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# Two post-harvest treatments for the reduction of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in eastern oysters (*Crassostrea virginica*)

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TWO POST-HARVEST TREATMENTS FOR THE REDUCTION OF *VIBRIO VULNIFICUS*  
AND *VIBRIO PARAHAEMOLYTICUS* IN EASTERN OYSTERS (*CRASSOSTREA*  
*VIRGINICA*)

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
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Master of Science

in

The School of Renewable Natural Resources

by  
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## ABSTRACT

*Vibrio vulnificus* and *V. parahaemolyticus* are naturally occurring estuarine bacteria and the leading causes of seafood-borne illness in the United States. Multiple outbreaks due to raw oyster consumption in the last decade has lead to much research to remediate these bacteria from oysters destined for the half-shell market. The focus of this research was to investigate the efficacy of icing and high salinity exposure as two post-harvest treatments for the reduction in numbers of *V. vulnificus* and *V. parahaemolyticus* in commercial quantities of shellstock oysters.

The icing experiment was conducted in the summer of 2006, and the high salinity trials were done in September 2007. Treatments for the icing experiment include: (1) on-board icing immediately after harvest exposed to minimal handling and shipping; (2) on-board icing immediately after harvest exposed to typical industry shipping and handling practices; (3) dockside icing approximately 1-2 hours prior to docking; and (4) no icing. In most instances during icing and cold storage, there were no statistically significant differences in *V. vulnificus* and *V. parahaemolyticus* counts by treatment or time. The only exceptions occurred in August samples, in which case *V. vulnificus* and *V. parahaemolyticus* counts in dockside and non-iced oysters were significantly higher than the immediately iced on-board samples. Treated (iced) oysters had significantly higher gaping after one week in cold storage than did non-iced oysters.

For the high salinity exposure research, oysters were relocated to an area of full strength sea water (>30 ppt) to measure change in both *V. vulnificus* and *V. parahaemolyticus*. Oysters placed at ambient air temperatures over night had significantly higher numbers of both *V. vulnificus* and *V. parahaemolyticus* than when they were first harvested. Due to safety concerns and equipment failure, the experiment could not be completed, but preliminary results indicate a significant decrease in *V. vulnificus* and *V. parahaemolyticus* counts after one week of exposure

to sea water. However, one week was insufficient time to reduce either *V. vulnificus* or *V. parahaemolyticus* to non-detectable numbers.

Overall, post-harvest icing did not substantially reduce *V. vulnificus* or *V. parahaemolyticus* in oysters, and icing negatively impacted oyster survival during subsequent cold storage. High salinity exposure showed promise in *V. vulnificus* and *V. parahaemolyticus* reduction; however, the logistics of relocating oysters to full strength sea water need to be closely evaluated.

## CHAPTER 1 INTRODUCTION

Oyster harvesting and processing are crucial components of Louisiana's coastal economy. Louisiana oyster harvests constituted 33% of the nation's catch from 1997 to 2005 and had a dockside value of \$30 million in 2006 (LDWF 2007). Despite the importance of the Louisiana oyster industry, recently it has come under heavy scrutiny because of food-borne illnesses associated with consuming Gulf Coast oysters that contain *Vibrio spp.* Freshly shucked raw oysters are considered a delicacy, but there are risks when one eats any uncooked meat product. The controversy surrounding Gulf Coast oyster safety has resulted in a sales ban in California unless they are treated post-harvest to reduce *V. vulnificus* and *V. parahaemolyticus* numbers to safe levels (Wirth & Minton 2004).

*Vibrio vulnificus* and *V. parahaemolyticus* are under greatest study because they are the leading causes of seafood-related illness and death (Mead et al. 1999). *Vibrio vulnificus* and *V. parahaemolyticus* are members of the vibrionaceae family, which are naturally occurring, obligate, halophilic, motile gram-negative, rod-shaped bacteria (Oliver 2005). These bacteria are most prevalent during the warmer months (i.e. May through October) in Gulf inshore coastal waters from where the majority of the nation's oysters are harvested and distributed (Rippey 1994; Hlady et al. 1997; Cook et al. 2002). Being filter feeders, oysters accumulate internal *V. vulnificus* and *V. parahaemolyticus* populations through the bacteria's association with plankton, the main food source for oysters (Kaneko & Colwell 1973; Kelly & Dinuzzo 1985; Chowdhury et al. 1990; Groubert & Oliver 1994). In fact, both *V. vulnificus* and *V. parahaemolyticus* densities are higher in oysters than surrounding waters (Depaola et al. 1990; Lin et al. 2003).

*Vibrio vulnificus* infections are characterized by fever, diarrhea, nausea, cramps, and in severe cases, primary septicemia (Kaysner & Depaola 2004). Primary septicemia occurs when

the bacteria invade the bloodstream resulting in skin lesions, septic shock, and often death. Although there are many more prominent food-borne pathogens, *V. vulnificus* possesses the notoriety of having the highest case-fatality rate of 39% because of septicemia (Mead 1999). Those who developed septicemia typically consumed raw shellfish within 2 days of becoming ill and often have critical underlying medical conditions such as liver disease, alcoholism, and/or cancer (Shapiro et al. 1998).

The mechanism of *V. vulnificus*' survival in oysters and pathogenicity in humans is not well understood, but studies have shown that iron-overloaded mice have a higher susceptibility and death rate when infected (Wright et al. 1981). Iron overload may explain why immunocompromised individuals, especially those with liver disease, have a higher mortality rate from *V. vulnificus*-induced septicemia infections. Two morphological types of *V. vulnificus*, nonencapsulated (translucent) and encapsulated (opaque), have been documented, but pathogenicity has been linked only to encapsulation (Simpson et al. 1987). Moreno & Landgraf (1998) confirmed pathogenicity of encapsulated strains as well as the ability to produce proteases, hemolysins, DNase, lecithinase, and lipase. These enzymes are most likely crucial elements in *V. vulnificus*' ability to infect human systems. A study by Depaola et al. (2003) unsuccessfully utilized several methods to try and locate virulent gene markers in environmental strains compared to clinical strains. They concluded that all encapsulated strains of *V. vulnificus* should be considered potentially harmful and pathogenic.

*Vibrio parahaemolyticus* is not as deadly as *V. vulnificus* but is an equally widespread pathogen. *Vibrio parahaemolyticus* infections result in gastroenteritis, which symptoms include diarrhea, cramps, nausea, vomiting, and fever (Kaysner & Depaola 2004). Pathogenicity from *V. parahaemolyticus* is associated with an enterotoxin produced by strains that contain a

thermostable direct hemolysin (tdh<sup>+</sup>) gene (Shirai et al. 1990). The bacteria attach to the intestinal lining and cause an ion flux, which results in the aforementioned symptoms (Nichibuchi & Kaper 1995). A concern with *V. parahaemolyticus* infections is that they are not as severe as *V. vulnificus* illness; therefore, the cases are probably underreported, thus making it difficult to accurately predict the severity of the *V. parahaemolyticus* threat (Mead et al. 1999). An infective dose has not yet been determined for either *V. vulnificus* or *V. parahaemolyticus*, so raw shellfish consumption is strictly at one's own risk.

Due to recent *V. parahaemolyticus* outbreaks (CDC 1998; CDC 1999; CDC 2006) not to mention the severity of *V. vulnificus* infections, a great deal of research has been conducted to remediate *V. vulnificus* and *V. parahaemolyticus* numbers in oysters destined for human consumption, especially the half shell market. The goal of this research is to determine if various post-harvest icing and high-salinity exposure post-harvest treatments are appropriate in reducing *V. vulnificus* and *V. parahaemolyticus* to non-detectable levels (<1 CFU/0.1g) as defined by the National Shellfish Sanitation Program (2005).

### **Previous Work**

**Temperature Studies.** Thus far, research has found that temperature is the main factor in *V. vulnificus* and *V. parahaemolyticus* proliferation (Lin et al. 2003; Randa et al. 2004). This is supported by studies that show higher *V. vulnificus* and *V. parahaemolyticus* numbers during summer months through early October, followed by a notable decline over winter (Depaola et al. 1990; O'Neill et al. 1990; Jones & Brason 1998; Gooch et al. 2002; Depaola et al. 2003). To survive cold winter temperatures, *V. parahaemolyticus* overwinters in sediments and then reappears in the water column with spring plankton blooms (Kaneko & Colwell (1973). Due to

the low numbers of *V. vulnificus* and *V. parahaemolyticus* in winter samples, it has been suggested by Ruple & Cook (1992) that oysters should only be harvested during winter months.

The major factor causing dangerous *V. vulnificus* and *V. parahaemolyticus* levels in half shell oysters is time-temperature abuse. After oysters are harvested, they are sacked, placed on the boat's deck, and allowed to sit shaded at ambient air temperature until they are hauled to shore and transported to a processing plant. Cook (1994) first found that *V. vulnificus* in oysters sitting at ambient air temperatures will increase slightly more than one log unit in 30 hours. In a later study, Cook (1997) reported an approximate 2 log<sub>10</sub> increase in *V. vulnificus* after 14 hours sitting on deck. Similarly, Gooch et al. (2001) found a 3 log<sub>10</sub> increase in *V. parahaemolyticus* after 24 hours incubation at 26<sup>0</sup>C. A retail study by Ellison et al. (2001) showed that *V. parahaemolyticus* numbers are significantly higher in restaurant oysters than wholesale product, which further implicates time-temperature abuse.

Since oysters at ambient temperatures result in a *V. vulnificus* and *V. parahaemolyticus* increases, refrigeration should be a logical solution to cause their decrease. Cold storage has been shown to lower *V. vulnificus* and *V. parahaemolyticus* numbers (Table 1.1), but not to non-detectable levels. Nonetheless, Cook and Ruple (1992) were able to recover *V. vulnificus* from oysters stored at -20<sup>0</sup>C for 12 weeks, and Johnson et al. (1973) noted *V. parahaemolyticus* survival at 4<sup>0</sup>C after 3 weeks. When stored in cold temperatures, both *Vibrio* spp. can enter a viable but non-culturable state, which is a condition where the bacteria live but cannot grow (Johnson et al. 1973; Wolf & Oliver, 1992). Bang & Drake (2002) and Bryan et al. (1999) believe that both bacteria can produce adaptive cold proteins to aid in their survival at low temperatures. Consequently, refrigeration is best utilized to prevent *V. vulnificus* and *V. parahaemolyticus* from increasing prior to serving oysters (Kaspar & Tamplin 1993).

**Table 1.1** Previous studies on the effect of cold storage on reducing *Vibrio vulnificus* (Vv) and *V. parahaemolyticus* (Vp) in the Eastern oyster *Crassostrea virginica*.

Bacteria	Temp (°C)	Incubation Time	Change in Numbers (log)	Author(s)
Vv	4.0	14 days	-0.2	Cook & Ruple (1992)
Vv	-1.9	14 days	-1.2	Cook & Ruple (1992)
Vv	7.0	10 days	-0.5	Lorca et al. (2001)
Vv	4.0	2 hours	-1.0	Oliver (1981)
Vv	ice	7 days	-2.0	Ruple & Cook (1992)
Vp	3.0	14 days	-0.8	Gooch et al. (2002)
Vp	3.0	14 days	-0.9	Gooch et al. (2001)

**Salinity Studies.** Salinity is the second major factor in *V. vulnificus* and *V. parahaemolyticus* growth; however, unlike temperature, salinity displays a negative correlation (Oliver et al. 1982; Kelly & Stroh 1988; Lin et al. 2003). *Vibrio vulnificus* and *V. parahaemolyticus* are obligate halophiles that require salinities of at least 4 ppt, but optimum salinities have been reported between 17 and 23 ppt (Tamplin et al. 1982; Chowdhury et al. 1990; Kaspar & Tamplin 1993; Depaola et al. 2003). Both *V. vulnificus* and *V. parahaemolyticus* are well adapted to live in estuarine waters; however, Kaspar & Tamplin (1993) found a significant decrease (88%) in *V. vulnificus* when placed in 35 ppt water. Motes & Depaola (1996) discovered that oysters purged themselves of *V. vulnificus* (<10 MPN/g) in 7-17 days when relayed to high salinity waters (>30 ppt). Also, oysters from the higher salinity waters of the Atlantic Coast have been found to have near non-detectable numbers of *V. vulnificus* (Motes et al. 1998). High salinity exposure is not a well researched avenue of post-harvest treatment for *Vibrio* reduction, but one that has promising results from the few studies already performed.

