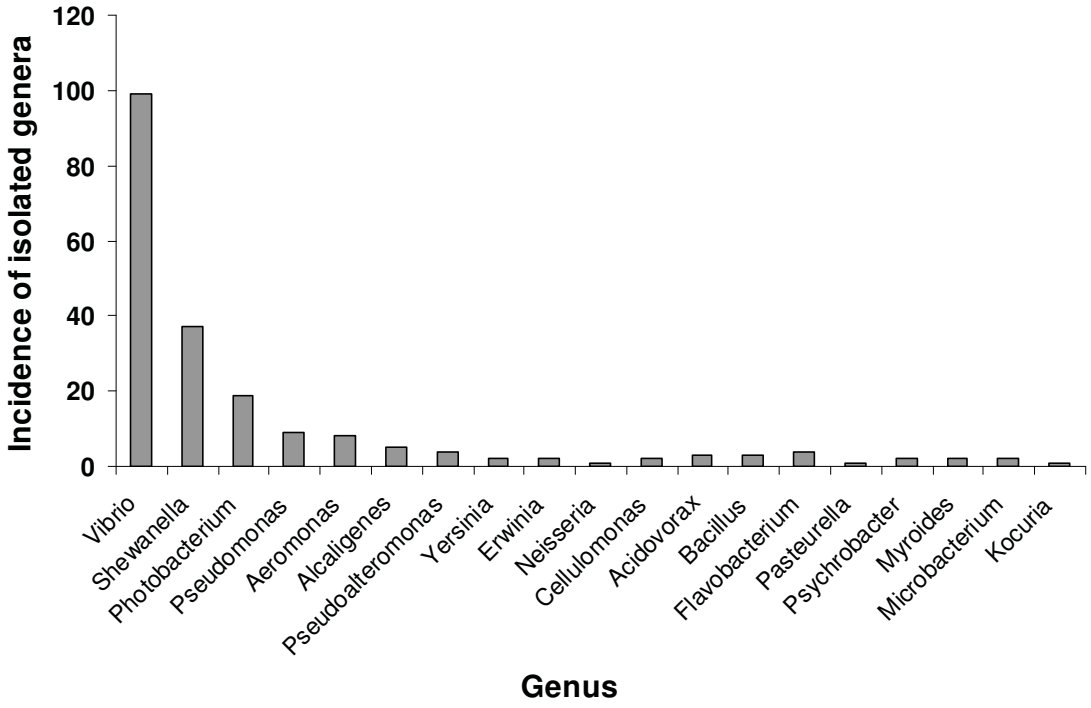


Figure 5. Incidence of isolated bacterial genera from freshly harvested oysters

Others have found a similar microbiological profile of fresh summer-harvested oysters (Colwell and Liston, 1960; Kueh and Chan, 1985). The dominance of vibrios in Gulf Coast oysters is not a surprise; according to Pujalte et al. (1999), *Vibrio* spp. were the dominant bacteria in Mediterranean oyster microflora, comprising around 65% of all bacterial isolates, with dominance declining to 50% during the cold season. They found that some *Vibrio* species appeared only in warm and late-warm season (*V. harveyi, V.pelagicus, V. mediterranei, V. tubiashii*), whereas *V. splendidus* dominance was restricted to winter and spring samples. Similarly, Guisande et al. (2004) found that microflora

of Spanish Galician bivalve mollusks consists of 80% facultative anaerobes and 20% aerobes. Moreover, 57% of facultative anaerobic bacteria were vibrios, including *V. aesturianus*, *V. splendidus*, *V. alginolyticus*, *V. anguillarum*, and *V. salmonicida*, while the predominant aerobic genera were *Shewanella*, *Pseudoalteromonas*, *Pseudomonas*, and *Alcaligenes*.

In the present study, 68% (67 isolates out of 99 isolated vibrios) were pathogenic *Vibrio* species, either *V. parahaemolyticus, V. vulnificus,* or *V. cholerae.* Moreover, a seasonal influence on incidence was observed (Figure 6), with a greater percentage of pathogenic *Vibrio* species occurrence during the period between July, 18, 2006, and Sept, 8, 2006. This result is in agreement with other studies (Kelly, 1982; O'Neill et al., 1992; Motes et al., 1998), which also showed abundance of pathogenic vibrios in Gulf Coast oysters during warm summer months.

Microflora that predominated in freshly harvested oysters after 2 weeks of refrigerated storage (4$^{o}$C) is shown in Figure 7. The predominance of vibrios was dramatically reduced compared to prevalence in oysters before storage (Figure 5). Instead, among 80 isolates, psychrotrophic *Pseudomonas* (25 isolates), *Aeromonas* (19), *Shewanella* (9), *Psychrobacter* (6), and *Pseudoalteromonas* (5) were the predominant genera (Figure 7).

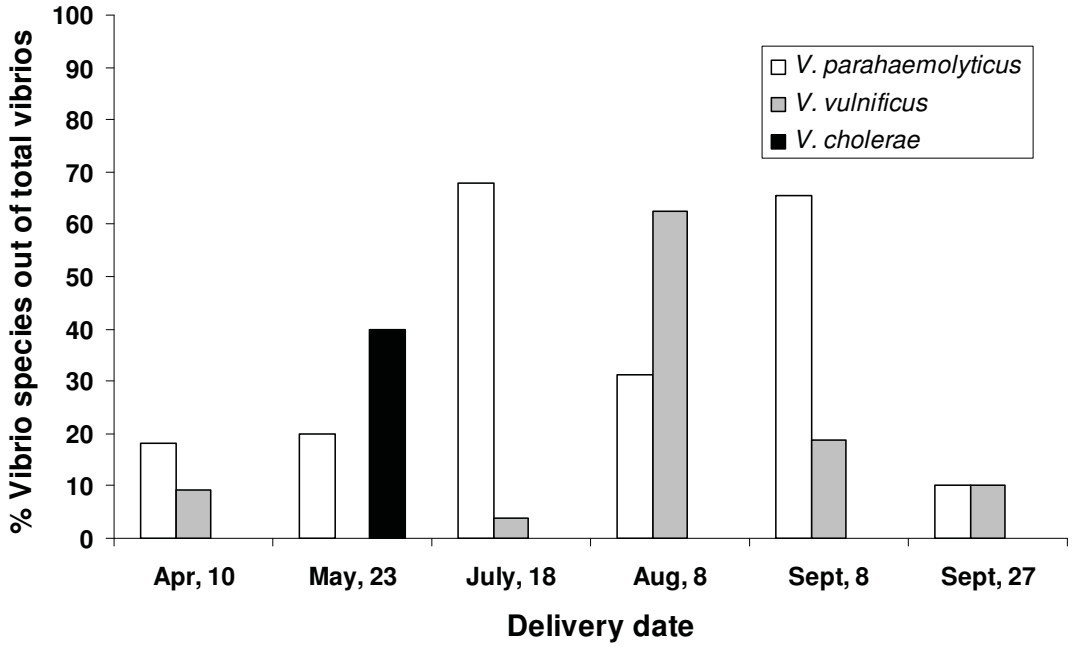


Figure 6. Percentage of pathogenic *Vibrio* species out of total vibrios isolated from fresh oysters on different days

One of four isolated *Vibrio* spp. from 2-week stored oysters was identified as a pathogenic species, *V. parahaemolyticus.*

The microflora of aerobically stored refrigerated oysters noticeably changes within time, favoring psychrotrophic spoilage-inducing bacteria such as *Pseudomonas* spp. and *Shewanella putrefaciens*, followed by appearance of enterococci, lactobacilli, and yeasts at later stages of spoilage (Jay, 2000). Others have reported similar genera from spoiled refrigerated oysters, such as *Pseudomonas, Shewanella, Enterobacter, Lactobacillus, Serratia*, and *Flavobacterium* (Cruz-Romero et al., 2008).

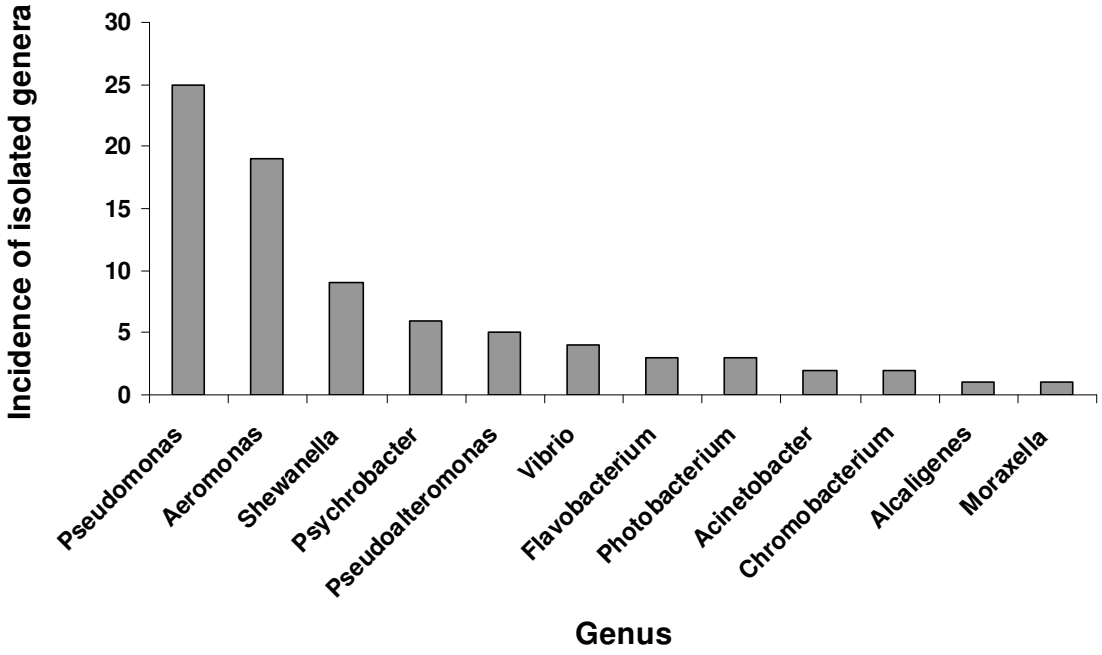


Figure 7. Incidence of isolated bacterial genera from freshly harvested oysters stored for 2 weeks at 4°C

The incidences of isolated genera from oysters depurated for 2 weeks at 10 or 22°C are shown in Figure 8. With 22°C depuration, oysters had *Vibrio* (31 isolates) and *Shewanella* (22 isolates) as predominant genera, while 10°C depuration yielded predominantly *Pseudoalteromonas* (28 isolates) followed by equal incidences of *Vibrio* and *Shewanella* (16 isolates each).

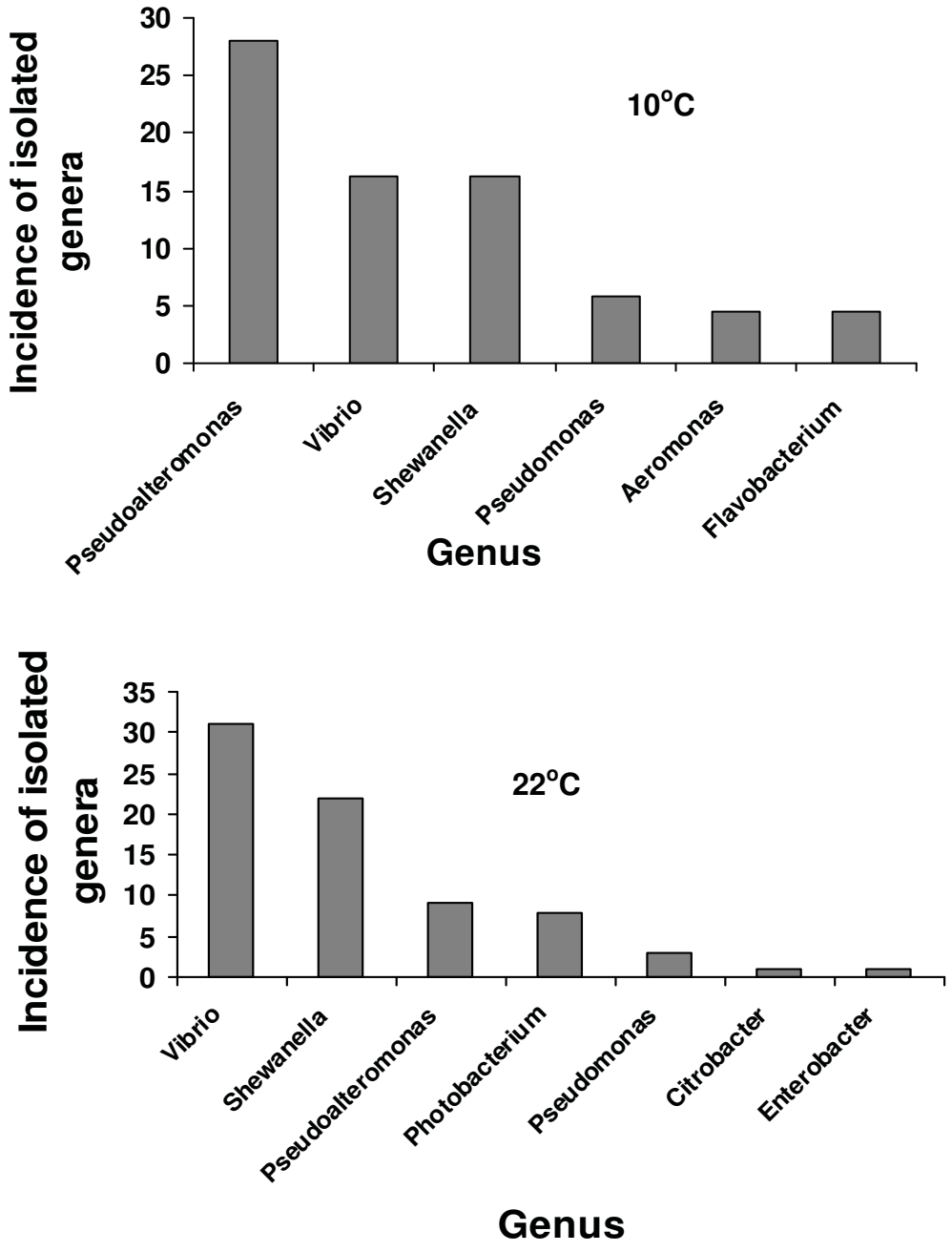


Figure 8. Incidence of isolated bacterial genera from oysters depurated for 2 weeks at 10 (top) and 22°C (bottom)

Vibrios isolated from oysters depurated at 10°C were mostly non-pathogenic species possibly due to the fact that pathogenic species prefer warmer temperatures (Jones et al., 1991) (Figure 9). *Vibrio parahaemolyticus* was the predominant pathogenic species in oysters depurated at 22°C (Figure 9). This result mimicked the predominance of this species in freshly harvested non-depurated oysters (Figure 6). Others have found that this species is the predominant pathogenic vibrio in Gulf Coast oysters (Jones et al., 1991).

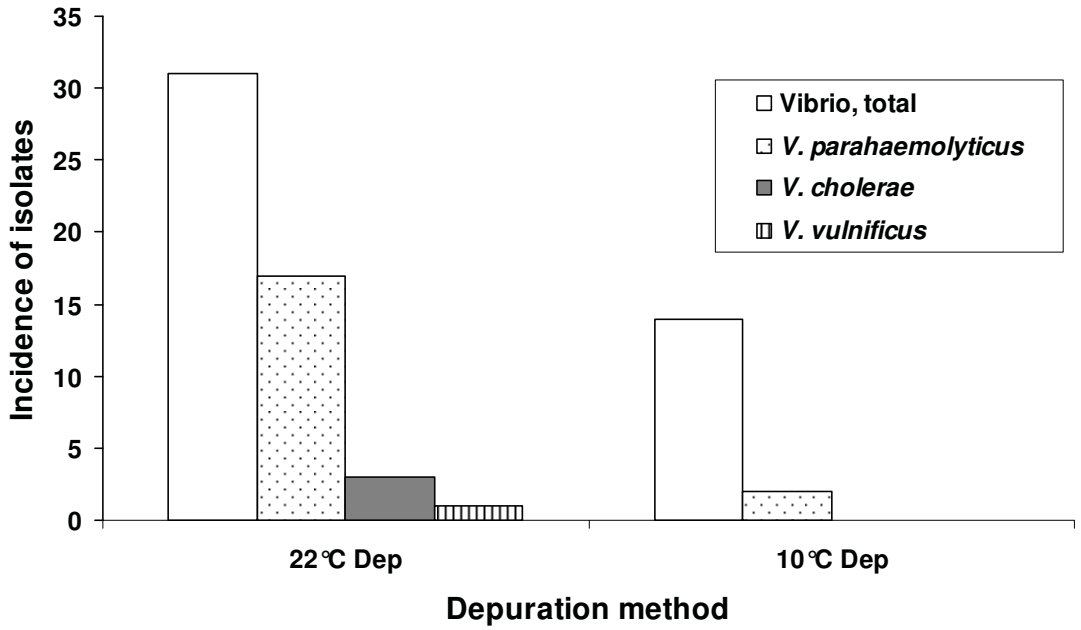


Figure 9. Incidence of pathogenic vibrios in oysters depurated for 2 weeks at 10 and 22°C (all salinities combined)

Depurated oysters that were subsequently stored at 4°C for 2 weeks had similar microbiological profile no matter what depuration temperature was used. In both cases, *Pseudomonas* predominated (Figure 10). The microbiological

profile of stored depurated oysters had a similar dominance of psychrotrophic bacteria as found with stored freshly harvested oysters (Figure 7).

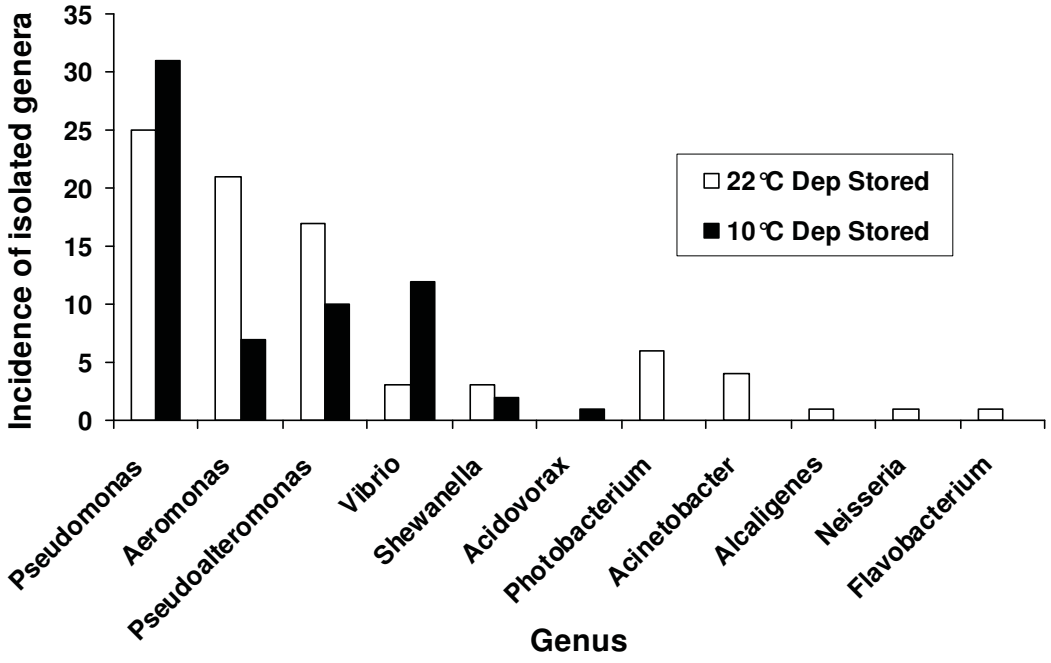


Figure 10. Microbiological profile of stored (4°C) ambient and cold depurated oysters

To summarize, depuration at 22°C was as effective for *V. vulnificus* reduction in oysters as was 10°C depuration. Depuration at 22°C resulted in oysters with lower Aerobic Plate Counts than oysters depurated at 10°C. Low temperature storage (4°C) or depuration (10°C) of warm-water harvested live oysters shifted microflora of oysters from mesophilic vibrios to psychrotrophic *Pseudomonas*, *Aeromonas*, and *Shewanella* spp. Depuration of live oysters at 22°C retained dominance of mesophilic vibrios with greater incidence of

pathogenic vibrios compared to 10°C depuration, though overall APC count was lower. Prolonged oyster depuration failed to reduce *V. vulnificus* numbers to non-detectable levels, which means more aggressive depuration conditions must be considered. Future studies should investigate the effect of gradual increases of depuration seawater salinity to 30 to 35 ppt, as this salinity level proved effective against *V. vulnificus* during relaying. Another possible intervention might be use of antimicrobial compounds in the depuration water. More research is needed to evaluate these possibilities and their influence on depuration time, since two weeks depuration will not be accepted by oyster processors because of economic reasons.

# CHAPTER IV

# CORRELATION OF *VIBRIO VULNIFICUS* POPULATION DENSITY WITH OPTICAL DENSITY AND FLUORESCENCE VIABILITY

## Introduction

A pragmatic definition of prokaryote viability as their ability to multiply when provided with optimal conditions for growth was revisited by Roszak and Colwell (1987). According to them, a new term should be introduced, namely, “live”, for cells showing no ability to multiply, but possessing other signs of viability, such as respiration, even if cells are unable to divide under present conditions.

One of the earliest viability counts methods used acridine orange direct staining (Roszak and Colwell, 1987). It was suggested that living cells will have a red fluorescence, while inactive cells will show green fluorescence. This differential staining is due to the fact that acridine orange fluoresces red in association with RNA and green in case of DNA. A high RNA to DNA ratio indicates active metabolism (RNA presence) and physiologically active cells.

Kogure et al. (1979) described a tentative method for counting viable but not culturable bacteria from marine environments, which included direct viable count of cells with potential for cell division and DNA replication inhibited by

nalidixic acid and growth enhanced by the addition of yeast extract. The resultant "viable" cells appear larger and elongated compared to those not capable of growth due to the added substrate and inhibition of division. Another method, described by Roszak and Colwell (1987), included indirect estimation of bacterial respiration activity by reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride to a substance that is deposited as optically dense, dark red, intracellular aggregates that are visible by light microscopy.

Molecular Probes, Inc. (Eugene, OR, USA) markets Live/Dead BacLight™ bacterial viability kits to differentiate between live and dead bacteria within the same population using fluorescence microscopy, fluorescence spectroscopy, fluorescence microplate readers, or flow cytometry. The kit provides a novel two-color fluorescence assay of bacterial viability that has proven useful for *Vibrio parahaemolyticus* (Chen et al., 2009). The kit contains two vials of two fluorescent dyes, namely, SYTO® 9 green-fluorescent nucleic acid dye and red-fluorescent nucleic acid dye propidium iodide. The difference between these two dyes is not only in their spectral characteristics, but their ability to penetrate healthy cell envelops. The excitation/emission optimum for SYTO® 9 and propidium iodide dyes are 480/500 and 490/635 nm. SYTO® 9 penetrates both healthy and damaged cell envelops, which results in nucleic acid staining of both live and dead cells causing green fluorescence. On the other hand, propidium iodide penetrates only damaged cell envelops, which reduces SYTO® 9 green fluorescence and counter-stains dead cells with red fluorescence when both dyes

are present. Therefore, live cells having intact cell envelops will be stained fluorescent green, while dead cells with damaged cell envelops will stain fluorescent red. The manufacturer warns that this cell viability kit might give questionable results, especially for cells having compromised cell envelops that may be resuscitated resulting in reproduction on microbiological media. Alternatively, some cells with intact cell envelops may be unable to proliferate using classical microbiological techniques.

## Materials and methods

### Isolation and confirmation of *V. vulnificus* environmental strain (Env-1)

A set of twelve oysters was shipped priority overnight on ice from the Mississippi State University Experimental Seafood Laboratory, Pascagoula, MS to the Department of Food Science, Nutrition, and Health Promotion, Mississippi State University, Mississippi State, MS on September, 27, 2008. The oysters were harvested by Crystal Seas Seafood (Pass Christian, MS) and included tag with harvest location of Louisiana harvest area No 3. The oysters were aseptically shucked as described in Chapter 3 and the meat was added to a sterile blender jar, diluted 1:2 with sterile Phosphate-Buffered Saline (PBS, pH 7.4, Sigma, St. Louis, MO), and blended at high speed in a Warring blender (model 31BL92, Warring Products Division, New Hartford, CT) for 90 seconds. One milliliter of obtained suspension was diluted in 9 ml of sterile Alkaline

Peptone Water (APW, pH 8.5) and incubated overnight at 35°C. After incubation, a loopful of APW enrichment was streaked onto modified Cellobiose Polymyxin Colistin agar (mCPC) prepared as described in FDA BAM (DePaola and Kaysner, 2004), and incubated for 24 hours at 35°C. Selective mCPC agar contained the following ingredients specifically designed for microbiological media or at least reagent grade: Bacto™ beef extract, agar, bromthymol blue (BD Diagnostic Systems, Sparks, MD), sodium chloride, peptone (FisherScientific, Pittsburgh, PA), cresol red (MP Biomedicals, Solon, OH), ethanol (Acros Organics, Morris Planes, NJ), cellobiose, colistin, and polymyxin B (Sigma). Typical *V. vulnificus* colonies (yellow with yellow halo) were re-streaked onto Trypticase Soy Agar supplemented with 2% NaCl (TSA-2, BD Diagnostic Systems) for purification purposes and further maintenance on TSA-2 slants at 25°C.

One presumptive *V. vulnificus* isolate was designated strain Env-1. This isolate was confirmed by growth patterns on mCPC agar and Difco™ Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (BD Diagnostic Systems), Gram staining (Gram-negative rod), motility test medium plus 1% NaCl (peptone, Bacto™ beef extract, agar, sodium chloride; BD Diagnostic Systems) growth, salt tolerance in $T_1N_0$, $T_1N_3$, $T_1N_6$, $T_1N_8$, and $T_1N_{10}$ tryptone broths (Bacto® Tryptone, BD Diagnostic Systems), oxidase (BD Diagnostic Systems), catalase tests, rapid biochemical API-20E test strips (bioMérieux, Durham, NC), and fatty acid methyl

ester (FAME) microbial identification (MIDI, Inc., Newark, DE) by gas chromatography (Duran and Marshall, 2005).

## ***V. vulnificus*** **cultures maintenance and initial preparation**

*V. vulnificus* strains Env-1 and ATCC 27562 (Lyfocults, Quality Technologies, LLC, Newbury Park, CA) were maintained at room temperature on TSA-2 slants with 2-week re-streaking period to a fresh TSA-2 slant. Both strains were of opaque phenotype. For experimentation, individual strains were streaked on TSA-2 and grown for 12 hours at 35$^{o}$C. Single colonies of each strain were transferred to 60 ml of Trypticase Soy Broth supplemented with 2% NaCl (TSB-2) and grown individually in an environmental shaker (PsychroTherm, New Brunswick Scientific Co, Inc, Edison, NJ) for 18 hours at 25°C under agitation (150 rpm) yielding a turbid broth, which served as working cultures for further studies.