**Other *Vibrio* Reduction Studies.** One focus in food safety is to develop a post-harvest treatment that can successfully reduce both *V. vulnificus* and *V. parahaemolyticus* to non-detectable levels, but leave the product safe for raw consumption. Son & Fleet (1980) reported that UV light treatment and relaying to pollution-free waters cleanses *V. parahaemolyticus* from oysters contaminated in a laboratory. Conversely, Eyles & Davey (1984) showed UV light treatments to be ineffective at deparating oysters naturally colonized by the bacteria. Birkenhauer & Oliver (2003) failed to reduce *V. vulnificus* numbers using diacetyl up to concentrations of 0.2%. Quevado et al. (2005) noted no significant change in *V. vulnificus* when

oysters were refrigerated after exposure to ice slurry. As already discussed, simple refrigeration is an inadequate post-harvest treatment for bacteria reduction.

To date, the only approved and successful methods for fully cleansing oysters of *V. vulnificus* and *V. parahaemolyticus* are heat shock and high hydrostatic pressure processing (Berlin et al. 1999; Hesselman et al. 1999). However, these treatments result in the death of oysters, which can be less desirable for the raw market.

### **Thesis Research**

This research focused on icing and high-salinity exposure as post-harvest treatments for the reduction of *V. vulnificus* and *V. parahaemolyticus*. For the icing experiment, the hypothesis tested was that icing oysters directly after harvest or prior to transportation to a wholesaler will not lead to a reduction in *V. vulnificus* and *V. parahaemolyticus* to non-detectable numbers. A similar study performed by Quevado et al. (2005) found that oysters placed on ice for 3 hours and then refrigerated for 2 weeks will not result in a decrease of *V. vulnificus*. However, this research differed from the Quevado et al. (2005) study by testing icing on a commercial scale, allowing the oysters to sit in ice for a longer period of time, and testing for *V. parahaemolyticus*. Immediate icing may reduce bacterial numbers through temperature shock and eliminate time-temperature abuse in oysters on deck. Oyster gaping and heterotrophic bacteria levels were also measured to determine additional side effects that icing may have on oysters.

Icing treatments consisted of immediate icing after harvest, dockside icing, and a control of no icing. Dockside icing was simulated by placing oysters on ice approximately 2 hours before harvest pick-up at the dock. Half of the immediately iced sacks were sent to a retailer via normal commercial standards to compare minimal to actual shipping and handling practices. At day 7 post harvest, all sacks were allowed to sit at ambient room temperature for 2 hours to

further mimic commercial handling methods. Two hours is the limit that a dealer has to place oysters under temperature control after receiving a shipment from a harvester (NSSP 2005).

A second hypothesis tested was that high salinity exposure would not be a sufficient post-harvest treatment for *V. vulnificus* and *V. parahaemolyticus* reduction to non-detectable levels. This aspect of the project was an advancement of the relaying study by Motes & Depaola (1996). This research built upon their analysis by eliminating an acclimation period, testing for *V. parahaemolyticus* and heterotrophic bacteria, utilizing DNA probes for *V. vulnificus* and *V. parahaemolyticus* detection, constant salinity and water temperature monitoring, checking for *Perkinsus marinus* (Dermo), and measuring condition index (CI). Healthy oysters were expected to survive high salinity exposure; however, late summer oysters may be weak from spawning and show increased mortality. Therefore, CI and Dermo were analyzed to gauge their role in mortality.

Through this research, a greater understanding of icing and high salinity exposure as post-harvest treatments was gained. Should these methods prove adept at lessening *V. vulnificus* and *V. parahaemolyticus* they could be instituted as food safety techniques. Nonetheless, the logistics, public opinion, and economics to make such methods standard practice was not addressed in this study.

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## CHAPTER 2 ICING AS A POST-HARVEST TREATMENT

*Vibrio vulnificus* and *V. parahaemolyticus* are the leading causes of seafood-borne illness in the United States (Mead et al 1999). Both organisms can cause gastroenteritis; however, *V. vulnificus* may cause primary septicemia with high mortality in consumers with underlying medical conditions (Shapiro et al. 1998; Daniels et al. 2000; Kaysner & DePaola 2001). Unfortunately, the annual incidence of *V. vulnificus* and *V. parahaemolyticus* infection in the U.S. has actually risen over the last decade (CDC 2006). This has prompted increased efforts to adopt effective methods to reduce the risk of human illness associated with the consumption of oysters, particularly those destined for the half shell market.

As members of the *Vibrionaceae* family, *V. vulnificus* and *V. parahaemolyticus* are naturally occurring obligate halophilic bacteria that thrive in Gulf Coast waters where the majority of the nation's oysters are harvested (Cook et al. 2000; Wirth & Minton 2004; Oliver 2005). Oysters harvested in the warmer months of the year typically contain high levels of the pathogenic *Vibrio vulnificus* and *V. parahaemolyticus*. Because the organisms grow readily at ambient temperatures, the National Shellfish Sanitation Program (NSSP) requires oysters to be placed under cold storage within specific time frames post-harvest, depending on air temperature at time of harvest, and within 2 hours after the dealer receives the harvest (NSSP 2005). Dealers are also responsible for maintaining shellstock (unshucked live oysters) meat temperature at  $\leq 10^{\circ}\text{C}$  during points of transfer (unloading at a dock or restaurant) and at  $\leq 7.2^{\circ}\text{C}$  if a post-harvest treatment is applied. Oyster shipments are to be rejected if internal meat temperatures rise above  $15.6^{\circ}\text{C}$  after appropriate cooling. Despite these regulations, it has been documented that the vast majority of *V. vulnificus* and *V. parahaemolyticus* proliferation still occurs during the harvesting phase of the farm-to-fork continuum, in which naturally occurring *V. vulnificus*

and *V. parahaemolyticus* concentrations can increase more than 1 log<sub>10</sub> when oysters are stored on deck under ambient summer and autumn temperatures (Ford & Tripp 1996; Cook 1997).

In early studies, Cook & Ruple (1992) reported that *V. vulnificus* and *V. parahaemolyticus* levels decreased to nearly non-detectable numbers when oysters were placed on ice for 2 weeks. This study and others have prompted regulatory interest in the feasibility of immediate post-harvest icing of oysters (Cook 1997; Gooch et al. 2001; Gooch et al. 2002; Quevado et al. 2005). Accordingly, the purpose of this research was to evaluate whether on-board icing could be used as an effective post-harvest treatment on a commercial scale to control the levels of *V. vulnificus* and *V. parahaemolyticus* in shellstock oysters. Differences in *V. vulnificus* and *V. parahaemolyticus* levels between treated (iced) and untreated (non-iced) oysters, intended for either wholesale or retail markets, were monitored at harvest and for up to 2 weeks of refrigerated storage. The effect of icing on oyster gaping was also evaluated.

## **Materials and Methods**

**Sample Collection.** Oyster samples were harvested by dredge from approved shellfish growing waters in Louisiana Harvest Area Number 11 (29°27'22''N, 89°46'45''W) with the assistance of an industry collaborator during June and August of 2006. Salinity, water temperature, and weather conditions were recorded during each sampling period using a refractometer, thermometer, and visual observation, respectively. Each collection trip lasted 8 to 9 hours.

The first 3 dozen market-sized (>75 mm shell height) oysters harvested were placed in mesh crawfish bags and cooled as a single layer on ice which was covered with a burlap bag to prevent direct contact. These were considered the “time 0” control samples, representing the *V. vulnificus* and *V. parahaemolyticus* levels in oysters immediately after they were removed from

the water. Thereafter, a total of 12, 19 L (ca. 5 gal) burlap sacks were filled with freshly harvested oysters (approx. 100 specimens per sack, no more than 10 min filling time per sack). A Smartbutton data logger (ACR Systems, Inc., British Columbia, Canada) was taped on the outer shell of one oyster in each sack to record temperature changes over the experimental period. The oysters with Smartbuttons were randomly placed in different locations of each sack so as to obtain a full picture of temperature fluctuations that might occur as a function of oyster location in the sack.

These 12 sacks represented 4 treatments of 3 sacks each. Six of these sacks were exposed to on-board icing, in which they were buried immediately after harvesting in 159 L coolers filled with ice. The oysters were protected from direct contact with ice by the burlap sack and the coolers were unplugged to allow drainage of ice melt. The other six sacks remained shaded on the boat deck at ambient air temperature, as currently practiced by most commercial harvesters. Three of these sacks, designated “non-iced,” received no further treatment. To mimic “dockside icing,” three sacks were placed on ice approximately 1 to 2 hours before docking. Upon docking, all 12 sacks were loaded onto a refrigerated truck via conveyor and shipped to the first receiver (shucking house). The six sacks of iced oysters were subdivided to represent the other two treatments, reflecting two commercial storage practices. One set of three sacks remained at the first receiver and was labeled “on-board iced, wholesale storage.” The other three sacks were shipped to a second receiver and then to a restaurant where they were refrigerated; these were designated “on-board iced, retail storage.” To determine the numbers of *V. vulnificus* and *V. parahaemolyticus* after initial treatment and over the shelf life of the product, triplicate sub-samples (consisting of 12-15 oysters each) were collected upon docking and after 7 and 14 days of commercial storage (samples designated “day 0,” “day 7,” and “day 14,”

respectively). The sub-samples were stored on ice and transported to Louisiana State University in Baton Rouge, LA for microbiological analyses. To mimic minor temperature fluctuations that might occur during loading, unloading, or shucking of oysters, all treatment samples were placed at room temperature for a 2 hour period after 1 week of cold storage just prior to microbiological analysis. Aside from this, the oysters were handled under normal commercial practices for the entire study.

**Microbiological Testing.** Microbiological analyses for all samples were initiated on the morning after harvest. This constituted a holding period of <24 hours for all samples except the time 0 control, which slightly exceeded this time because these samples were collected at the beginning of the harvest trip. The colony lift hybridization method in the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM), with minor modifications, was utilized for detection and enumeration of *V. vulnificus* and *V. parahaemolyticus* (Cook et al. 2000; Kaysner & DePaola 2001). The oyster subsamples were rinsed, aseptically shucked and pooled to obtain a weight of 150-250 g; they were then diluted with an equal volume of alkaline peptone water, and homogenized in a filter stomacher bag (Nasco, Fort Atkinson, WI) under high speed for 2 min. The resulting filtrate was serially diluted in phosphate buffered saline ( $10^{-1}$  to  $10^{-4}$ ) and spread plated to *Vibrio vulnificus* agar (VVA) and thiosulfate-citrate-bile salt (TCBS) plates. The VVA and TCBS plates were incubated overnight at 35°C and 37°C, respectively. Dilutions were also plated onto tryptic-soy agar supplemented with 2% NaCl (TSAN<sub>2</sub>) and incubated at 37°C overnight to measure total estuarine bacterial counts.

Colony lift hybridizations were created from the VVA and TCBS plates as previously described (Cook et al. 2000; Kaysner & DePaola 2001). Control strips, including *V. vulnificus* (strains 1002 and 1007 obtained from Dr. Simonson, Louisiana State University Agricultural

Center) and *V. parahaemolyticus* (ATCC strains 70802 and 45929) were included in the analysis. Hybridization was done using 5 µmol of alkaline phosphatase conjugated 5' amine-C6 (designated X) DNA probes targeting either the *V. vulnificus* cytotoxin gene (*vw*, 5'-XGA GCT GTC ACG GCA GTT GGA ACC A-3') or the *V. parahaemolyticus* thermolabile hemolysin gene (*tlh*, 5'-XAA AGC GGA TTA TGC AGA AGC ACT G-3') (DNA Technology A/S, Denmark). After washing and color development, enumeration was done by visual counting of positive (purple) colonies.