## **Fluorescence measurements for *V. vulnificus* ATCC 27562 using Live/Dead® BacLight bacterial viability kit**

Twelve microliters of 50:50 ratio mix of fluorescence dyes (3.34 mM Syto® 9 in DMSO and 20 mM propidium iodide in DMSO, Molecular Probes Inc, Eugene, OR) were added to 2 ml of distilled, filter-sterilized (0.2 µm) water to prepare working dye mix. Prepared solution was used within 1 hour and stored in the dark until use to avoid fluorescent compound degradation. One hundred

microliters of bacterial culture was added to three wells of 96-well black fluorescent plates (Whatman Polyfiltronics Inc, Rockland, MA) followed by addition of equal volume of working dye mix. The microplate was incubated for 15 minutes in the dark and fluorescence readings were taken using a Series 4000 Cytofluor® multi-well plate reader (PerSeptive Biosystems, Framingham, MA) with excitation filter at 490 nm and emission filter at 530 nm for green wavelength response ("viable" cell response, $F_{530}$) and with excitation filter at 490 nm and emission filter at 630 nm for red wavelength response ("non-viable" cell response, $F_{630}$). A "viability" ratio of $F_{530}/F_{630}$, known as a Fluorescence Viability Index (FVI), was calculated for each of the three wells and averaged.

## **Optical density (OD 600 nm) measurements for *V. vulnificus* ATCC 27562 in artificial seawater**

A microtiter plate reader (Spectramax 250, Molecular Devices Corp, Sunnyvale, CA) with 96-well microplates was used for measuring turbidity (O.D. 600 nm) of *V. vulnificus* populations in 20 ppt seawater. Wells of the microtiter plate were filled with 250 µl of different populations of *V. vulnificus* and optical density (600 nm) was read after five seconds of automatic shaking. Results of turbidity from three wells of each population were averaged and recorded.

## Fluorescence viability index and optical density (ATCC 27562) standard curves

Six milliliter aliquots of the working broth culture of each strain were transferred to separate 15-ml tubes, which were centrifuged for seven minutes at 1,380 x g (Centrific Model 228, ThermoFisher Scientific, Fairlawn, NJ). Cell pellets were re-suspended in 6 ml sterile 20 ppt artificial seawater and centrifuged again under the same conditions. This procedure was repeated to obtain double-washed cells in 6 ml 20 ppt seawater. *Vibrio vulnificus* enumeration was done through serial dilutions in PBS and plating three consecutive dilutions on duplicate TSA-2 plates (25℃, 48 hours). Double-washed cells were diluted 1:2, 1:4, 1:6, 1:8, 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100 ratios in sterile seawater (20 ppt). Each dilution was analyzed for optical density (Spectramax 250) in three separate wells. Each dilution also was analyzed for fluorescence (Cytofluor®) to calculate viability index (FVI, $F_{530}/F_{630}$) in three separate wells. This experiment was conducted four times on four different days for both optical density standard curve construction and fluorescence viability index standard curve construction.

## Preparation of *V. vulnificus* for fluorescence measurements of dead/live cell ratio mixes

The procedure described by the manufacturer (Invitrogen, Inc.) was used to standardize fluorescence response to viable and non-viable cell ratio in

artificial seawater. One-ml aliquot of working culture was centrifuged for 1 min at 20,800 x g in a microfuge tube to obtain a cell pellet (Centrifuge 5417C, Eppendorf, Hinz GmbH, Hamburg, Germany). The cell pellet was washed twice with 1 ml 20 ppt sterile artificial seawater with centrifugation steps in between as described earlier. Final re-suspension was done in 0.5 ml 20 ppt seawater to achieve a 2X cell concentration increase. Two 0.1-ml aliquots of this suspension were transferred to 0.9 ml 20 ppt seawater and 0.9 ml 70% isopropanol (Sigma). Each mixture was vortexed and incubated for 1.5 hours at 25°C with 5-sec mixing every 15 minutes. Dilution in seawater was a positive control and served as a source of live cells, while exposure to 70% isopropanol was used to mortalize cells to non-detectable levels. Both samples were double washed in 1 ml 20 ppt artificial seawater with pelletization conditions of three minutes at 24,500 x g in between. Both samples were diluted 1:10 in 20 ppt seawater and adjusted to 0.16 $OD_{600}$ (8.3 $\log_{10}$ cfu/ml) with the same diluent. Absorbance readings were taken in three wells (250 µl analyzed volume) for each sample in a 96-well plate (Spectramax). *Vibrio vulnificus* numbers were confirmed by serial dilutions in PBS and spread plating (TSA-2, 48 hours, 25°C) for viable cell sample. Non-viable cell sample confirmation was done by direct spread plating of three 0.1 ml aliquots on separate plates (TSA-2, 48 hours, 25°C), with no growth observation confirming loss of viability.

Viable and non-viable cell samples were mixed to achieve 100:0 and 10:90 ratios, representing 100% live (~8.3 $\log_{10}$ cfu/ml) and 10% live (~7.3 $\log_{10}$

cfu/ml, or 1 $\log_{10}$ reduction in viable cell population). Fluorescence measurements of each ratio mix were performed in three wells as described previously in this chapter and results were averaged. The experiment was conducted five times on five different days.

**Statistical analysis**

Regression analysis using a cubic model was used to predict OD 600 nm values based on calculated $\log_{10}$ cfu/ml of 1:2, 1:4, 1:6, 1:8, 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100 diluted *V. vulnificus* ATCC 27562 cells in sterile 20 ppt seawater. Similarly, regression analysis using a cubic model was used to predict $\log_{10}$ cfu/ml counts based on OD 600 nm values.

Regression analysis using a quadratic model was used to predict fluorescence viability indices based on calculated $\log_{10}$ cfu/ml of 1:2, 1:4, 1:6, 1:8, 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100 diluted *V. vulnificus* ATCC 27562 cells. Similarly, regression analysis using a quadratic model was used to predict $\log_{10}$ cfu/ml counts based on fluorescence viability values.

One-way ANOVA procedure was applied to analyze the effect of the live cell percentage (100% or 10%) on Fluorescence viability index. Means were separated using Fisher's Least Significant Difference (LSD) test if the influence of live cell percentage factor was significant ($p<0.05$).

Statistical analysis, including standard regression coefficients and $R^2$ values estimation, as well as one-way ANOVA procedure for live:dead cell mixes, was done using SAS ver 9.2 software (SAS Institute Inc., Cary, NC).

## Results and discussion

White (2000) wrote that turbidity measurements to estimate bacterial concentration rely on the principle of light scattering that is proportional to the total cell mass in a solution. Optical density (OD) is a logarithm (base 10) of a ratio between incident light and transmitted light through an unknown solution. Optical density is directly proportional to cell density and is predictive of cell concentration. It is important to note that a linear model cannot be applied to high cell densities because of deviation from linearity (White, 2000).

Statistical analysis of regression models revealed excellent correlation between the independent variable *V. vulnificus* counts and the dependent variables $OD_{600}$ ($R^2$=0.97) and fluorescence viability index ($R^2$=0.99) (Figure 11). The regression equation to predict $OD_{600}$ based on bacterial counts (x) was $y = 0.17783x^3 - 4.0571x^2 + 30.905x - 78.498$, while the regression equation to predict fluorescence viability index based on bacterial counts (x) was $y = 10.739x^2 - 154.55x + 559.49$.

Similarly, statistical analysis of regression models revealed excellent correlation between the dependent variable, *V. vulnificus* counts, and independent variables $OD_{600}$ ($R^2$=0.95) and fluorescence viability index

($R^2$=0.97). The regression equation to predict bacterial counts based on $OD_{600}$ (x) was $y = 6.5260x^3 - 13.491x^2 + 9.3375x + 6.8549$, while the regression equation to predict bacterial counts based on fluorescence viability index (x) was $y = - 0.0008921x^2 + 0.08566x + 7.0889$. For all regression models, all values for y-intercept, slope, the second power of slope, and the third power of slope were significant ($p<0.0001$).

The lowest detection level for bacterial counts estimation was chosen as 7.85 $\log_{10}$ cfu/ml with corresponding $OD_{600}$ of 0.11 and fluorescence viability index of 10 (Figure 11).

Mixes of 100% live (~8.3 $\log_{10}$ cfu/ml) and 10% live (~7.3 $\log_{10}$ cfu/ml, or 1 $\log_{10}$ reduction in viability) were significantly different ($p<0.05$) from each other, based on fluorescence viability index values. A mix of 100% live cells had an average FVI of 14.27, while a mix of 10% live cells had an average FVI of 6.26, or 56% less. Based on FVI standard curve, 100% live mix corresponded to 8.13 $\log_{10}$ cfu/ml, while 10% live mix corresponded to 7.59 $\log_{10}$ cfu/ml, or 0.54 $\log_{10}$ less instead of 1.0 $\log_{10}$ less. The $OD_{600}$ value of both mixes was the same (0.16), implying the same total cell concentration.

To summarize, both OD 600 and FVI measurements were sufficient to predict *V. vulnificus* counts in artificial seawater down to 7.85 $\log_{10}$ cfu/ml level. FVI was able to differentiate between 100% and 10% live cell mixes; however, the observed 0.54 $\log_{10}$ cfu/ml decrease in viable cell count was less than the expected 1.0 $\log_{10}$ cfu/ml decrease.

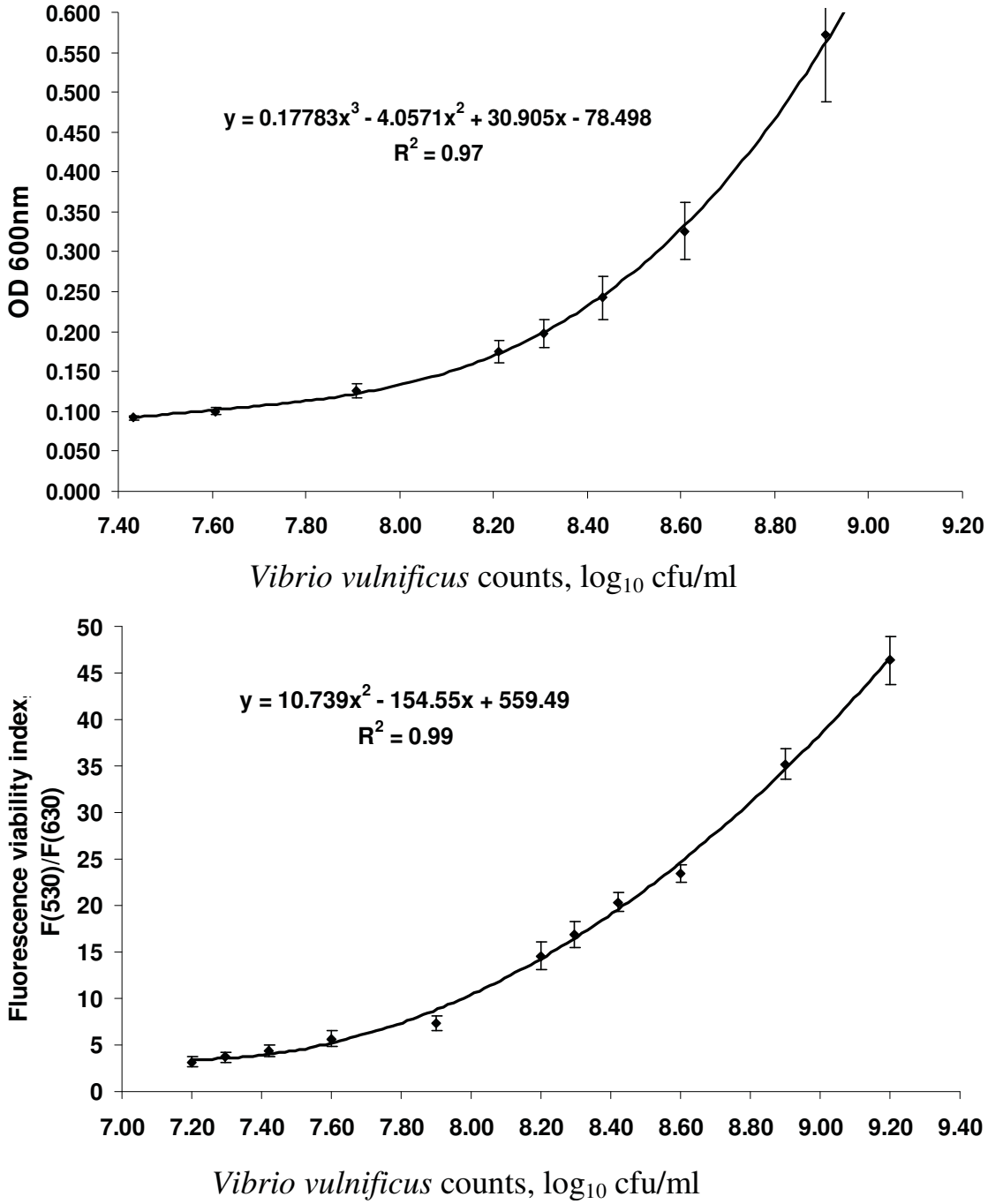


Figure 11. Standard curve of OD 600 nm (top) and Fluorescence Viability Index (FVI, bottom) dependence on *V. vulnificus* counts in 20 ppt artificial seawater

The correlations obtained in this experiment were used to distinguish between total plate count and viable plate count of *V. vulnificus* populations in Chapter V experiments dealing with *V. vulnificus* behavior in seawater at different salinities and temperatures. The experiments in Chapter V mimic *V. vulnificus* behavior in artificial seawater of depuration tanks with no UV treatments available, hopefully shedding some light on what might happen during long-term depuration treatments.

# CHAPTER V

# INFLUENCE OF TEMPERATURE (4, 10, AND 25°C) AND SALINITY (12, 20, AND 35 PPT) ON SURVIVAL OF STARVED *VIBRIO VULNIFICUS*

## Introduction

The objective of this study was to evaluate the influence of artificial seawater salinity (12, 20, and 35 ppt) and temperature (4, 10, and 25°C) on the ability of pre-starved (2 hours at room temperature) *V. vulnificus* to survive and retain culturability on general purpose media over a fourteen day storage period. Two-hour starvation adaptation before depuration mimics the time ambient temperature oysters may require when immersed into refrigerated depuration tanks to reach chill temperatures. Organic carbon load of natural seawater is usually low and varies from 0.3 to 2 mg/L, depending on the season and location, while estuarine waters have organic load between that of seawater and fresh water from rivers and streams (0.3 to 10 mg/L) (Thurman, 1995). Organic load of seawater is influenced by pollution and phytoplankton bloom (Thurman, 1995). Low organic load of depuration tank water is controlled by filtration, low water turbidity (<20 NFU's), and high dissolved oxygen requirements (50% saturation) (FDA-CFSAN, 1995). Low organic load could induce a *V. vulnificus* starvation response when the bacterium is first introduced to depuration tank water. During

the experiments in artificial seawater, cells counts were monitored by plating on solid medium (TSA-2% NaCl), by turbidity measurement ($OD_{600}$), and by viability assaying using LIVE/DEAD BacLight fluorescent dyes.

## Materials and methods

### Preparation of starved *V. vulnificus* in artificial seawater

Artificial seawater (12, 20, and 35 ppt) was freshly prepared for each replication by dissolving 12, 20, and 35 g of seasalt (CrystalSea MarineMix, Bioassay Laboratory formula, Marine Enterprise International, Baltimore, MD) in 1 L of distilled water followed by filter-sterilization (0.45 µm cellulose acetate, Corning Costar, Corning, NY). Artificial seawater pH was measured using an Accumet AR-60 pH meter (ThermoFisher Scientific).

Three 6-ml aliquots of a working broth culture of *V. vulnificus* strains ENV-1 and ATCC 27562 (see Chapter IV) were transferred to 15-ml tubes and centrifuged (Centrific Model 228, ThermoFisher Scientific) for seven minutes at 1,380 x g. Cell pellets were re-suspended in 6 ml of sterile seawater (12, 20, or 35 ppt), and centrifuged again under the same conditions. This procedure was repeated to obtain double-washed cells. Three 2-ml aliquots from double-washed cell suspensions were transferred to three 18-ml quantities of corresponding sterile seawater (12, 20, or 35 ppt) to achieve a 1:10 dilution. These samples were held at 25°C for 2 hours and then incubated at 4, 10, and 25°C for 14 days.

## Enumeration of starved *V. vulnificus* in artificial seawater

*V. vulnificus* enumeration was performed on days 0, 7, and 14 by spread-plating on duplicate TSA-2 plates (48 hours, 25°C) after appropriate dilutions in PBS. All samples were hand shaken within a 25-cm arc for 15 seconds before aliquot withdrawal for enumeration and then immediately returned back to the appropriate incubator. Turbidity and fluorescence measurements (Spectramax 250) of each *V. vulnificus* ATCC 27562 sample were performed on day 0, 7, and 14. A 1.5-ml aliquot from samples stored at 4 and 10°C was equilibrated first to 25°C for 30 minutes before turbidity and fluorescence measurements. Based on average values of three measurements of $OD_{600}$ and FVI, bacterial counts were calculated using regression equations from Chapter IV.

## Statistical analysis

A two-factorial experimental design was used with salinity (12, 20, and 35 ppt) and storage day (day 0, 7, and 14) being factors influencing bacterial counts in 4, 10, and 25$^{o}$C artificial seawater. The experimental setup included three replications for *V. vulnificus* ENV-1 and five replications for *V. vulnificus* ATCC 27562, which were carried out on three and five different days, respectively. Fisher's Least Square Difference (LSD) test was utilized to separate means if differences ($p<0.05$) occurred among treatments. Statistical analysis was performed using SAS ver. 9.2 statistical software.

## Results and discussion

Results revealed that storage day, but not seawater salinity, had a significant influence ($p<0.05$) on counts of *V. vulnificus* ENV-1 (Figure 12). Chill storage temperatures (4 and 10$^{o}$C) did not cause a significant decrease ($p>0.05$) in counts for this environmental strain on day 7. All three temperature treatments caused a significant decrease ($p<0.05$) in counts on day 14, with 0.6, 0.3, and 1.1 $\log_{10}$ cfu/ml decreases for 4, 10, and 25$^{o}$C, respectively (Figure 12).

For *V. vulnificus* ATCC 27562, both storage day and seawater salinity significantly influenced ($p<0.05$) bacterial counts (Figure 13). At both 10 and 25$^{o}$C counts decreased during the 14-day storage period, with greatest decrease seen in 35 ppt seawater. At 4$^{o}$C high salinity level (35 ppt) caused ($p<0.05$) a large 4 $\log_{10}$ cfu/ml count decrease after 14 days, which might be caused at least partially due to the switch of the cell population to VBNC based on APC, turbidity, and fluorescence staining data (Figure 13). This count reduction was greater than 2 logs more than what was seen at 10 and 25 $^{o}$C after 14 days. It is generally believed that *V. vulnificus* will not enter into VBNC state at temperatures at or exceeding 10$^{o}$C (Oliver et al., 1991; Whitesides and Oliver, 1997). Oliver et al. (1991) showed a 5 $\log_{10}$ reduction from 6 to 1 $\log_{10}$ cfu/ml within fourteen days at 5$^{o}$C in artificial seawater, while acridine orange direct count and viability (respiration activity) direct counts showed less than a 1 log reduction.

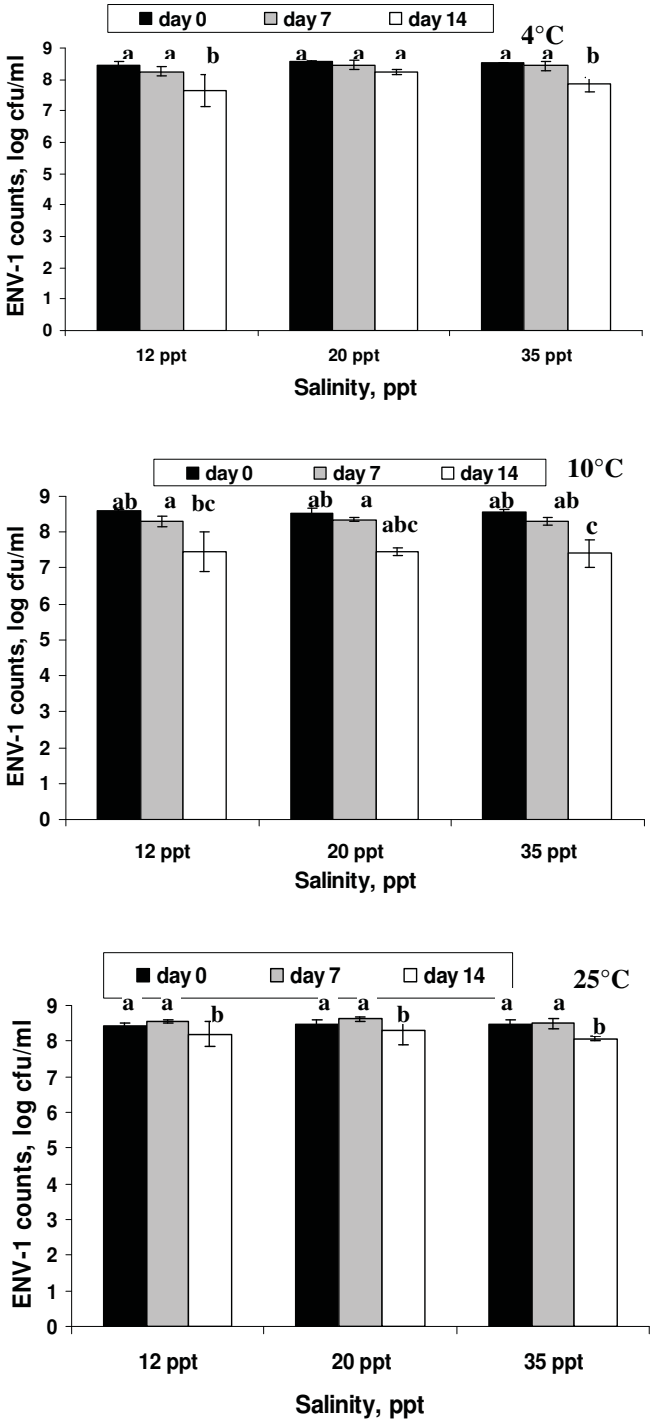


Figure 12. Changes in *V. vulnificus* ENV-1 numbers after starvation at 4 (top), 10 (middle), and 25°C (bottom) seawater for 14 days

NOTE: Means with the same letter are not significantly different ($p>0.05$)

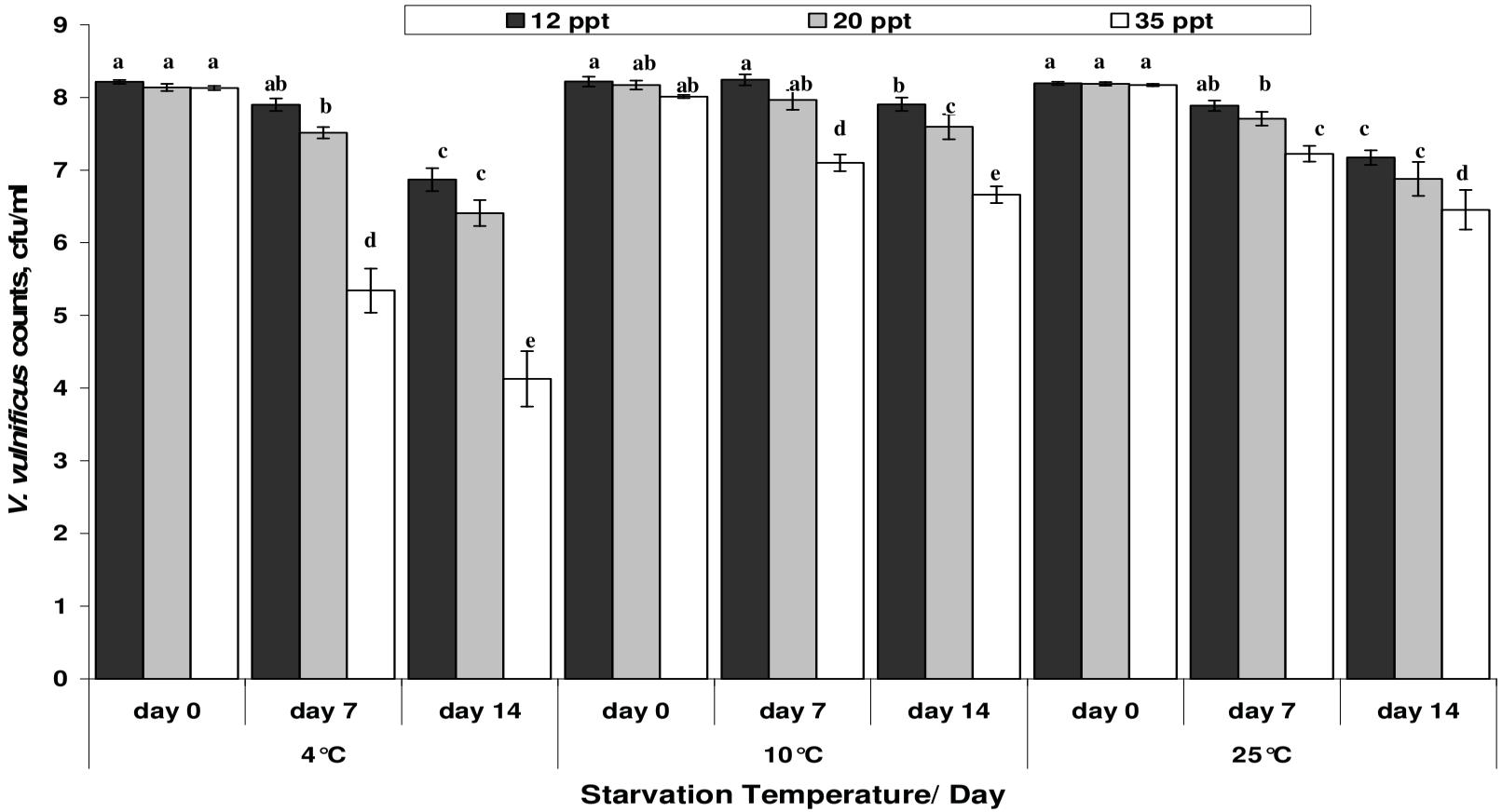


Figure 13. Changes in *V. vulnificus* ATCC 27562 numbers after starvation in 12, 20, and 35 ppt seawater at 4, 10, and 25°C for 14 days

NOTE: Means with the same letter within the same starvation temperature are not significantly different ($p>0.05$)

Studies by Bryan et al. (1999) noted that immediate shifting of *V. vulnificus* culture from 35 to 6$^{o}$C caused transition to non-culturable state, while cells adapted to 15$^{o}$C before 6$^{o}$C storage remained viable and culturable. Similar studies were performed by Bang and Drake (2002), who noted that cold adaptation of *V. vulnificus* ATCC 27652 made the culture more resistant to VBNC entry at 5$^{o}$C.