**Oyster Gaping.** Gaping percentage was measured for each treatment after 7 and 14 days of refrigerated storage as determined by inspecting and tapping every oyster in all replicates with a shucking knife handle. Oysters that did not close when handled or sounded hollow when tapped were considered gaped and discarded (ISSC 2007).

**Statistical Analyses.** Bacterial numbers were converted to log<sub>10</sub> values for statistical analysis. If no *V. parahaemolyticus* or *V. vulnificus* colonies were detected on the least dilute sample, then half of the lower limit of detection was used to estimate counts. The data were analyzed using SAS version 9.1 (SAS Institute, Cary, NC) with a 95% confidence interval. A one-way ANOVA with Tukey-Kramer adjustments (Proc Mixed) was used to test significance between the effect of the treatments on initial (time 0) counts of *V. vulnificus* and *V. parahaemolyticus*. A 3x4 factorial analysis with a Tukey-Kramer adjustment (Proc Mixed) was used to evaluate differences in *V. vulnificus* and *V. parahaemolyticus* counts within treatments (on-board iced, wholesale & retail, dockside, and non-iced) and over the 2 weeks of cold storage (days 0, 7, and 14). To determine significance for the gaping data, a logistic analysis (Proc Glimmix) of the treatments' effect on gaping was performed.

## Results

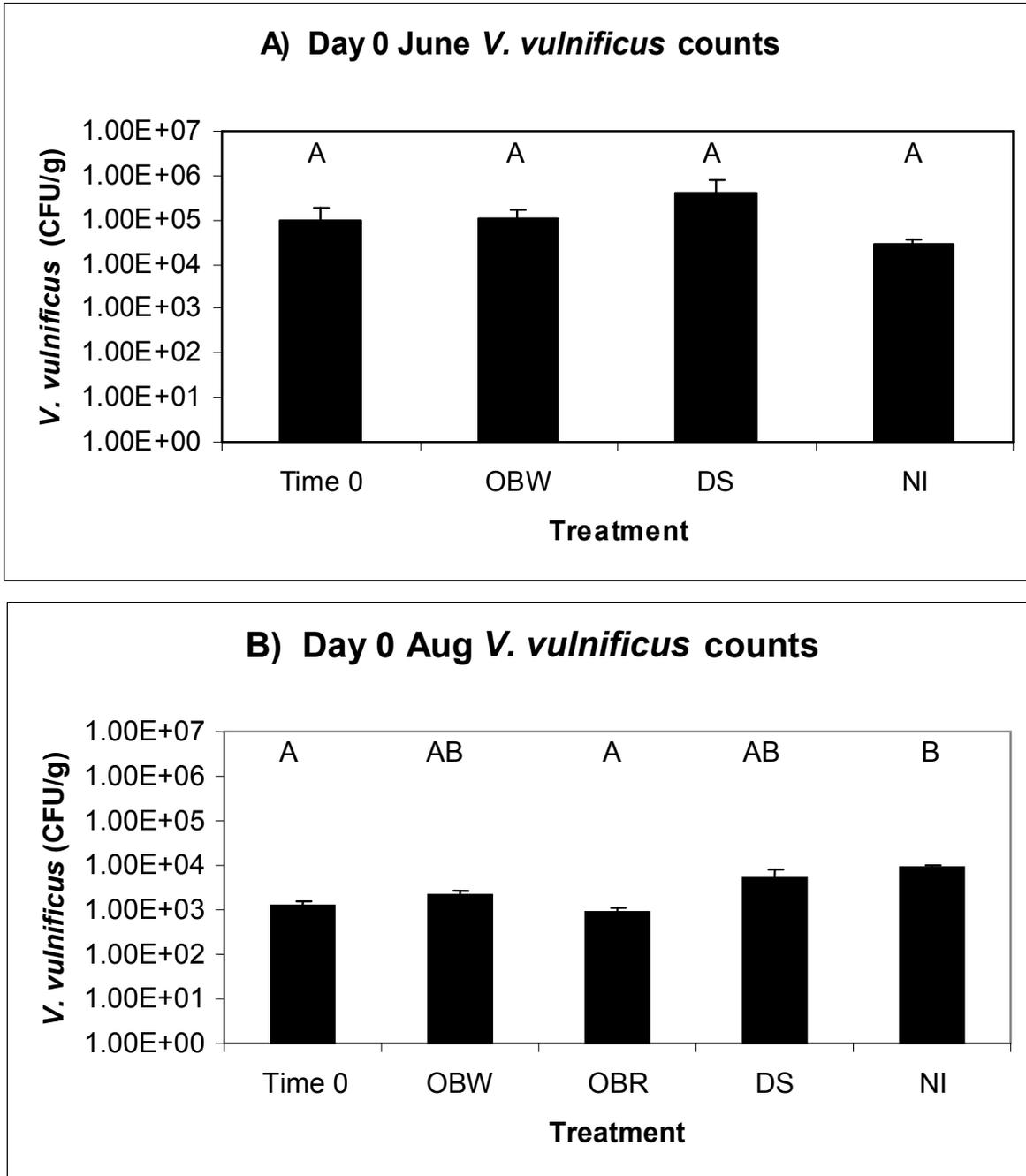
**Weather Conditions during Sample Collection.** Weather and water conditions were similar for both sampling periods. Water temperature was 30 and 31°C and salinity was 16 and 17 ppt in June and August, respectively. Wind speed was 8-16 km/h (5-10 mph) with clear skies.

**Smartbutton Temperature Data from Oysters in Cold Storage.** Based on data obtained using the ACR Smartbuttons, all on-board iced oyster samples reached the NSSP required post-harvest treated target temperature of 7.2°C between 96 to 270 min (June) and 148 to 358 min (Aug). It required 76 to 197 min (June) and 103 to 274 min (Aug) for these same samples to reach the NSSP shellstock storage temperature of 10°C. None of the dockside iced replicates reached 7.2°C before loading onto the refrigerated truck; however, two replicates in June and one in August reached 10°C prior to loading. For at least 7 hours, the non-iced oysters in both months remained at ambient air temperatures between 23 and 28°C until loaded onto the refrigerated truck. The monitors showed that occasionally the oysters' temperature were >7.2°C during transportation or storage, but <7.2°C was maintained for the majority of storage.

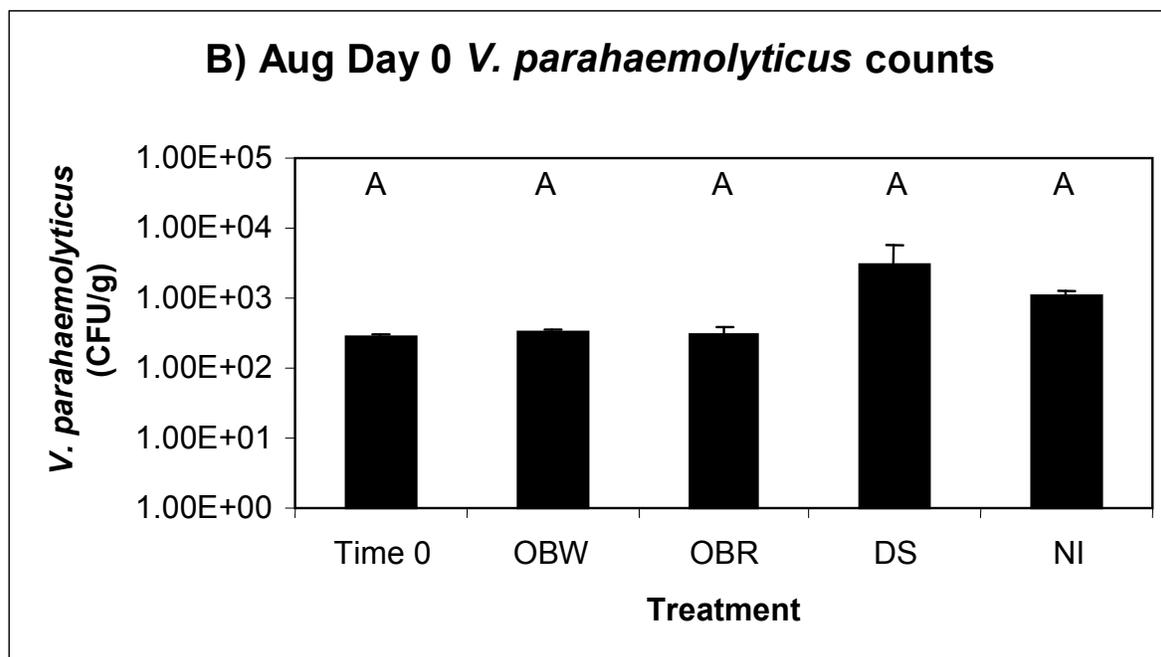
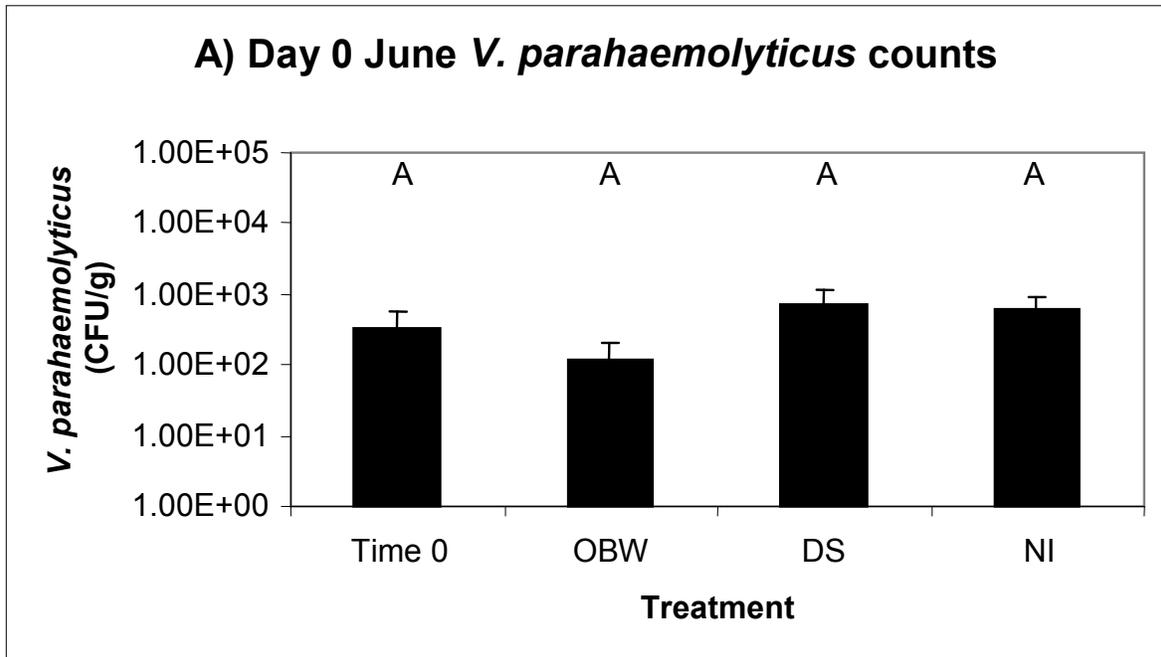
**Microbiological Data.** For both sampling months, *V. parahaemolyticus* counts were less than *V. vulnificus* counts for “time 0” samples (Figures 2.1 & 2.2). In June, the difference was 2-3 log<sub>10</sub>, while in August it was approximately 0.5-1.0 log<sub>10</sub> CFU/g. Only *V. vulnificus* counts for August samples showed statistically significant differences (p<0.05) by treatment (Figure 2.1). Specifically, the levels of *V. vulnificus* in the non-iced oysters were significantly higher than the levels in “time 0” and “on-board iced, retail storage” oysters. Icing and cold storage did not result in decreases in total estuarine bacterial counts, regardless of sampling month. After the 2 weeks in cold storage, the total estuarine bacterial counts in the oysters were greater than 6 log<sub>10</sub> CFU/g for all treatments.

When analyzing counts over the entire 14 day storage period, *V. vulnificus* counts in June samples were significantly different by sample day ( $p < 0.001$ ) but not by treatment, and no significant day by treatment interaction was found. For all treatments in June, *V. vulnificus* counts declined  $< 3 \log_{10}$  CFU/g from day 0 to 7, but increased by  $< 0.5 \log_{10}$  CFU/g from day 0 to 14 (Figure 2.3). Conversely, *V. vulnificus* levels in August showed a significant difference between treatments ( $p < 0.001$ ) but not sampling day, and no significant day by treatment interaction was detected. There were only slight *V. vulnificus* decreases ( $< 0.5 \log_{10}$  CFU/g) in the on-board iced treatments from day 0 to 14, while “dockside” and “non-iced” treatments increased  $< 0.5 \log_{10}$  CFU/g between days 0 and 14 (Figure 2.3). Only “on-board, wholesale storage” *V. vulnificus* counts on days 0 and 7 in June showed significant difference between days within treatments ( $p < 0.005$ ); however, the “dockside” and “non-iced” *V. vulnificus* counts were significantly higher than the on-board iced treatments in August ( $p < 0.001$ ).

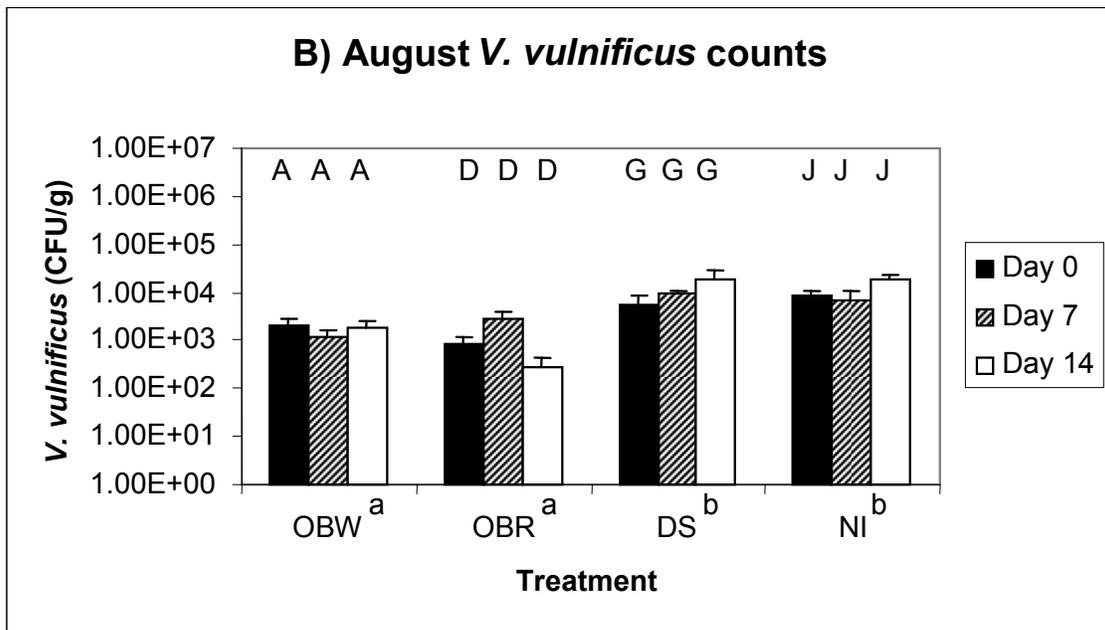
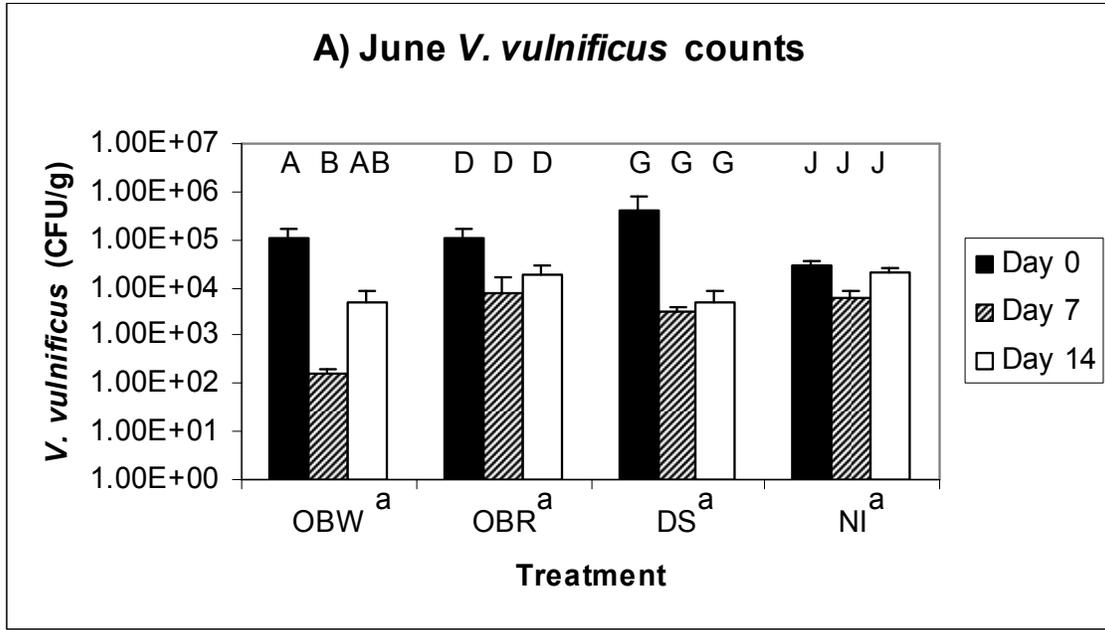
*Vibrio parahaemolyticus* levels for both June and August samples showed significance by treatment ( $p < 0.03$  [June],  $p < 0.001$  [August]) and sample day ( $p < 0.02$  [June],  $p < 0.001$  [August]); although, a significant day by treatment interaction was detected only in June ( $p < 0.03$ ). No significance in *V. parahaemolyticus* counts between treatments or days within treatments were found in June; however, the “dockside” and “non-iced” treatments were significantly higher than the on-board iced treatments in August ( $p < 0.03$ ) (Figure 2.4). The only significant change in *V. parahaemolyticus* counts between days within a treatment occurred for the “non-iced” oysters on days 0 and 14 in August ( $p < 0.01$ ). Except for the “on-board iced, retail storage” samples in August, *V. parahaemolyticus* levels were higher at day 14 than at day 0, but increases were generally limited to 1-2  $\log_{10}$  CFU/g. For both *V. vulnificus* and *V. parahaemolyticus*, counts were more variable in June.



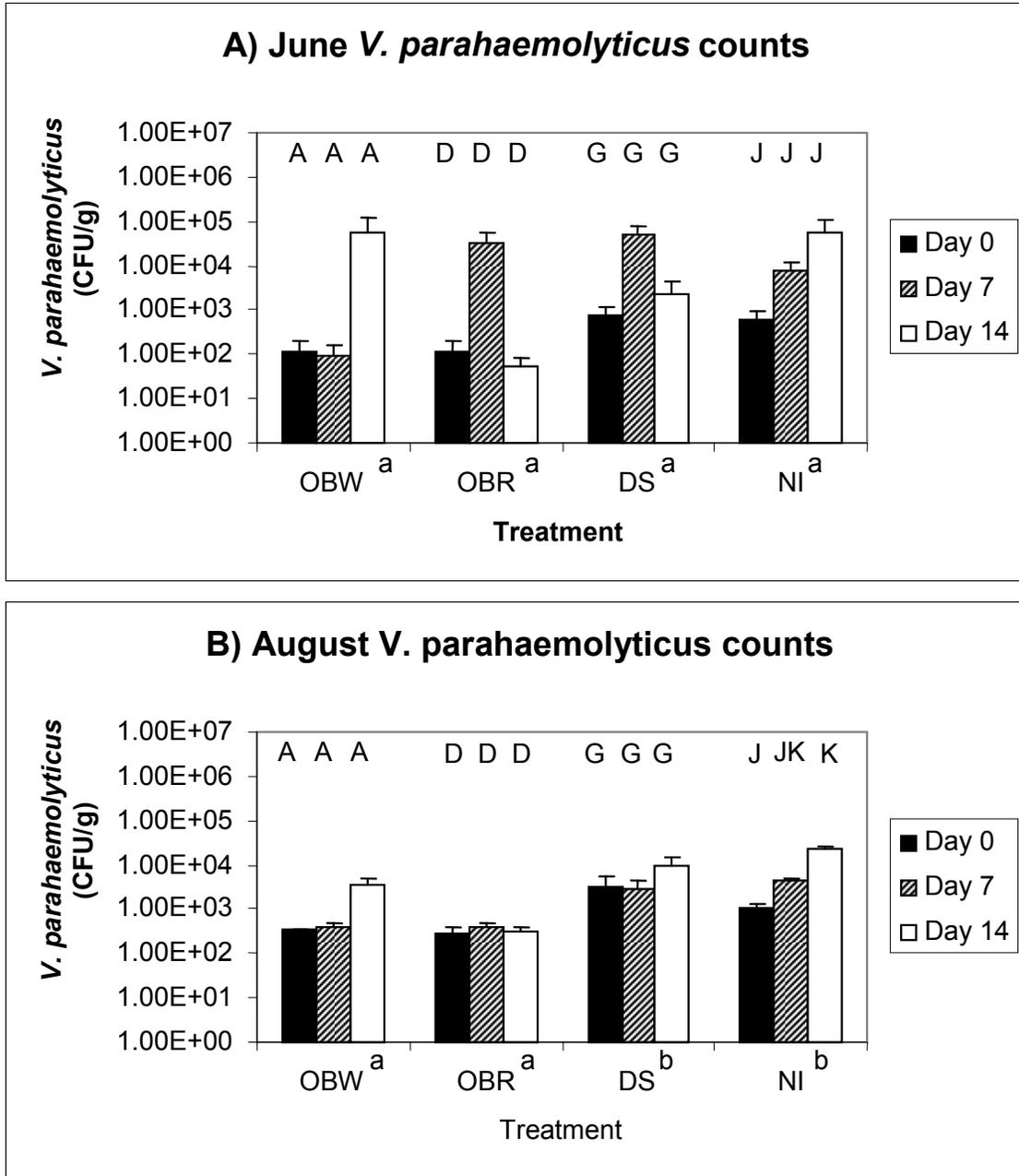
**Figure 2.1** Counts of *V. vulnificus* in June (A) and August (B) for oysters taken immediately from the water (time 0) and after docking for treatments consisting of non-iced (NI), dockside iced (DS), on-board iced, wholesale storage (OBW) and on-board iced, retail storage (OBR). OBR counts after docking were not obtained in June. Values reflect the mean and standard error of three samples in log<sub>10</sub> CFU/g. Different upper case letters indicate statistically significant differences in *V. vulnificus* counts when comparing treatments within each harvest month.



**Figure 2.2** Counts of *V. parahaemolyticus* in June (A) and August (B) for oysters taken immediately from the water (time 0) and after docking for treatments consisting of non-iced (NI), dockside iced (DS), on-board iced, wholesale storage (OBW) and on-board iced, retail storage (OBR). OBR counts after docking were not obtained in June. Values reflect the mean and standard error of three samples in log<sub>10</sub> CFU/g. Different upper case letters indicate statistically significant differences in *V. parahaemolyticus* counts when comparing treatments within each harvest month.



**Figure 2.3** Counts of *V. vulnificus* in June (A) and August (B) immediately after docking (day 0) and after 7 and 14 days of commercial refrigerated storage. Treatments consisted of non-iced (NI), dockside iced (DS), on-board iced, wholesale storage (OBW) and on-board iced, retail storage (OBR). OBR counts after docking were not obtained in June, so values from OBW samples were used for statistical comparisons. Values reflect the mean and standard error of three samples in log<sub>10</sub> CFU/g. Different upper case letters indicate statistically significant differences in *V. vulnificus* counts (by factorial analysis) when comparing days within each treatment, while different lower case letters indicate statistically significance differences between treatments across the entire product storage period.