Oliver et al. (1991) showed that stationary-phase *V. vulnificus* cells were significantly more resistant to VBNC entry (2.5 $\log_{10}$ cfu/ml reduction within 14 days) compared to logarithmic cells (6 $\log_{10}$ cfu/ml reduction within 14 days). In the same study, Oliver at al. (1991) wrote that pre-starvation of *V. vulnificus* for as little as 4 hours before temperature downshift to 5$^{o}$C enabled the pathogen to maintain culturable cell density of at least 5 $\log_{10}$ cfu/ml without entering VBNC state within 13 days. Several groups (Morton and Oliver, 1994; Paludan-Müller et al., 1996; Jones et al., 2008) showed that *V. vulnificus* produced starvation-induced proteins to cope with the lack of carbon and nitrogen nutrients. These proteins are believed to help in long-term survival of *V. vulnificus* at low temperature. Similarly, Biosca et al. (1996) found that logarithmic-phase cells lost their culturability faster (>5 $\log_{10}$ reduction after 10 days) than stationary phase cells (1 $\log_{10}$ reduction after 10 days) at 5$^{o}$C in artificial seawater. However, pre-starvation of logarithmic-phase cells for 48 hours in artificial seawater before shifting to 5$^{o}$C improved survival to the level of stationary phase cells.

In the present study *V. vulnificus* was grown under conditions similar to natural conditions found in Gulf Coast seawater (25$^{o}$C with shaking). Cells used here were double-washed in artificial seawater to avoid growth medium nutrient transfer. Cells were pre-starved in seawater for two hours before transfer to incubators. The present study also used a proprietary seasalt blend (CrystalSea MarineMix), which contained numerous macro- and microelements, such as magnesium, sulfur, calcium, bromide, strontium, manganese, iodide, molybdate, vanadium, selenium, and boron. Other researchers used chemically-defined simplified seasalt prepared from scratch, which might have lacked those macro- and microelements. Bryan et al. (1999) showed that macroelements, such as iron, might have an important role for improving cold temperature adaptation of *V. vulnificus*.

Similar to present results, Oliver et al. (1991) showed a 2.5-log decrease in stationary-grown *V. vulnificus* cells counts within 14 days at 5$^{o}$C. Cook and Ruple (1992) investigated survival of five *V. vulnificus* strains in different media at 4$^{o}$C. They found that growth in rich medium (TSB) had the greatest inactivation rate, ranging from 0.29 to 0.47 $\log_{10}$ cfu/ml per day. Nutrient-free medium (PBS and PBS plus 2%NaCl) improved survival rates, with two strains having survival rate declines of 0.13 $\log_{10}$/day and one strain having 0.16 $\log_{10}$/day survival. On the other hand, Bang et al. (2007) showed similar inactivation D-values for *V. vulnificus* at 5$^{o}$C storage temperature, ranging from 1.61 to 4.36 days/log. Such rapid culture inactivation might be attributed to the toxic effect of residual

nutrients transferred from the culture broth to artificial seawater or to the fresh broth used as a refrigerated substrate and for inoculation of the pre-chilled media.

Few studies are available on the influence of temperature and salinity on survival of *V. vulnificus* in artificial seawater. For example, Marco-Noales et al. (1999) found that 25°C was optimal for *V. vulnificus* biotype 2 survival in 3 to 5 ppt artificial seawater, while 12°C was optimal for survival in 15 to 38 ppt seawater. Similarly, the present investigation found that 10°C was better than 4 or 25 °C for the survival of starved *V. vulnificus* strains ENV-1 and ATCC at 12 and 20 ppt salinity (Figures 12 and 13). Marco-Noales et al. (1999) found less than a 1 $\log_{10}$ reduction in three strains of *V. vulnificus* stored for 20 days in 15 ppt artificial seawater at 25°C, while direct viable count method (Kogure et al., 1979) and acridine orange direct count showed no count reduction. Present results were similar with the exception of 35 ppt salinity seawater, where the decrease was more dramatic.

Kaspar and Tamplin (1993) investigated survival of triple-washed *V. vulnificus* in artificial seawater at different salinities and temperatures. They found that population numbers decreased by 93% and 71% (around 1 $\log_{10}$) after 7 days and by 100% and 92% after 14 days when incubated between 0.5 and 6.0°C in 10 ppt seawater.

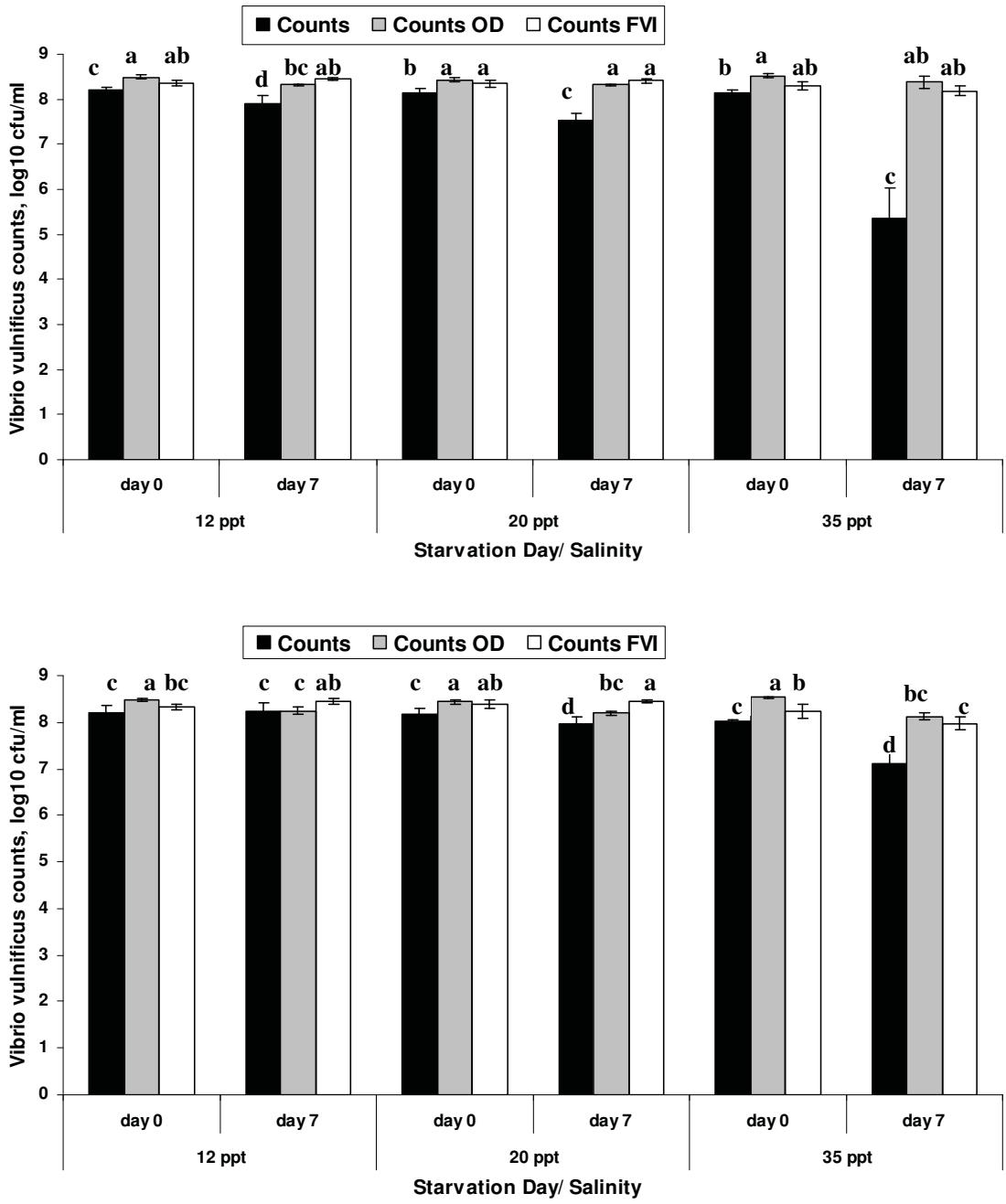


Figure 14. *Vibrio vulnificus* counts estimated by Aerobic Plate Count (Counts), Optical Density at 600 nm (Counts OD), and Fluorescence Viability Index (Counts FVI) after starvation in artificial seawater at 4°C (top) and 10°C (bottom)

NOTE: Means with the same letter within the same salinity are not significantly different ($p>0.05$)

More interestingly, they found that *V. vulnificus* numbers slightly increased in 5 to 25 ppt seawater at 14°C within 6 days, while 30, 35, and 35 ppt seawater caused 58 to 83% decrease within the same time frame. These results are similar to present findings, where 35 ppt salinity was more detrimental to culturability of *V. vulnificus* than 12 and 20 ppt.

Figure 14 shows *V. vulnificus* ATCC 27562 counts at 4 and 10°C estimated by Aerobic Plate Count, Optical Density at 600nm, and Fluorescence Viability Index methods. APC counts significantly decreased at 20 and 35 ppt on day 7, while counts estimated by optical density and fluorescence measurements did not change compared to day 0 counts. Optical density measures total cell concentration, while fluorescence viability index also depends on the ratio of viable/dead cells. If OD remains the same, total *V. vulnificus* cell count did not change on day 7 compared to day 0. As shown in Chapter IV, a 1 log reduction in viable cell count (10% viable/90% dead) caused a theoretical decrease of FVI corresponding to only a 0.5 $\log_{10}$ cell count reduction (based on FVI standard curve). Strain ATCC 27562 APC significantly decreased from 8.1 to 5.3 $\log_{10}$ in 35 ppt seawater after 7 days at 4°C even though FVI and $OD_{600}$ estimated counts remained unchanged (8.3 $\log_{10}$ vs. 8.2 $\log_{10}$) and (8.5 $\log_{10}$ vs. 8.4 $\log_{10}$) (Figure 13 and 14). This observation suggests that higher salinity (35 ppt), along with refrigeration facilitates entry of *V. vulnificus* into a non-culturable state followed by cell death.

The results from this chapter indicate that *V. vulnificus* might retain culturability in 12 to 20 ppt salinity seawater under refrigeration. Greater salinity levels (>35 ppt) appear to be needed to reduce pathogen numbers. Count reductions seen in depuration tanks over prolonged storage might be attributed to the effect of dilution and UV light bactericidal effect.

# CHAPTER VI

# FATTY ACID PROFILE ANALYSIS OF STARVED *VIBRIO VULNIFICUS* IN ARTIFICIAL SEAWATER

## Introduction

A microbial identification system that utilizes the knowledge that bacterial species grown on a specified medium under specified conditions produce a unique set (both qualitatively and quantitatively) of fatty acids in their cell membrane phospholipids is the subject of this chapter. In this method, pure culture cells are harvested after appropriate growth conditions to collect a biomass of at least 40 mg, followed by saponification to release triglycerides from the cell envelop and to hydrolyze them into sodium salts of fatty acids. Sodium salts of fatty acids are then methylated to obtain fatty acid methyl esters, which are volatile compounds that can be analyzed by gas chromatography. Fatty acid methyl esters are then extracted with organic solvents (mixture of hexanes and methyl-tert butyl ether), followed by extract wash with sodium hydroxide solution to remove contaminants. A sample is injected into a column with hydrogen as a carrier gas, where fatty acid methyl esters are separated based on their structure and molecular weight. They elute from the column at different retention times under conditions of increasing temperature. When fatty acid methyl esters reach

the end of the column, a signal is detected using a flame ionization detector and recorded as an area beneath each peak to quantify individual fatty acids in a sample. Peaks are identified using proprietary software as specific fatty acids based on a previous run of fatty acid standard mix, which is supplied by the company. Fatty acids extracted from unknown cultures are automatically identified, quantified, and fatty acid profile is compared to those profiles present in the software library. Unknowns can be identified down to the genus/species/subspecies level based on similarity indexes. The only commercially available gas chromatography system for bacterial identification is a bundle that includes a gas chromatograph and analysis software marketed by Microbial ID, Inc., Newark, DE.

In the FDA Bacteriological Manual, DePaola and Kaysner (2004) suggested that fatty acid profile analysis was an alternative method to confirm *V. vulnificus* cultures based on a published collaborative AOAC study (Landry, 1994). This study resulted in first action adoption by AOAC International. Thirteen collaborators found that among 126 *V. vulnificus* isolates, 118 (93.7%) were correctly identified. Sixty-five negative controls yielded one non-viable sample, one was misidentified as *V. vulnificus*, and two were misidentified as *V. parahaemolyticus*. Moreover, 95.3% of negative controls were correctly identified. Overall, statistical analysis showed a sensitivity rate of 0.872, specificity rate of 0.982, false positive rate of 0.010, and false negative rate of 0.206.