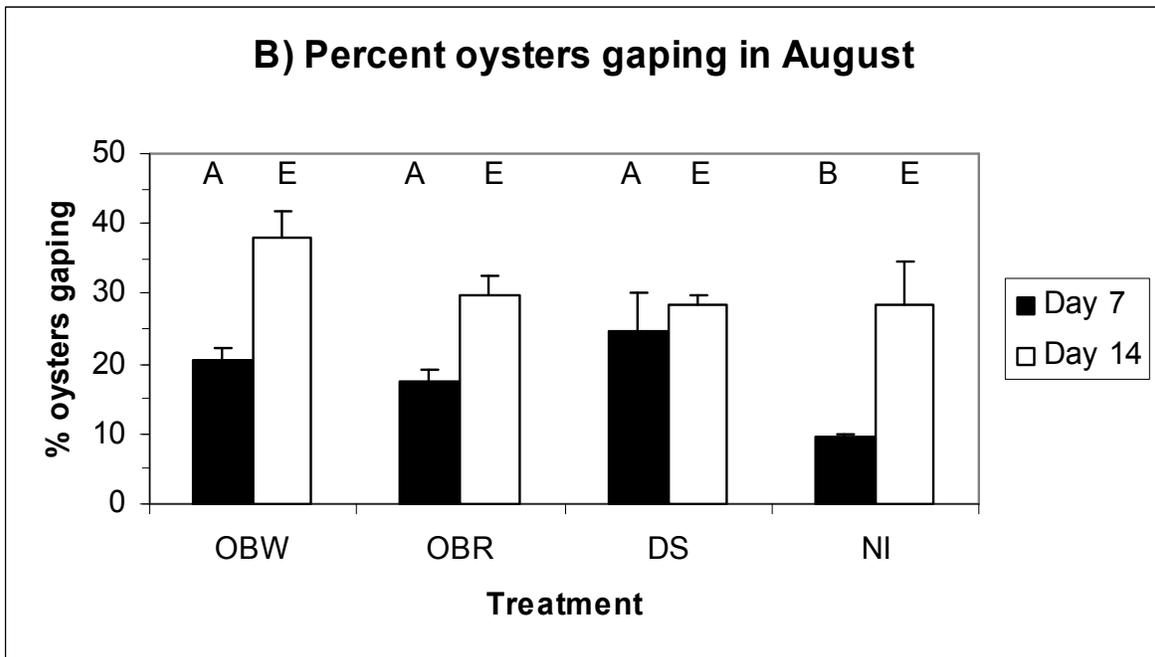
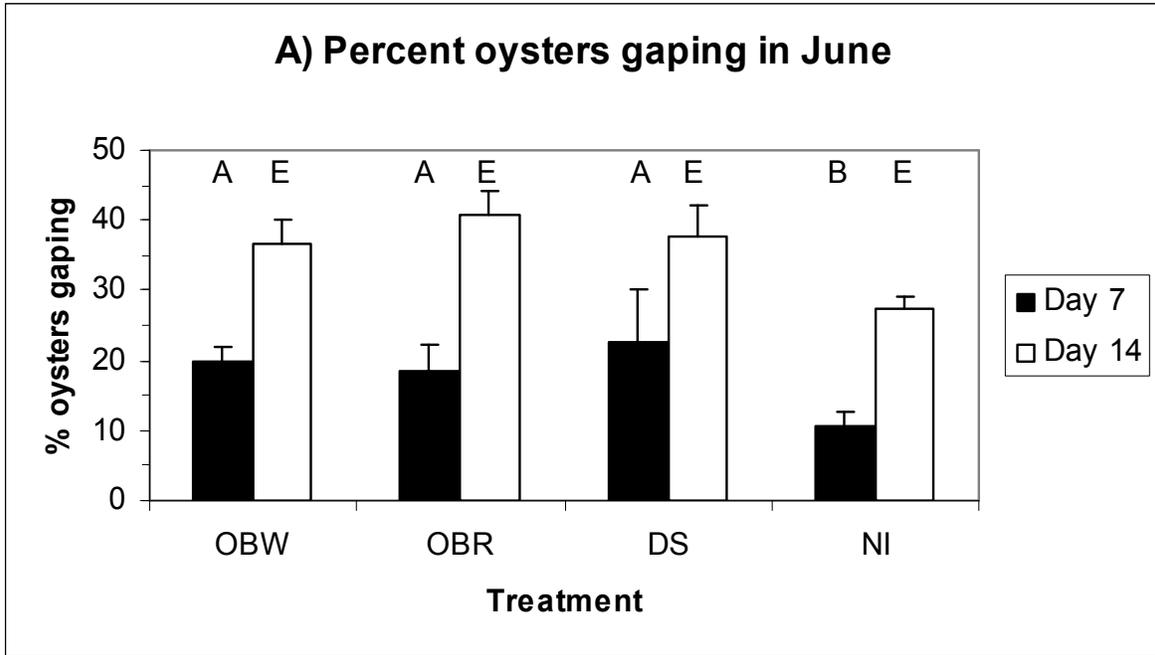


**Figure 2.4** Counts of *V. parahaemolyticus* in June (A) and August (B) immediately after docking (day 0) and after 7 and 14 days of commercial refrigerated storage. Treatments consisted of non-iced (NI), dockside iced (DS), on-board iced, wholesale storage (OBW) and on-board iced, retail storage (OBR). OBR counts after docking were not obtained in June, so values from OBW samples were used for statistical comparisons. Values reflect the mean and standard error of three samples in log<sub>10</sub> CFU/g. Different upper case letters indicate statistically significant differences in *V. parahaemolyticus* counts (by factorial analysis) when comparing days within each treatment, while different lower case letters indicate statistical significance differences between treatments across the entire product storage period.

**Gaping after Post-Harvest Treatment and Cold Storage.** Iced oysters showed a higher gaping percentage than did “non-iced” oysters after 7 and 14 days; however, this relationship was only statistically significant at day 7 ( $p < 0.005$ ) (Figure 2.3). After 1 week of cold storage in both June and August, the “dockside” oysters had the highest gaping percent, yet the on-board iced oysters, both wholesale and retail conditions, had the highest gaping percent after day 14 in June and August. Day 14 gaping percentages were higher than day 7 percentages for all treatments in both months.

### Discussion

In early studies, Cook & Ruple (1992) reported that *V. vulnificus* and *V. parahaemolyticus* levels decreased to nearly non-detectable numbers when oysters were placed on ice for 2 weeks. Others have reported that after 2 weeks of storage at 0°C, the level of naturally occurring *V. vulnificus* in oysters decreased by 2-2.5  $\log_{10}$  units but still remained above 4  $\log_{10}$  MPN/100 g (Kaspar & Tamplin 1993). *Vibrio parahaemolyticus* studies have shown 0.9 and 0.8  $\log_{10}$  reductions when oysters were stored at 3°C for 2 weeks (Gooch et al. 2001; Gooch et al. 2002). In the only previous icing study to be published to date, Quevado et al. (2005) observed that when oysters were iced for 3 hours and then placed in cold storage, *V. vulnificus* levels did not differ significantly from those of control (refrigerated) oysters. In most instances, the study showed that the process of ice immersion produced  $\leq 10\%$  reductions in the levels of *V. vulnificus* and did not appear to cause any sustained effect on levels of the organism during prolonged (2 week) refrigerated storage. Overall, the investigators concluded that ice immersion resulted in relatively small *V. vulnificus* declines and interestingly, increased fecal coliform counts. This experiment’s design differed from that of Quevado et al (2005) in that commercial quantities of oysters followed by standard commercial storage conditions were



**Figure 2.5** Mean percent of oyster gaping at days 7 and 14 for non-iced (NI), dockside iced (DS), on-board iced, wholesale storage (OBW) and on-board iced, retail storage (OBR) oysters harvested in June (A) and August (B). Different upper case letters indicate statistically significant differences in gaping between days 7 and 14 within each treatment category.

analyzed for bacterial counts. The results presented here further demonstrate that on-board or dockside icing followed by refrigeration does not reduce *V. vulnificus* or *V. parahaemolyticus* counts in shellstock oysters to non-detectable levels. While in some instances on-board icing prevented time-temperature increases for both *V. vulnificus* and *V. parahaemolyticus* during harvest, the overall effect on both initial counts and those over the shelf-life of the product were minimal.

Historically, gaping has been used for determining relative oyster mortality, although it does not necessarily reflect total mortality (i.e. no cardiac movement) of the harvest. From a commercial perspective, gaping is a concern because it results in loss of oyster liquor and shortened product shelf-life. After 1 week of refrigerated storage, “non-iced” oysters exposed to ambient air temperature during harvest displayed approximately half the gaping of oysters treated with ice. Between the first and second week of storage, the additional gaping was similar between all treatments. The degree of gaping observed may seem somewhat high, but this is probably associated with the season of harvest. In the Gulf region, oysters are physiologically weaker during the warmer months because of spawning and gaping can be further exacerbated by oyster disease (Ford & Tripp 1996; Supan & Wilson 2001). Nonetheless, it was observed that the combined gaping percentage (days 7 and 14) was always higher for the iced oysters.

The results indicate that icing oysters either immediately post-harvest or just prior to truck loading does not predictably reduce *V. vulnificus* or *V. parahaemolyticus* counts. Hence, it is unlikely that ice immersion alone will result in the dramatic reductions in *V. vulnificus* and *V. parahaemolyticus* levels currently sought by the Interstate Shellfish Sanitation Conference (ISSC); although, combinations of treatments have not yet been evaluated. In addition, on-board icing appeared to cause significantly higher oyster gaping, which could lead to economic losses

unacceptable to the industry. Taken together, the data suggest that other post-harvest treatments, including but not limited to high pressure processing or commercial heat shock, may be more promising alternatives for achieving substantial reductions in pathogenic *V. vulnificus* and *V. parahaemolyticus* and producing a safer “raw” oyster product while maintaining shelf-life and viability of the oyster industry (Berlin et al. 1999; Hesselman et al. 1999).

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### CHAPTER 3 HIGH SALINITY EXPOSURE AS A POST-HARVEST TREATMENT

*Vibrio vulnificus* and *V. parahaemolyticus* are obligate halophilic bacteria that occur naturally in estuarine waters along the Gulf Coast and many other areas of the world (Oliver 2005). These bacteria accumulate in oysters via filter feeding and can result in severe infections within human consumers when oysters are eaten raw (Kelly & Dinuzzo 1985; Daniels et al. 2000). Typically, infections develop within a few days of consumption and both *V. vulnificus* and *V. parahaemolyticus* can lead to gastroenteritis; however, *V. vulnificus* may cause primary septicemia, which is fatal in one-third of reported cases (Hlady 1997; Shapiro et al. 1998). Fatal infections tend to be limited to older individuals with compromised immune systems from liver disease, hepatitis, alcoholism, cancer, etc.; however, there is concern that *V. vulnificus* and *V. parahaemolyticus* infections are under- or misreported and are actually a more prevalent disease (Shapiro et al. 1998; Mead et al 1999).

One method of reducing the levels of *V. vulnificus* and *V. parahaemolyticus* in oysters has been relaying. Relaying is defined as removing oysters from restricted growing areas and moving them to approved growing waters to allow the oysters to depurate naturally (NSSP 2005). Son and Fleet (1980) reported that 6 days of relaying could reduce *V. parahaemolyticus* from two-thirds the original count to non-detectable. Motes and DePaola (1996) found that relaying oysters to higher salinity (>30 ppt) waters could reduce *V. vulnificus* to non-detectable numbers with minimal mortality.

Studies show that both *V. vulnificus* and *V. parahaemolyticus* grow optimally at salinities around 17 ppt, but can be detected in ranges of 5-25 ppt (Kaspar & Tamplin 1993; DePaola et al. 2003; Oliver 2005). Koh et al. (1994) reports that *V. vulnificus* and *V. parahaemolyticus* cannot be detected below 5 ppt, and other studies found that their numbers are greatly reduced or non-

detectable in waters >30 ppt (Motes et al. 1998; Kaspar & Tamplin 1993; Parvathi et al. 2004). Based on this information it is hypothesized that high salinity exposure, similar to relaying, could be a treatment to reduce *V. vulnificus* and *V. parahaemolyticus* to non-detectable numbers to make oysters more safe for the half-shell market. Motes and DePaola (1996) showed the process to work for *V. vulnificus* within 7-17 days; however, their study did not explore changes in oyster physiology.