Cellular fatty acid profile of bacterial species fluctuates under different physiological conditions (Yuk and Marshall, 2005). For example, the ability of bacteria to multiply under suboptimal conditions (low pH, in the presence of toxic chemicals, refrigeration or elevated temperatures) requires membrane fluidity modification, a phenomenon known as homeoviscous adaptation. It has been shown that membrane fluidity can be altered through membrane fatty acid profile modification, with shorter chain or unsaturated fatty acids making the cell membrane more fluid (Tokarskyy and Marshall, 2008). Studies by Linder and Oliver (1989) examined changes in membrane fatty acids in viable but nonculturable state of *V. vulnificus*. According to them, on day zero of pathogen incubation in artificial seawater at 5$^{o}$C, C16:0 and C16:1 fatty acids predominated. No significant changes in fatty acid profile were observed on day 13, although the profile showed significant change at the point of cell non-culturability (day 24). Notably, both C16:0 and C16:1 percentages decreased, the percentage of fatty acids with chain lengths less than 16 increased, while long-chain fatty acids (C19:0, C20:0, and C22:1) appeared.

The objective of this study was to investigate *V. vulnificus* fatty acid profile changes to generate data that might be used to explain results observed in oyster depuration tanks and *in vitro* systems. The hypothesis was that a decrease in seawater temperature will cause an increase in short-chain and unsaturated fatty acids present in the cell envelop of *V. vulnificus*. In addition, the

influence of high salinity (35%) on cell envelop fatty acid composition was of interest to investigate.

## Materials and methods

Working broths of two *V. vulnificus* strains, ATCC 27562 and ENV-1, were prepared as described previously (Chapter IV). Seven 6-ml aliquots of the working broth culture were transferred to 15-ml tubes and centrifuged for seven minutes at 1,380 x g (Centrific model 228). Cell pellets were re-suspended in 6 ml of sterile seawater (12, 20, or 35 ppt) and centrifuged again under the same conditions. Double-washed cells were finally re-suspended in 6 ml of sterile seawater (~$10^9$ cfu/ml). One sample was analyzed for fatty acid profile after a 2-hour room temperature cell adaptation period (day 0), while three 2-sample sets were immediately transferred to 4, 10, and 25°C incubators. One sample from each incubator was withdrawn on day 7 and day 14, and analyzed for change in fatty acid profile. In order to assess bacterial fatty acid profile, each analyzed sample was centrifuged for 7 minutes at 2,700 x g to obtain a cell pellet. Procedures for cell lysis and fatty acid saponification, methylation, extraction, and alkali wash were performed as described previously (Yuk and Marshall, 2005).

## Statistical analysis

The experiment was repeated three times for each of the two strains for each salinity and temperature on three different days. Statistical analysis was done using SAS ver. 9.2 (SAS Institute Inc., Cary, NC).

A two-factorial design with three replications was utilized to determine the influence of seawater salinity (12, 20, and 35 ppt) and storage day (day 0, day 7, and day 14) on percentage of each fatty acid found at three different storage temperatures (4, 10, and 25$^{o}$C). Fisher's Significant Difference (LSD) Test was utilized to separate treatment means when differences ($p<0.05$) occurred among treatments.

A multifactorial design was used to test the effects of treatment temperature (fresh, 4, 10, and 25$^{o}$C), seawater salinity (12, 20, and 35 ppt), and starvation day (0, 7, and 14), as well as their interaction, on cis-vaccenic acid (C18:1ω7C) fatty acid percentage for *V. vulnificus* isolates ENV-1 and ATCC 27562. Fisher's Least Significant Difference (LSD) Test was utilized to separate treatment means when differences ($p<0.05$) occurred among treatments.

## Results and discussion

Nine major fatty acids were recovered from cellular membranes of *V. vulnificus*; notably, 12:0 3OH, 14:0, 16:1ω9c, 16:0, 18:1ω7c, 18:1ω6c, 18:0, summed feature 2 (16:1 ISO I & 14:0 3OH), and summed feature 3 (15:0 ISO 2OH & 16:1ω7c). Minor fatty acids with abundance percentage of 0 to 0.7 were

not reported since they were not recovered consistently due to the low amounts present.

Significance of influence of starvation day and salinity on individual fatty acid percentage is shown in Table 2. Starvation day, but not salinity, significantly influenced ($p<0.05$) individual fatty acid percentages for most treatments. The notable exceptions were 18:1ω7c (cis-vaccenic acid) and 18:1ω6c fatty acids, where both starvation day and salinity influenced their percentage. Though not analyzed, it appeared that lower temperatures together with starvation prevented changes in fatty acid profile of *V. vulnificus*.

Table 2. Statistical influence of starvation day (0, 7, and 14) and salinity (12, 20, and 35 ppt) on percentage of individual fatty acids for different treatments (bacterial strain and starvation temperature).

| Treatment | | Significance of starvation day/salinity influence on *V. vulnificus* fatty acid abundance. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial culture | Storage temp | 12:0 3OH | 14:0 | 16:1 ω9c | 16:0 | 18:1 ω7c | 18:1 ω6c | 18:0 | sum feature 2 | sum feature 3 |
| ATCC 27562 | 4°C | +/-* | +/- | -/- | -/- | +/- | +/+ | +/- | +/- | +/- |
| ATCC 27562 | 10°C | +/- | +/- | -/- | -/- | +/+ | +/- | +/- | +/- | +/- |
| ATCC 27562 | 25°C | +/+ | +/- | +/- | +/- | +/- | +/- | +/- | -/- | +/- |
| ENV-1 | 4°C | -/- | -/- | -/- | -/- | +/+ | +/+ | +/- | -/- | +/- |
| ENV-1 | 10°C | -/- | -/- | -/- | -/- | +/+ | +/+ | -/- | -/- | +/- |
| ENV-1 | 25°C | +/- | -/- | +/- | -/- | +/- | +/- | +/- | -/- | +/- |

NOTE: Plus (+) denotes significant influence ($p<0.05$), while minus (-) denotes non-significant influence ($p>0.05$). Slash character separates the influence of starvation day (left) from salinity (right).

Results for fatty acid profile analysis for *V. vulnificus* ATCC 27562 and ENV-1 are shown in Figures 15 and 16. All salinities were combined for a specific starvation day because the influence of salinity was not significant ($p>0.05$) in most cases. In the case of starved *V. vulnificus* ATCC 27562, increased storage time at any temperature caused a decrease ($p<0.05$) in the amount of C14:0 and summed feature 3 and an increase ($p<0.05$) in C18:0 (Figure 15). Storage at 25$^{o}$C also caused a significant increase ($p<0.05$) in C16:0. Similar trends were observed with *V. vulnificus* isolate ENV-1 (Figure 16).

As noted by Linder and Oliver (1989), a decrease in incubation temperature should cause bacterial cells to increase the percentage of unsaturated fatty acids and short-chain fatty acids in their cell envelops. At the same time, the percentage of saturated and long-chain fatty acids should decrease in membrane phospholipids in order to maintain sufficient membrane fluidity. Apparently, under starvation with no nutrients available, cells of *V. vulnificus* did not adjust their membrane fluidity as a response to storage temperature during the time frame studied in the present experiment, with a notable exception of a shift from 18:1ω7c to 18:1ω6c fatty acid. The shift involved relocation of a single double bond in 18:1 unsaturated fatty acid from ω7 carbon to ω6 carbon position. This trend was noted for both strains grown at 25$^{o}$C where significant amounts of C18:1ω7c fatty acid were found initially (18 to 19%) followed by transformation into C18:1ω6c during room temperature starvation.

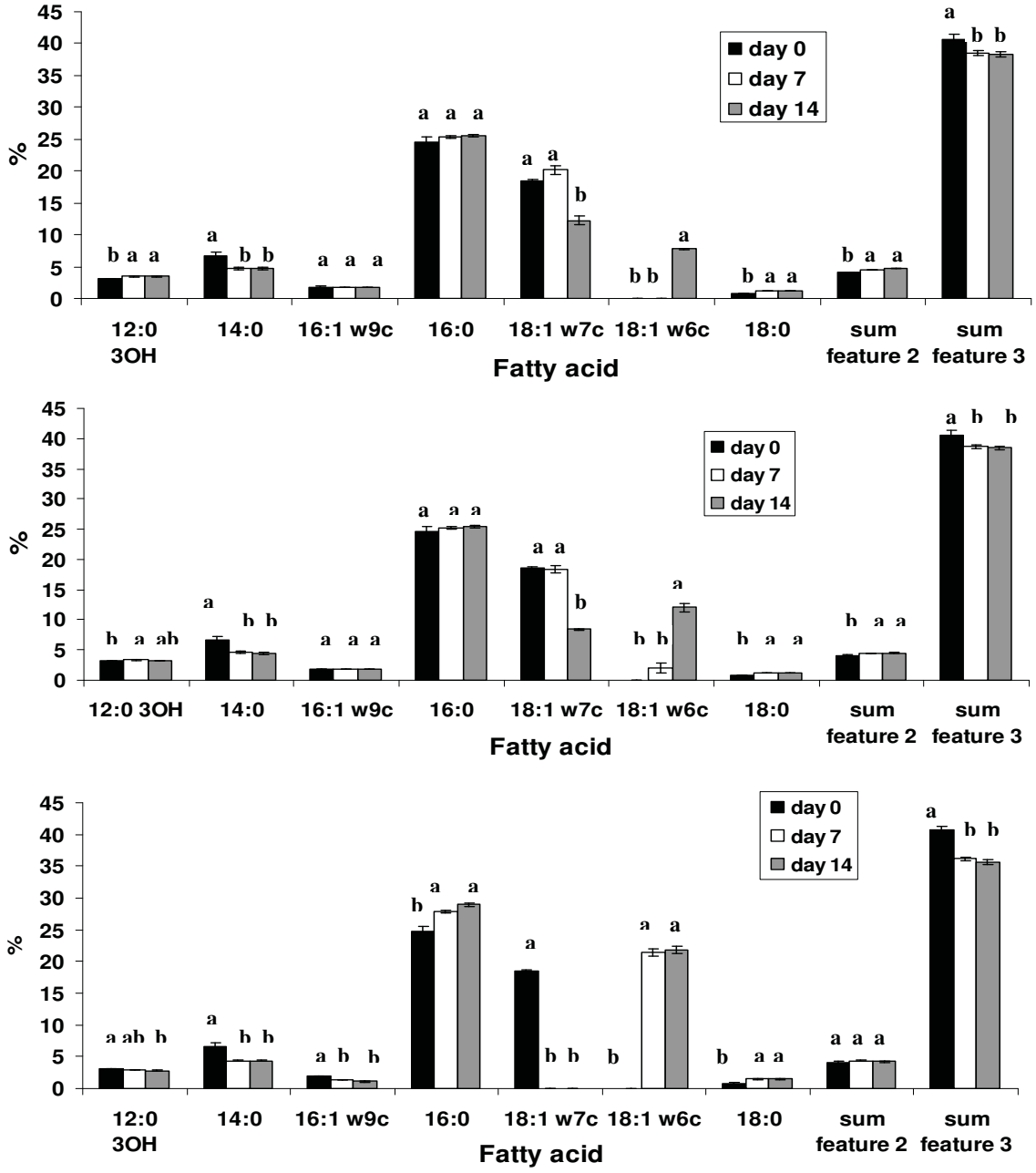


Figure 15. Fatty acid profile analysis of starved *V. vulnificus* ATCC 27562 in artificial seawater with all salinities combined at 4°C (top), 10°C (middle), and 25°C (bottom)

NOTE: Means with the same letters within the same fatty acid are not significantly different ($p>0.05$)

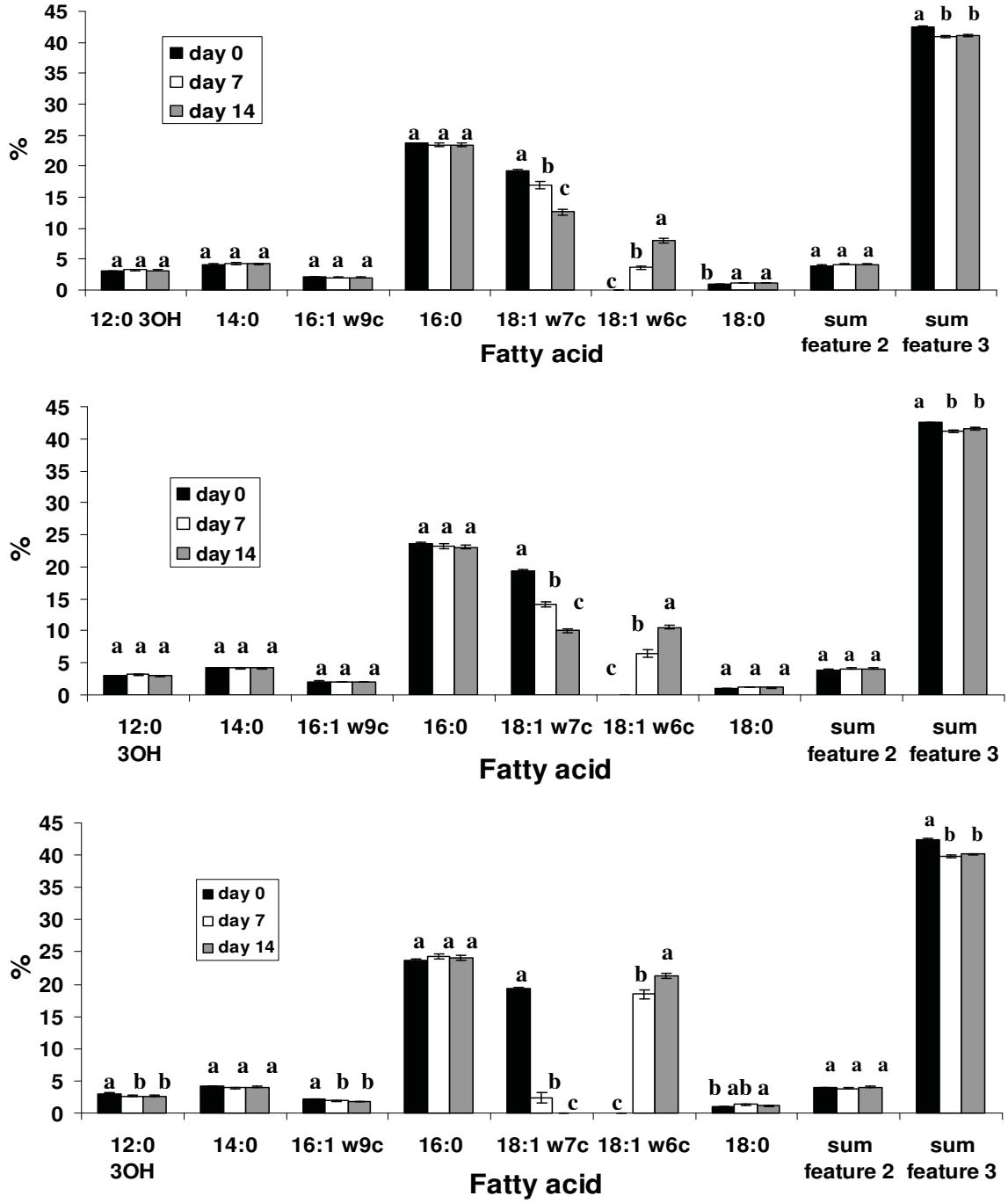


Figure 16. Fatty acid profile analysis of starved *V. vulnificus* ENV-1 in artificial seawater with all salinities combined at 4°C (top), 10°C (middle), and 25°C (bottom)