This study aims to build on the knowledge from Motes and DePaola's (1996) research by measuring changes in *V. vulnificus* and *V. parahaemolyticus* prevalence and eliminating the acclimation period that they used prior to relaying. Although they reported 6% oyster mortality, Motes and DePaola (1996) did not explain what may have been factors in mortality. Condition index and *Perkinsus marinus* levels were measured to determine if general health or disease were factors in mortality. It is believed that high salinity exposure could be a quick and natural means of controlling *V. vulnificus* and *V. parahaemolyticus* levels in oysters destined for the half-shell market.

## **Materials and Methods**

**Sample Collection.** Oysters were harvested from Hackberry Bay (29° 24' 693''N, 90° 2' 929''W) north of Grand Isle, LA with the assistance of an industry cooperator. Temperature and salinity of the harvesting water were measured using a thermometer and refractometer, respectively, while weather was recorded using visible cues.

The first 30 oysters harvested were placed immediately on ice, labeled as "Start" (ST), and used as a baseline comparison for initial *V. vulnificus* and *V. parahaemolyticus* counts, condition index, and *Perkinsus marinus* (Dermo) levels. Three ~ 50 kg LA measure sacks (1.5 bu each) of oysters were collected and placed in the shade on the deck at ambient air

temperatures during the harvesting trip and overnight until they were individually placed into three 81x61x28 cm (32x24x11 in) plastic chicken coops (Kuhl Corporation, Flemington, NJ) the following morning. The coops were then placed in a 119x84x69 cm (42x33x27 in) welded steel frame rack (Figure 3.1) and transported approximately 14.5 km south of Grand Isle where they were placed on-bottom at a depth of approximately 15 m near the “Sulphur Mine” artificial reef (29° 11’ 36’’N, 89° 11’ 36’’W). Prior to placing the rack with oysters in the water, sub-samples of 10 oysters were taken in triplicate on day 0 and later at day 7 from each of the replicated coops designated top (T), middle (M), and bottom (B). Day 0 represents the time the oysters were placed in the water. A Kemmerer 1.2 L water sampler (Wilco Supply Co. Inc, Buffalo, NY) and YSI (YSI Inc., Yellow Springs, OH) probe were used to measure dissolved oxygen (DO) at the depth in which the oysters were placed. An YSI 600 LS (YSI Inc., Yellow Springs, OH) data logger was placed within the top coop to measure temperature and salinity every six hours for the duration of the project. Once sub-samples were collected, they were placed on ice to arrest bacterial growth and were transported to Louisiana State University in Baton Rouge, LA to measure *V. vulnificus* and *V. parahaemolyticus* levels, condition index, and Dermo incidence.

The experiment was terminated after day 7 due to equipment malfunction and safety concerns; however, the project was to be conducted until *V. vulnificus* and *V. parahaemolyticus* levels reached non-detectable numbers. Had the experiment been completed as planned, all remaining oysters were to be removed and mortality measured.

***Vibrio* DNA Probing.** *Vibrio vulnificus* and *V. parahaemolyticus* were detected and counted using a modified protocol developed by Cook et al. (2000). For each replicate (ST, T, M, B), six oysters were washed under running water, disinfected with 70% ethanol, and aseptically shucked into stomacher bags. The oysters were then diluted with equal parts



**Figure 3.1** Photograph of oyster rack with oysters in chicken coops. The steel frame was 119x84x69 cm (42x33x27 in) and raised 30.5 cm (12 in) off the ground. The chicken coops contained a bushel of oysters and weighed approximately 100 lbs. each. The coops plus the frame weighed approximately 850 lbs.

alkaline peptone water, homogenized in a stomacher, and filtered in Whirl-Pak (Nasco, Atkinson, AR) bags to separate liquid from unhomogenized meat. The filtrate was then serially diluted in phosphate buffered saline ( $10^{-1}$  to  $10^{-4}$ ) and spread plated on *Vibrio vulnificus* agar (VVA), thiosulfate-citrate-bile salt (TCBS), and tryptic-soy agar supplemented with 2% sodium chloride (TSAN<sub>2</sub>) plates. The VVA plates were incubated overnight at 35°C, and the TCBS and TSAN<sub>2</sub> were incubated at 37°C, respectively. The TSAN<sub>2</sub> plates were used to measure change in aerobic bacteria numbers.

All formulations mentioned in the remainder of this section can be referenced in Cook et al. (2000). Whatman #541 filter disks (Whatman International Ltd., Maidstone, England) were used to make colony lifts by saturating the filter papers with distilled water, pressing the moist paper onto VVA and TCBS plates, placing the lifts colony-side up into lysis solution, and microwaving the papers dry. The dried lifts were washed in ammonium acetate buffer and rewashed in 1X standard saline solution (SSC) at room temperature. Control strips of *V. vulnificus* (strain 1009) and *V. parahaemolyticus* (strain 33837) were made in the same fashion as the colony lifts and included in the process as a reference. Both *V. vulnificus* and *V. parahaemolyticus* strains were provided by Dr. Janet Simonson of the Department of Agricultural Chemistry, Louisiana State University in Baton Rouge, LA.

The colony lifts (5 lifts per bag plus a control strip) were placed in bags of 1X SSC and stock proteinase K solution (Sigma-Aldrich Inc., St. Louis, MO) for 30 min and incubated at 42°C with shaking. Three subsequent 1X SSC washes were performed to remove excess proteinase K. The colony lifts were then incubated at 54°C for 30 minutes in hybridization buffer. Next, the colony lifts were exposed to fresh hybridization buffer supplemented with alkaline phosphatase conjugated 5' amine-C6 DNA probes. VVA colony lifts received *V.*

*vulnificus* cytolysin (vvh) probe and TCBS lifts received thermostable labile hemolysin (tlh) probe (DNA Technology A/S, Denmark). The colony lifts, hybridization buffer, and probes were incubated and shaken for 1 hour at 54°C. Excess probe and buffer were removed by two 1X SSC/Sodium Dodecyl Sulfate washings at 54°C. Finally, all colony lifts were washed five times with 1X SSC at room temperature and placed in 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt and Nitro-Blue Tetrazolium Chloride (NBT/BCIP) (Roche Applied Science, Germany) solution for color development.

Prior to enumeration, the colony lifts were rinsed in distilled water three times and allowed to dry. Enumeration was performed by visual counting of positive colonies. Positive colonies tended to be dark purple and well-rounded; however, the control strips were used as a reference in case of ambiguous coloration.

**Condition Index.** An index was calculated to determine the relative body condition of the oysters from beginning to end of the experiment. Five oysters from each replicate coop were shucked into individual aluminum pans, and the meat and shells were dried separately for 48 hours at 65°C as outlined by Lucas and Beninger (1985). Condition index was determined by:

$$(\text{Dry tissue weight} / \text{Dry shell weight}) * 100$$

Unlike Lucas and Beninger (1985), the dry tissue/dry shell value was multiplied by 100 not divided by 100 to produce an easier number to report. Condition index was measured only on the first samples, but would have been done on the last samples if the experiment had not been ended prematurely.

**Dermo Disease Detection.** *Perkinsus marinus* (Dermo) infection has been shown to result in poor body condition and survival and was measured as an explanatory cause to potential mortality. *Perkinsus marinus* was detected by utilizing a protocol developed by Ray (1952).

Five individual oyster rectums from each coop were excised and incubated in Rays's Fluid Thioglycollate Media (RFTM) in the dark at room temperature (Bushek et al. 1994). Chloramphenicol and nystatin (Sigma-Aldrich Inc., St. Louis, MO) were added to the RFTM to prevent bacterial and fungal growth, respectively. After 1 week of incubation, the rectums were placed on individual slides, macerated, and mixed with Lugol's Iodine to stain the *P. marinus* hypnospores (functional prezoosporangia). Disease prevalence and intensity was determined by counting the stained (black in color) hypnospores under a compound microscope at 40x and 100x magnification. A modified Mackin's (1962) scale was used to rank infection intensity. No hypnospores in the tissue received a score of 0 while a very heavy infection was given a score of 5. Testing was performed only on the first samples, but would have been done on the last samples if the experiment had not been ended prematurely.

**Statistical Analyses.** Bacterial numbers were converted to  $\log_{10}$  values for statistical analysis. A 2x4 factorial analysis with a Tukey-Kramer adjustment (Proc Mixed) was utilized to detect significant differences in *V. vulnificus* and *V. parahaemolyticus* counts between "ST", "T", "M", and "B" replicates and days 0 and 7. A one-way ANOVA with Tukey-Kramer adjustments (Proc Mixed) was used to compare condition index and Dermo levels between replicates. All analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

## Results

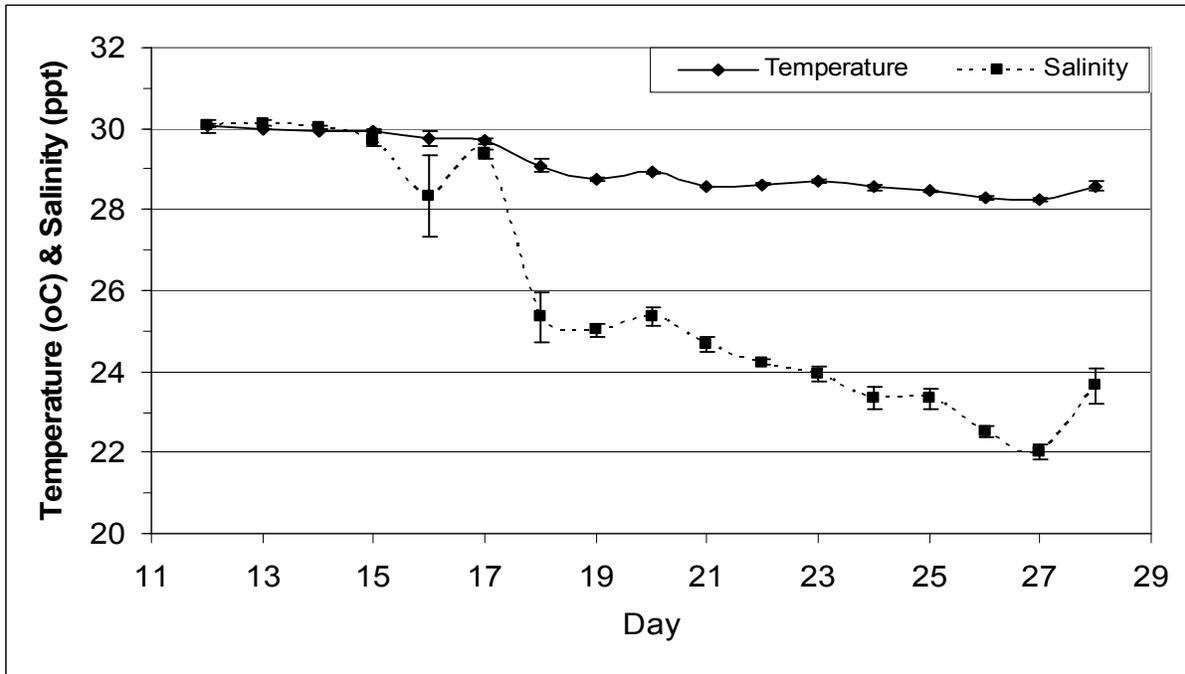
Due to equipment malfunction and safety issues, the experiment had to be terminated after the first week; however, some preliminary bacteria, condition index, and Dermo data was gathered.

**Sampling.** The skies were clear and sunny and the wind was blowing approximately 8-16 km/h (5-10 mph) from the south while harvesting oysters from Hackberry Bay, LA with

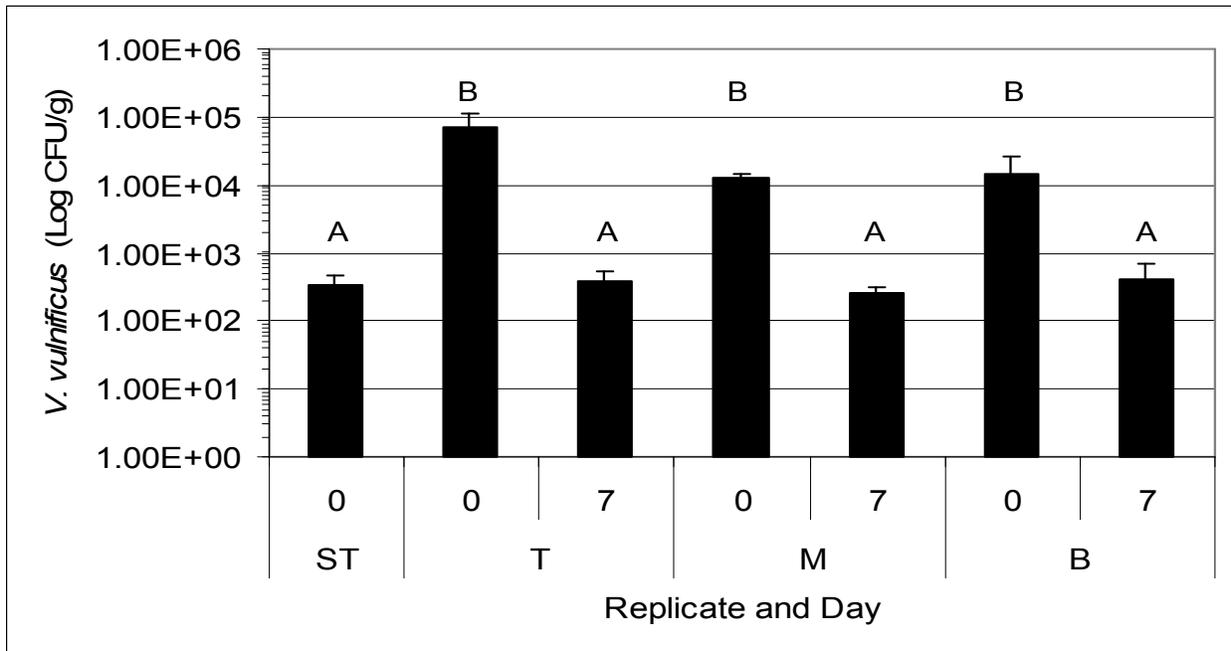
ambient water temperature and salinity of 28°C and 15 ppt, respectively. The oysters for the experiment remained at ambient air temperature for approximately 26 hours until they were deployed at the test site. However, bacterial testing did not commence until 28 hours after harvesting. For the initially iced oysters (ST), this was an acceptable timeframe as outlined by Cook et al. (2000). The DO in the water strata where the oyster rack was placed at the time of deployment was 1.98 mg/L. Temperature was steady between 28 and 30 °C, and salinities ranged from 25 to 30 ppt during the first and only sampling period (Figure 3.2).

***Vibrio* DNA Probe Results.** Although the experiment was terminated before *V. vulnificus* and *V. parahaemolyticus* numbers reached non-detectable levels, the results from days 0 and 7 indicate that moving oysters from medium salinities (15 ppt) to higher salinities (30 ppt) can cause a significant reduction in both bacteria counts (Figures 3.3 & 3.4). The “T”, “M”, and “B” replicates for both *V. vulnificus* and *V. parahaemolyticus* at day 0 were significantly higher ( $p < 0.05$ ) than the “ST” samples which were iced immediately after harvesting. After a week at the experiment site, the “T”, “M”, and “B” counts for both *V. vulnificus* and *V. parahaemolyticus* were not significantly different from the “ST” samples. One week of high salinity exposure was not enough to reduce either species to non-detectable numbers.

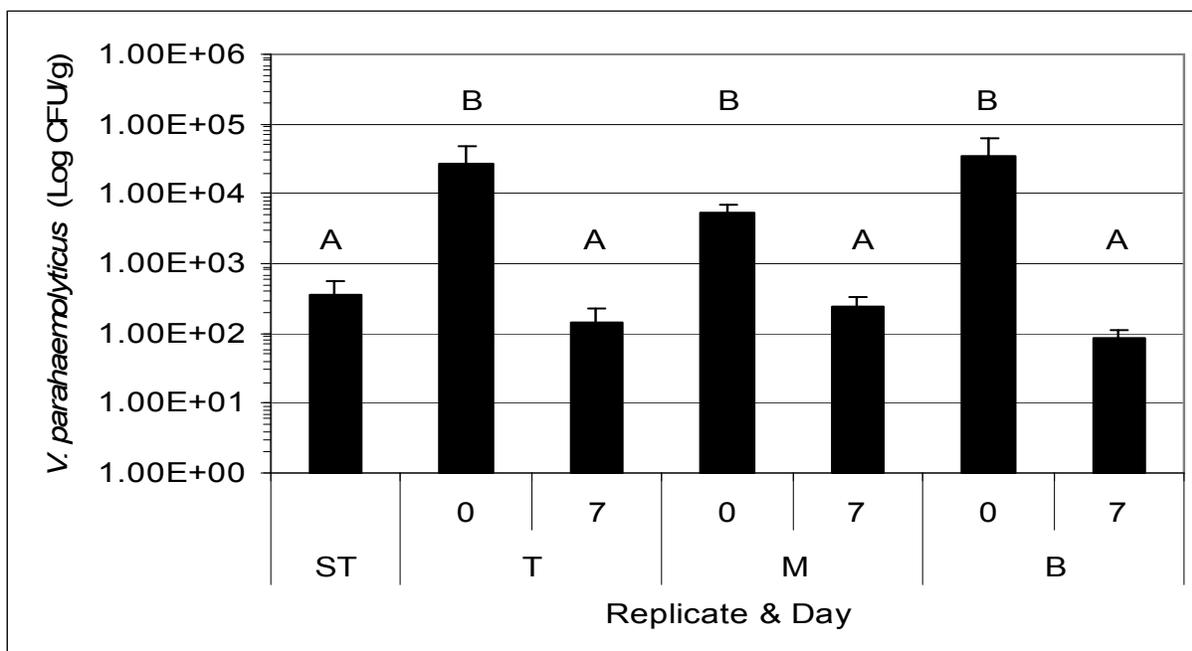
**Condition Index and Dermo.** The mean condition index (CI) and Dermo values for the day 0 samples ranged from 0.976-1.153 and 0.9-1.8, respectively (Figures 3.5 & 3.6). However, there were no significant differences between replicates for either parameter. There was 100% Dermo prevalence in the “ST”, “T”, and “M” samples; however, the “B” samples had 80% prevalence. The “T” replicate had one oyster with a high Dermo infection intensity (3), which explains the higher weighted incidence than the other replicates. Although the project was not



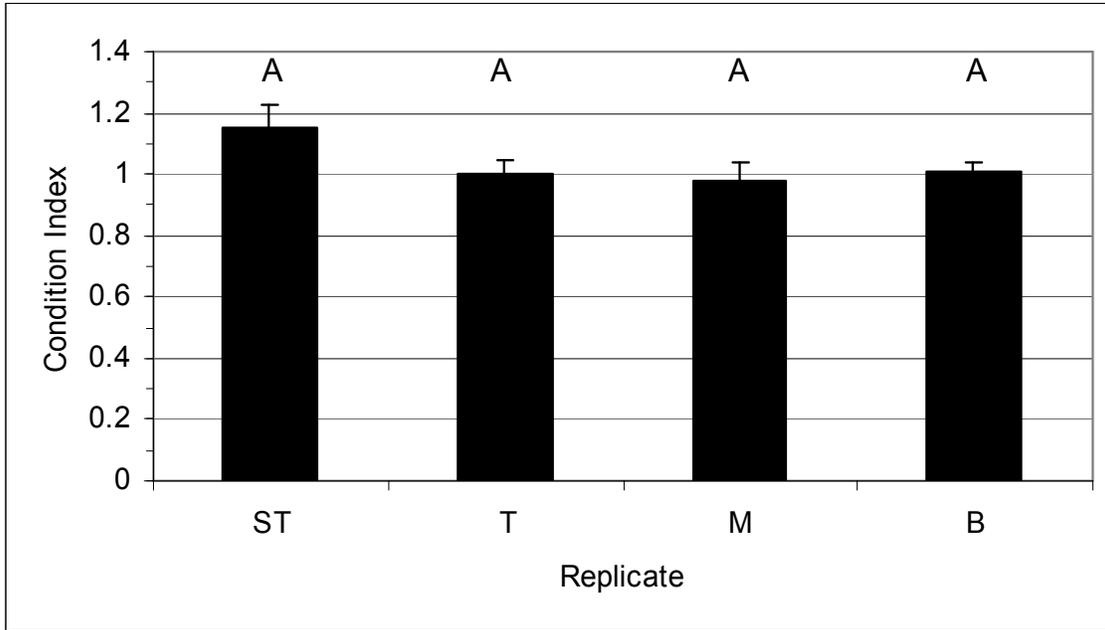
**Figure 3.2** Mean (n=4) temperature (°C) and salinity (ppt) with standard error bars data from September 12-28, 2007. The temperature showed little fluctuation; however the salinity decreased below the desired 30 ppt after Sep-15-2007.



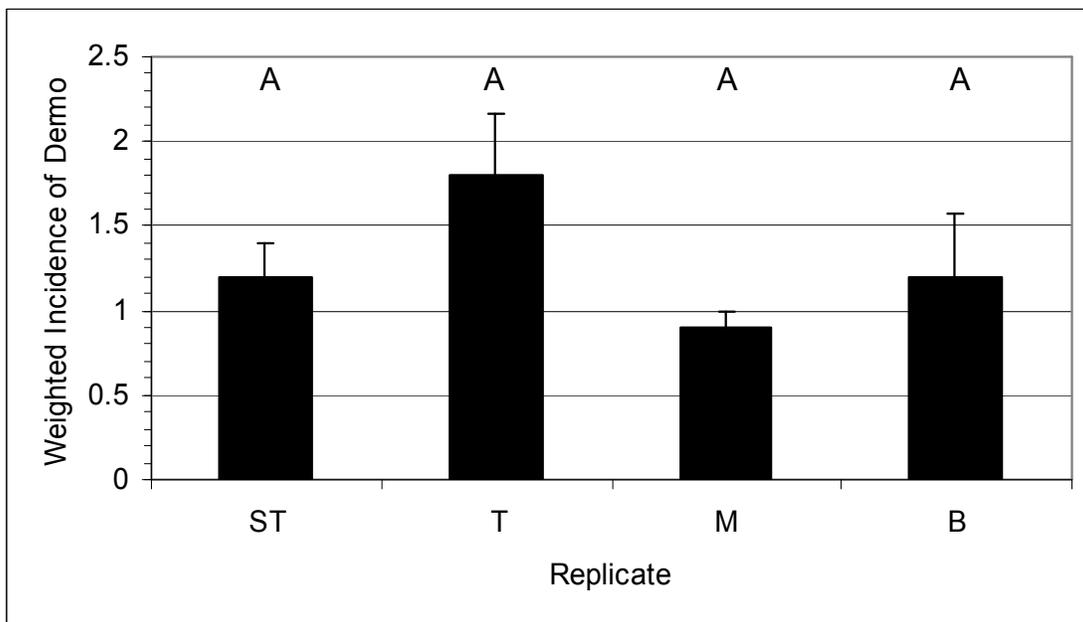
**Figure 3.3** Mean (n=3) with standard error bar day 0 and day 7 *V. vulnificus* counts in September 2007 for oyster sub-samples taken immediately at harvest (ST) and for top (T), middle (M), and bottom (B) replicates. Day 7 top (T), middle (M), and bottom (B) replicates were not significantly different from the initial (ST) samples; however, the day 0 oysters stored at ambient air temperature for over 24 hours were significantly higher than ST or day 7 *V. vulnificus* numbers.