NOTE: Means with the same letters within the same fatty acid are not significantly different ($p>0.05$)

At 4 and 10°C, transformation of C18:1ω7c to 18:1ω6c was slower, with larger amounts of C18:1w7c retained on day 7. It appears that *V. vulnificus* cells, while starved and not growing, are trying to "preserve" their original 25°C-grown state while refrigerated with a notable exception of C18:1ω7c to 18:1ω6c shift (Figures 15 and 16).

A multifactorial design was used to test the effects of treatment temperature, seawater salinity, starvation day, as well as their interactions on cis-vaccenic (18:1ω7C) fatty acid percentage. There was a significant influence ($p<0.05$) due to starvation day, treatment temperature, and their interaction on *V. vulnificus* ATCC 27562. All three factors (starvation day, treatment temperature, and salinity) and treatment-salinity interaction had significant effects ($p<0.05$) on cis-vaccenic fatty acid percentage for ENV-1.

Figure 17 shows the amounts of cis-vaccenic fatty acid in strains ATCC 27562 and ENV-1 under the different treatments. It appeared that ATCC 27562 strain preserved cis-vaccenic fatty acid better at lower temperature (4 and 10°C), while transition to C18:1ω6c was already complete at 25°C after day 7. On the other hand, ENV-1 strain had faster transition at lower temperature (4 and 10°C), but slower transition at 25°C. Interestingly, higher salinity (35 ppt) significantly slowed the transition of C18:1ω7c into C18:1ω6c for ENV-1 at 4 and 10°C (Figure 17).

Studies by Linder and Oliver (1989) examined changes in membrane fatty acids in viable but nonculturable state of *V. vulnificus*. According to them, C16:1,

C16:0, and C18:1 fatty acids predominated on day zero in artificial seawater at 5°C, comprising 46, 32, and 12% of total extracted fatty acids, respectively. Only slight changes (<2%) were observed in these fatty acids on day 13, while *V. vulnificus* cells lost their culturability from 7 $\log_{10}$ cfu/ml to 3.5 $\log_{10}$ cfu/ml. The present study obtained similar percentages for C16:1, C16:0, and C18:1 fatty acids, which were 47, 24, and 19%, respectively. Fatty acid C16:1 percentage was estimated as a sum of C16:1ω9c, summed feature 2, and summed feature 3. According to Linder and Oliver (1989), *V. vulnificus* fatty acid profile showed a drastic change only at the point of complete non-culturability (5°C, day 24, 0 $\log_{10}$ cfu/ml). Notably, both C16:0 and C16:1 percentage decreased from 32 and 46% to 11 and 8%, respectively. At the same time, the percentage of fatty acids with chain length of less than 16 carbons increased from 5.4 to 28.9% with the concomitant appearance of long-chain fatty acids (C19:0, C20:0, and C22:1).

Results from the present study are similar to those seen by Linder and Oliver (1989). Fatty acid profiles were similar on day 14 (present study) versus day 13 (Linder and Oliver study). On the other hand, present results did not reveal reductions in C16:0 and C16:1 or appearance of C19:0, C20:0, and C22:1 fatty acids during storage. This is possibly explained by the shorter incubation period used (14 days vs. 24 days) and the lack of nutrients.

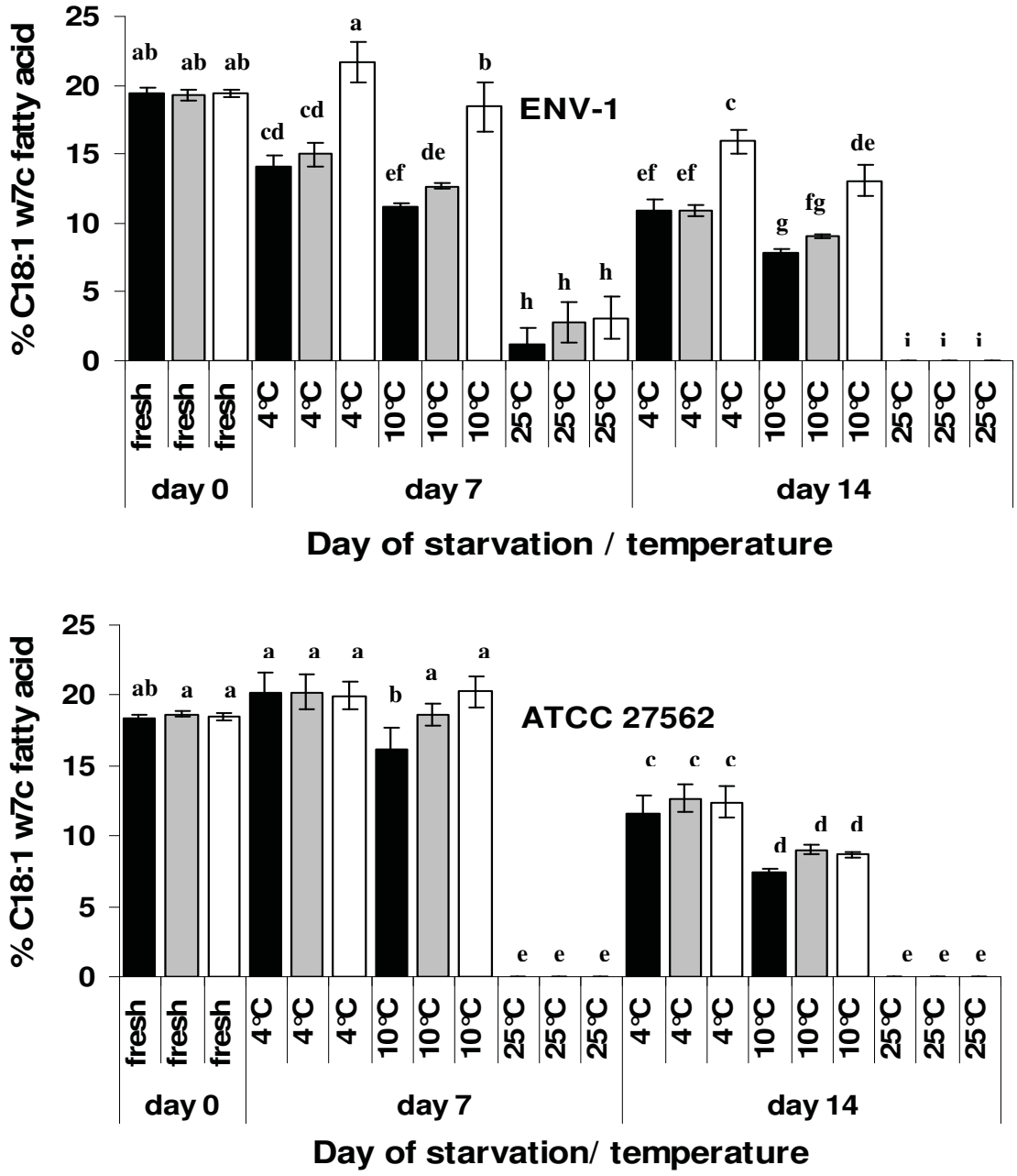


Figure 17. Changes in C18:1ω7c fatty acid percentage of starved *V. vulnificus* ENV-1 (top) and ATCC 27562 (bottom) at different temperatures in artificial seawater

NOTE: 12 ppt – black bar, 16 ppt – grey bar, and 35 ppt – white bar. Means with the same letters are not significantly different ($p>0.05$)

The present GC-FAME microbial identification system is more sensitive for identification of not only specific fatty acids with defined chain length and number of double bonds, but also for differentiation of the same fatty acid with different double bond location. For example, Linder and Oliver (1989) did not observe any significant changes in C18:1 fatty acid during 13 days storage at 5$^{o}$C, while the present effort identified a significant relocation of the double bond from ω6c to ω7c position as discussed earlier.

The significance of double bond relocation is difficult to explain. The length of the carbon backbone and number of unsaturated double bonds did not change in octadecenoic acid (C18:1). These two factors have been shown to be important in influencing membrane fluidity of *Escherichia coli* O157:H7 (Yuk and Marshall, 2005). Similar shift of C18:1ω7c into C18:1ω6c was shown by Kahng (2002) for *Pseudomonas* sp. strain KK1 capable of growing on naphthalene source. *Pseudomonas* KK1 while growing in the presence of naphthalene had greater C18:1ω6c contents at the expense of C18:1ω7c. The author concluded that such switch might be a physiological adaptation of *Pseudomonas* sp. in response to toxic chemicals and to the utilizable substrate, naphthalene. The same switch was observed in the current study, and its significance might be demonstrated in the case of ENV-1 grown in 35 ppt seawater at 4 and 10$^{o}$C. The ENV-1 population slowed the transition of C18:1ω7c into C18:1ω6c and also remained culturable compared with ATCC 27562 under the same conditions (Chapter V).

# CHAPTER VII

# SURVIVAL OF *VIBRIO VULNIFICUS* IN SIMULATED GASTRIC FLUID (SGF) AFTER PROLONGED STORAGE IN 20 PPT SEAWATER

## Introduction

There are several reports that *V. vulnificus* adaptation to certain environmental conditions can induce cross-adaptation to other stressful environmental conditions (Rosche et al., 2005b; Bang and Drake, 2002). For example, Bang and Drake (2002) investigated resistance of cold- and starvation-stressed *V. vulnificus* to freeze-thaw exposure and cold temperature tolerance. Cold stress had no effect on freeze-thaw resistance, but starvation enhanced freeze-thaw resistance for one strain. Cold adaptation (holding for 4 hours at 15$^{o}$C) enhanced cold temperature tolerance (5$^{o}$C) of *V. vulnificus*.
Bang and Drake (2005) found that *V. vulnificus* acid adaptation in Trypticase Soy Broth supplemented with 2% NaCl and acidified with either hydrochloric acid (pH 5.0), citric acid (pH 5.5), or acetic acid (pH 5.5) improved survival in the same medium acidified to pH 3.5 with hydrochloric acid.

The ability of starved and cold-stored *V. vulnificus* to cause infectious disease has been investigated as well, mostly focusing on VBNC state of the bacterium. For example, Kaysner et al. (1989) found that lethality of *V. vulnificus*

(iron-treated adult mouse injection) was not adversely affected by cold storage within oyster tissues for five days at 0.5$^{o}$C. Similarly, Marco-Noales et al. (1999) found that *V. vulnificus* cells from saline microcosms were able to infect eels and mice by intraperitoneal injection even after starvation storage for two years. Oliver and Bockian (1995) showed that *V. vulnificus* VBNC cells retained their virulence against mice by confirming their death after injection of inoculated refrigerated (5$^{o}$C, 7 days storage) artificial seawater containing <0.04 CFU of culturable cells but >$10^5$ cells in VBNC state. Moreover, they recovered culturable *V. vulnificus* cells from blood and peritoneal cavities of dead mice.

Despite this evidence, questions remain about the ability of cold-starved *V. vulnificus* to cause foodborne infections. Interestingly, Shapiro et al. (1998) found that among seventy cases of *V. vulnificus* infection from 1988 to 1996 caused by raw oyster consumption, 89% were tracked back to when seawater harvesting temperatures was above 22$^{o}$C. The few cases caused during cold months leads to the question of whether cold-starved *V. vulnificus* can cause foodborne infection. To investigate this question, a study was designed to determine the ability of simulated gastric fluid (pH 4.1) to inactivate *V. vulnificus*. The high acidity of gastric fluid is the main barrier infectious bacteria must overcome in order to establish infection in the human gastrointestinal tract (Koo et al., 2000a, 2000b). A report showing that low pH was bactericidal to *V. vulnificus* at 21$^{o}$C when hydrochloric acid was used as acidulant, with D-values of

30 to 50 minutes at pH 4.0, 1.5 to 4.5 minutes at pH 3.5, and 0.4 to 0.5 minutes at pH 2.0 (Anonymous, 2001).

## Materials and methods

Simulated gastric fluid was freshly prepared for each replication by dissolving 20 mg pepsin (from porcine stomach, 424 units/mg solid, Sigma), 0.7 g mucin (type II: crude, from porcine gastric mucosa, Sigma), and 1.7 g NaCl in 200 ml sterile distilled water. Sterile 1.0 N hydrochloric acid was used to adjust pH to 4.1.

*Vibrio vulnificus* (ATCC 27562) working culture was prepared as described earlier (Chapter IV). Six milliliters of the working culture was centrifuged at 1,380 x g for 7 min to a obtain cell pellet, which was re-suspended in sterile 20 ppt seawater, and the procedure was repeated to obtain double-washed cells. Two ml of double-washed cells were further diluted in triplicate tubes containing 18 ml of 20 ppt seawater to obtain inoculated seawater. All three tubes were incubated for two hours at 25°C. Inoculation population level was confirmed by serial dilutions in PBS and plating on TSA-2 (48 hours, 25°C). Following the 2-hour seawater adaptation, tubes were incubated at 4, 10, or 25°C for seven days.

*Vibrio vulnificus* survival in simulated gastric fluid after exposure to seawater under starvation was assessed on day 0 (after 2-hours adaptation in 20 ppt seawater at 25°C), and on day 7 for samples stored at 4, 10, or 25°C. Refrigerated samples were immediately returned to cold storage after aliquot

transfer. *Vibrio vulnificus* counts in stored samples were determined before exposure to simulated gastric fluid to estimate cell population in simulated gastric fluid at time point "0 min". To assess survival of the bacterium in simulated gastric fluid, 1 ml aliquots of inoculated stored seawater samples were transferred to 9 ml simulated gastric fluid equilibrated to 37°C in a water bath. These were vortexed for 10 seconds and immediately returned to the 37°C water bath. One ml aliquots from each treatment were transferred after 3, 6, 9, 12, and 15 minutes to 9 ml PBS blanks, vortexed, and further serially diluted as needed in 9 ml PBS. One tenth milliliter of each dilution was then plated in duplicate on TSA-2 (48 hours, 37°C) to evaluate vibrio survival at each time point. Counts were expressed as $\log_{10}$ cfu/ml of simulated gastric fluid. Addition of 1 ml of 20 ppt seawater to 9 ml simulated gastric fluid did not result in simulated gastric fluid pH change. Addition of 1 ml of simulated gastric fluid (pH 4.1) to 9 ml of phosphate-buffered saline (PBS, pH 7.4) did not result in PBS pH change.

**Statistical analysis**

The experiment was repeated five times on five different days. An experimental design with two factors was used to compare the effects of *V. vulnificus* storage conditions, exposure time to simulated gastric fluid, and their combined effect on *V. vulnificus* counts. The storage conditions were untreated day 0, 4°C day 7, 10°C day 7, and 25°C day 7. Exposure time in simulated gastric fluid was 0, 6, 9, 12, and 15 minutes. D-values in simulated gastric fluid

were expressed in minutes and reflected the time needed to reduce *V. vulnificus* population by 1 $\log_{10}$ cfu/ml. D-values were calculated for each replication and each storage condition based on 0 to 12 minutes time frame. Mean D-values were separated using Fisher's Least Significant Difference test when influence of storage condition was significantly different ($p<0.05$).

## Results and discussion

Figure 18 shows inactivation curves of *V. vulnificus,* either untreated or stored at different temperatures, during exposure to simulated gastric fluid.

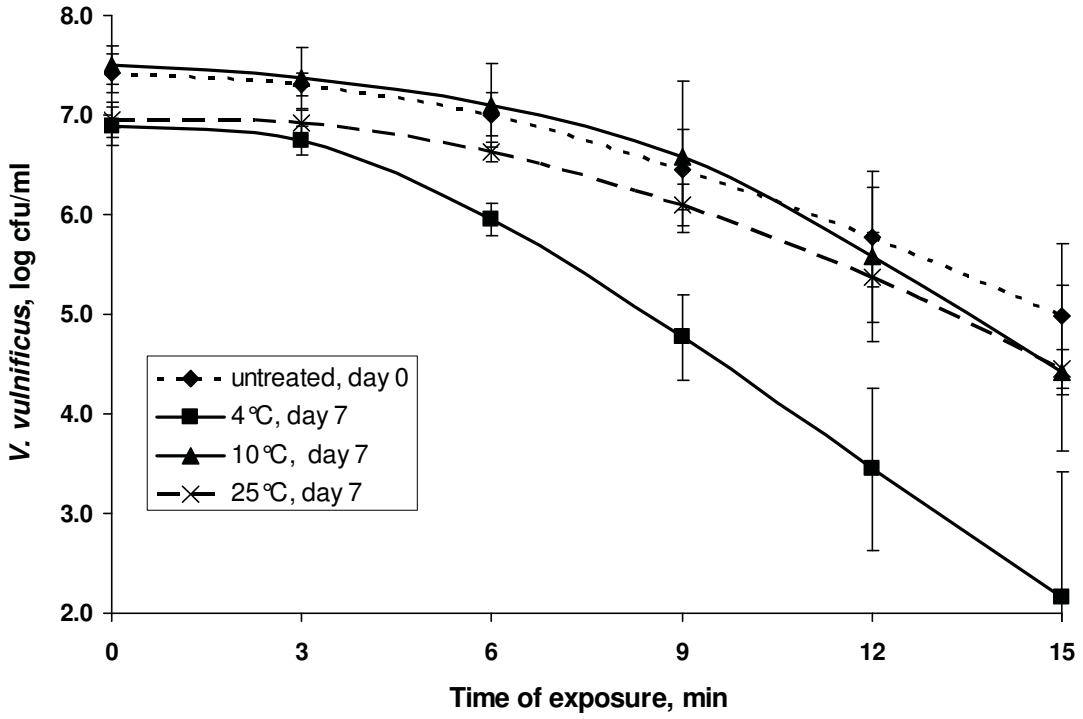


Figure 18. Survival of *V. vulnificus* ATCC 27562 in Simulated Gastric Fluid (SGF) after storage in sterile 20 ppt seawater for 7 days at 4, 10, and 25℃

There was a significant influence ($p<0.05$) of *V. vulnificus* storage condition and exposure time to simulated gastric fluid, as well as their combined effect, on *V. vulnificus* counts. There also was a significant treatment difference ($p<0.05$) between D-values of *V. vulnificus* inactivation by simulated gastric fluid (Figure 19). D-value for untreated *V. vulnificus* was 7.8 +/- 2.4 minutes, while the D-value for 7-day, 25°C stored *V. vulnificus* was 9.1 +/- 3.9 minutes. Koo et al. (2000b) found D-values for three non-starved *V. vulnificus* strains ranged from 2.8 to 4.1 minutes at pH 4.0 in the same simulated gastric fluid composition. It appeared that simulated gastric fluid had better bactericidal ability compared to acidified nutrient broth at the same pH level. For example, Koo et al. (2000a) showed D-values ranged from 26.7 to 51.8 minutes for three strains of *V. vulnificus* in acidified TSB-2%NaCl with hydrochloric acid at pH 4.0. Similarly, Bang and Drake (2005) observed a 5-log reduction within 1 hour for three strains of *V. vulnificus*, including strain ATCC 27562 (D-value of 12 minutes), in TSB-2%NaCl acidified with hydrochloric acid to pH 4.0.

Interestingly, as the storage temperature decreased, *V. vulnificus* became more susceptible to the bactericidal effect of low pH, with D-values of 3.7 +/- 0.89 minutes and 6.6 +/- 1.99 minutes for *V. vulnificus* stored at 4 and 10°C, respectively (Figure 19).

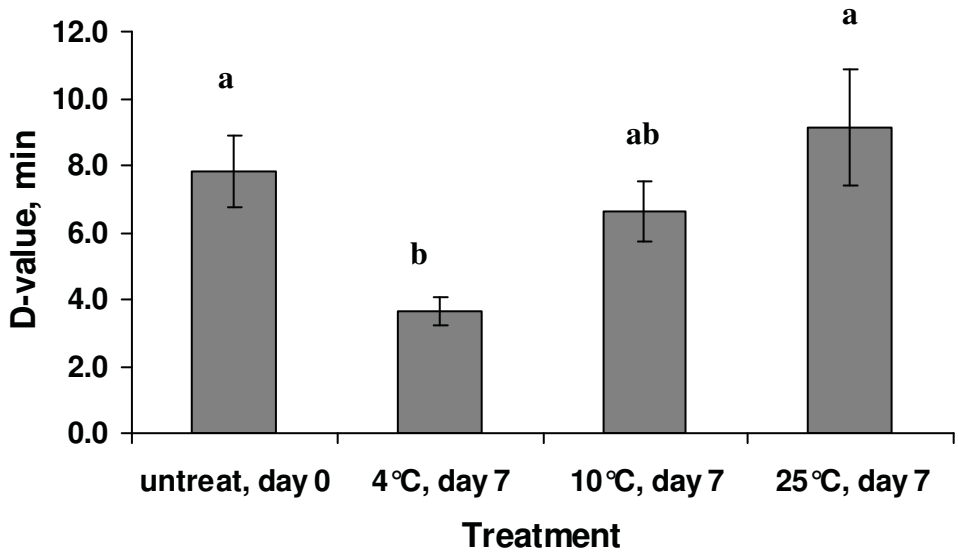


Figure 19. Inactivation D-values of starved *V. vulnificus* stored under different conditions after exposure to simulated gastric fluid

NOTE: Means with the same letter are not significantly different ($p>0.05$)

It appears that low temperature adaptation may offer less resistance to low pH that might be experienced by the bacterium during gastric transit. Bang and Drake (2002) found no effect of cold stress on heat-thaw resistance of *V. vulnificus*, while starvation of *V. vulnificus* enhanced freeze-thaw resistance and heat tolerance of only one strain. Based on the obtained data, *V. vulnificus* might be more susceptible to lethal effects of gastric fluid after starvation storage at refrigerated temperatures. This may explain why the majority of *V. vulnificus* cases (89%) associated with raw oyster consumption occur when seawater harvesting temperatures exceed 22$^{o}$C (Shapiro et al., 1998).

# CHAPTER VIII

## SUMMARY AND CONCLUSIONS

Prolonged depuration of live oysters in either ambient seawater (22$^{o}$C) or cold seawater (10$^{o}$C) significantly reduced ($p<0.05$) *Vibrio vulnificus* populations. Seawater temperature was not a significant factor ($p>0.05$) in population reductions. Either depuration scheme failed to reduce pathogen populations to non-detectable levels (<3 MPN/g), which favors the hypothesis of a close ecological relationship between the pathogen and bivalve mollusks. The overall microflora of freshly harvested oysters was composed mostly of mesophilic vibrios, while storage/depuration of live oysters at refrigerated temperatures shifted the microflora from mesophilic vibrios to a dominance of psychrotrophic *Pseudomonas*, *Aeromonas*, and *Shewanella* spp. In contrast, depuration of live oysters at ambient temperature retained mesophilic vibrio dominance with greater incidence of pathogenic vibrios compared to cold water depuration. Overall aerobic plate counts for ambient treated oysters was significantly lower ($p<0.05$) than cold treated oysters; therefore, prolonged cold water depuration did not have an advantage over ambient water depuration, since *V. vulnificus* population reduction was similar. Since neither method eliminated *V. vulnificus,* the economical justification of prolonged depuration remains questionable.

*In vitro* results showed that high salinity (35 ppt) rendered *V. vulnificus* unable to grow on microbiological media, even though viable cell population size remained unchanged at 4°C. Lower salinities (12 and 20 ppt) and lower temperatures (4 and 10°C) did not cause significant reduction in *V. vulnificus* numbers. Perhaps this is because pre-starvation acclimatized *V. vulnificus* to the new environment. It therefore appears that reduction in *V. vulnificus* populations in depuration tanks was due to dilution and UV light treatment sterilization of depuration seawater. It was also shown that natural environmental *V. vulnificus* isolates might be more resistant to harsh environmental conditions compared to archived ATCC *V. vulnificus* isolates.

Changes in *V. vulnificus* fatty acid profile revealed a significant switch ($p<0.05$) from C18:1w7c into C18:1w6c by double bond relocation under starvation conditions. The relocation occurred faster at ambient temperatures and was slower at refrigerated temperatures. Under starvation with no nutrients available, cells of *V. vulnificus* did not adjust their membrane fluidity to storage temperature within the investigated time frame. However, a faster fatty acid switch was observed for *V. vulnificus* strain ENV-1 at 4°C compared to *V. vulnificus* ATCC 27562 with the exception of 35 ppt seawater, where a significant slowdown was noted. The importance of this observation is difficult to interpret, but it might be related to ENV-1 strain's inability to enter a VBNC state at 4°C in 35 ppt seawater compared to ATCC 27562 strain.

It is generally known that the majority of *V. vulnificus* human infections occur during warm summer months, while during colder winter months pathogen counts are low either due to VBNC state, pathogen death, or combined effect of both. The ability of starved cold stored *V. vulnificus* to survive gastric fluid, which is the first barrier this pathogen has to overcome in order to establish infection, was investigated. Interestingly, *V. vulnificus* was less resistant to low pH of simulated gastric fluid after prolonged storage at refrigerated temperatures compared to control culture. This, together with low counts of the pathogen in winter harvested oysters, may impact the low number of foodborne infections during the winter period of the year.

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