**Figure 3.4** Mean (n=3) with standard error bar day 0 and day 7 *V. parahaemolyticus* counts in September 2007 for oyster sub-samples taken immediately at harvest (ST) and for top (T), middle (M), and bottom (B) replicates. Values are means (n=3) with standard error bars ( $\log_{10}$  CFU/g). Day 7 top (T), middle (M), and bottom (B) replicates were not significantly different from the initial (ST) samples; however, the day 0 oysters stored at ambient air temperature for over 24 hours were significantly higher than ST or day 7 *V. parahaemolyticus* numbers.



**Figure 3.5** Mean (n=5) and standard error condition index values for day 0 oysters at harvest (ST) and top (T), middle (M), and bottom (B) replicates in September 2007. No replicates were significantly different.



**Figure 3.6** Mean (n=5) and standard error weighted incidence of Dermo for day 0 oysters at harvest (ST) and top (T), middle (M), and bottom (B) replicates in September 2007. No replicates were significantly different.

completed as planned, the oysters collected on day 7 appeared to contain less glycogen than the oysters from day 0 which indicates a possible decrease in CI.

### Discussion

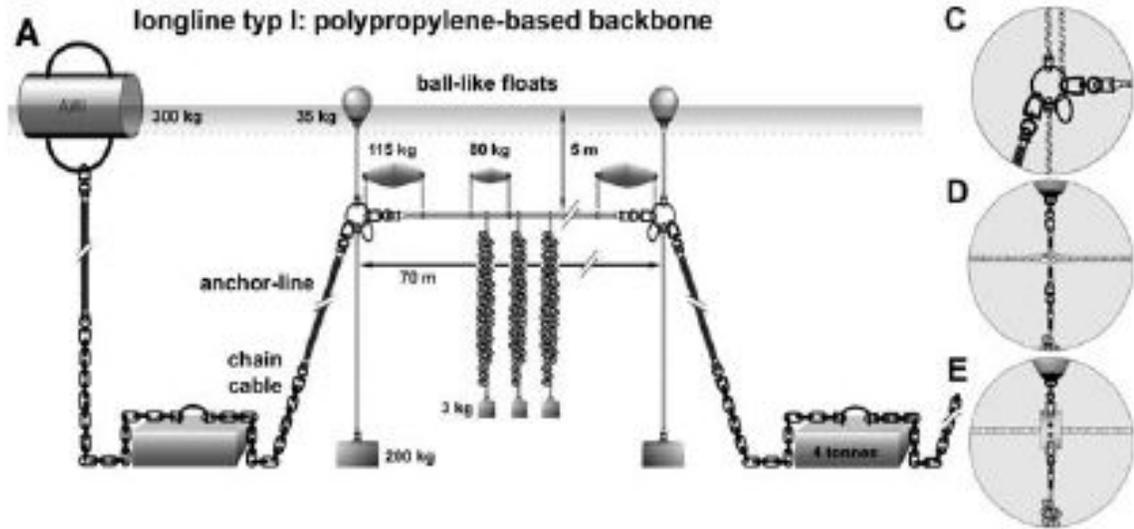
Despite promising preliminary findings with *V. vulnificus* and *V. parahaemolyticus* reduction, the rough seas and broken equipment prevented the collection of any conclusive data. The significant decrease in both *V. vulnificus* and *V. parahaemolyticus* is in agreement with Motes and DePaola (1996); however, they found that 7-17 days were needed for *V. vulnificus* to reach non-detectable levels. With over a log unit<sub>10</sub> decrease in 7 days for all replicates, it is possible that *V. vulnificus* and *V. parahaemolyticus* would have reached non-detectable counts in a similar time frame. Kaspar and Tamplin (1993) found a 50 to 88% decrease in *V. vulnificus* when exposed to salinities above 30 ppt; however, they also reported that *V. vulnificus* grows well in waters up to 25 ppt. This is a concern because the salinities declined to less than 30 ppt during the first week and what would have been subsequent sampling periods. It is possible that with these salinity decreases, the decline in *V. vulnificus* and *V. parahaemolyticus* may have stopped or the bacteria may have even increased. Other studies have found that both *V. vulnificus* and *V. parahaemolyticus* exhibit a negative correlation between growth and increasing salinities (Oliver et al. 1982; Lin et al. 2003). Despite water temperature being the main factor in both *V. vulnificus* and *V. parahaemolyticus* growth, the constant water temperature during the experiment would have caused salinity to be the limiting factor in growth (Kelly & Stroh 1988). The data also supports previous studies of the drastic increase of *V. vulnificus* and *V. parahaemolyticus* when exposed to ambient air temperature for extended periods of time (Cook 1997; Cook et al. 2002).

Condition index and Dermo were measured as potential factors in mortality. Since the experiment was not completed, it can not be stated if oyster condition or disease prevalence could have been factors in mortality. Dermo was measured because it is a disease that is positively correlated with increasing salinities (Hoffman et al. 1995; Calvo et al. 2001). Ragone and Burreson (1993) noted that there is high oyster mortality from Dermo when oysters are moved from low salinities to high salinities. When compared to June 2006 numbers of Dermo prevalence and intensity in the same harvest area, the incidence in this project was comparable but slightly higher for market-sized oysters; however, the prevalence and intensities were much lower in June 2007 (LDWF 2007). It is worth noting that the LDWF (2007) report utilized 30 oysters as opposed to the five per replicate used in this study. Dissolved oxygen was also a concern, but the Kemmerer 1.2 L water sampler was broken during day 7, so DO was not measured and no conclusion can be surmised on its potential effect on mortality.

In the future, it is highly recommended that a much larger boat and stronger winches be utilized. The RV Percy Viosca was not appropriate for retrieving the oysters on rough seas. One miscalculation was that the rack plus oysters would weigh approximately 385 kg (850 lbs). However, that figure was an estimate and the oysters may have contributed more weight plus the suction from mud was not taken into consideration. A combination of rough seas and unaccounted weight resulted in the breaking of two winches. The rack was tipped onto its side during one failed retrieval attempt, and it was nearly 2 weeks after that before the oysters were recovered. Once the rack was on board the boat, it was noted that oysters were buried in sediment and suffered almost 100% mortality. The sedimentation compromised any results that may have been found in the remaining oysters.

Overall, high salinity exposure showed promising signs of *V. vulnificus* and *V. parahaemolyticus* reduction; however, the logistics of this experiment were not appropriate for a more permanent solution to the process. A vessel such as a jack-up barge, commonly used in off-shore oil and gas activities, would be better suited for retrieving oysters from the water bottom, or the oysters could be suspended from a structure such as an artificial reef or oil platform. Nonetheless, if high salinity exposure were to be pursued as a post-harvest treatment, the oysters would need to be easily transported and accessed, or else the cost of the process may overshadow the value of the oysters.

The goal of this project was to utilize high salinity exposure as a method for reducing *V. vulnificus* and *V. parahaemolyticus* to non-detectable numbers, which would result in a safer product for raw oyster consumers. Theoretically, oyster harvesters could gather oysters, place them in high salinity waters for the necessary depuration period, and sell a harvest free of *V. vulnificus* and *V. parahaemolyticus*. Should future studies show that high salinity exposure result in non-detectable *Vibrio* numbers, a sunken longline system utilized in other countries as reviewed by Quayle and Newkirk (1989) and Menzel (1991) could be the solution because high salinity waters (> 30 ppt) in LA are typically found offshore. Longline systems similar to the one utilized in Australia and Grand Isle, LA could be ideal for such an endeavor. Maxwell (2007) reviewed the initial costs for construction and materials of a 0.4 hectare inshore longline system and estimated the cost to be approximately \$41,000. However, the longline system proposed in this work would only require the purchase of bags, clips, end caps, cable, and shipping along with extra cable, anchors, and buoys for mooring and location purposes (Figure 3.7). Instead of riser posts to support the bags, float balls suitable for immersion could be utilized to offset the sinking weight of the oysters.



**Figure 3.7** Diagram of a proposed underwater offshore longline system for remediation of *V. vulnificus* and *V. parahaemolyticus* in oysters. Diagram is reprinted from Buck (2007).

**Table 3.1** Cost for construction of a 100 m underwater offshore longline system. Values are based on those presented by Maxwell (2007) and Rotonics Manufacturing, Inc (Rotonics, 2005). The total value does not take into account anchoring material, marking buoys, or deployment and retrieval costs.

Item	Number	Cost	Total
Bags	3	273.17	819.51
Bag clips	198	0.42	83.16
End caps	198	1.42	281.16
Cable (100 m)	1	510.00	510.00
Floats	31	89.00	2760.00
Shipping			3000.00
<b>Total</b>			<b>7453.83</b>

Using values provided by Maxwell (2007) and a price list for float balls (Rotonics, 2005), it is estimated that one 100 m offshore longline would cost approximately \$7500 (Table 3.1). This value does not factor in the cost of large marking/mooring buoys, deployment (fuel and labor), or anchoring material because these items can be variable in price depending on availability and size. The longline system could be constructed on shore, transported by boat to the appropriate high salinity site, and deployed by either a diver attaching the cable with oysters and floats to a spliced loop in a permanent anchor line or the anchoring cable with attached oysters and floats to could be deployed simultaneously. A strong boat and winch set-up would be necessary for retrieval of the entire system or a diver could release the oysters and floats from the anchor line via the aforementioned spliced loop and allow the line to float to the surface.

A study by Buck (2007) showed that a flexible polypropylene longline could be utilized for offshore mussel culture; however, the study revealed that predation, fouling, and boat traffic are great concerns when a longline structure is left offshore for an extended time of six months or more. The proposed usage of an offshore oyster depuration longline as previously described would greatly reduce predation and fouling because the oysters would be placed in enclosed bags rather than on strings open to the water (typical to mussel culture) and would only be deployed for the amount of time necessary to reduce *V. vulnificus* and *V. parahaemolyticus* to non-detectable numbers. Also, zoning areas for offshore mariculture would be necessary prior to placement of the longlines to negate user conflict.

Despite the potential for high salinity exposure and an offshore underwater longline, little data is published on either aspect; therefore, it is highly recommended that more research be performed before high salinity offshore longlines are approved as a feasible *Vibrio* reduction method.

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## CHAPTER 4 SUMMARY AND CONCLUSIONS

The focus of this research was to test icing and high salinity exposure as post-harvest treatments for the reduction of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in commercial amounts of shellstock oysters. The icing treatments included on-board icing immediately after harvest, dockside icing within two hours of docking, and no icing. The on-board iced oysters were split into two additional treatments, minimal and retail handling, to measure the effect that industry shipping and handling practices may have on iced oysters. The high salinity post-harvest treatment simply consisted of harvesting oysters and placing them in a high salinity (>30 ppt) environment such as an offshore artificial reef site.

The results from the icing experiment indicate that icing does not have a significant effect on *V. vulnificus* and *V. parahaemolyticus* reduction, especially to non-detectable numbers as anticipated. Despite the variable fluctuations in bacteria numbers, the August samples showed a significant increase in both *V. vulnificus* and *V. parahaemolyticus* counts for the dockside and non-iced oysters when compared to the on-board iced oysters. This difference supports other studies which report time-temperature abuse post-harvest as the leading factor in *V. vulnificus* and *V. parahaemolyticus* increases (Hood et al. 1983; Cook 1994; Cook 1997). When harvesters sack oysters and store them on deck for the duration of the harvesting trip, the warm ambient air provides ideal temperatures for *V. vulnificus* and *V. parahaemolyticus* proliferation. These increases are what concern the industry and consumers at risk of becoming ill from raw oyster consumption. Recently, the United States Food and Drug Administration and the National Shellfish Sanitation Program (NSSP) have discussed icing as a post-harvest treatment for both *V. vulnificus* and *V. parahaemolyticus* reduction. The results of this project (discussed in Chapter 2) indicate that icing, either on-board or dockside, is not an appropriate post-harvest treatment

for *V. vulnificus* or *V. parahaemolyticus* reduction to non-detectable numbers. At best, icing may be used as a method of preventing *V. vulnificus* and *V. parahaemolyticus* counts from drastically increasing while the harvest remains on deck. However, the results also show that oyster gaping is significantly increased when ice is used as a post-harvest treatment. The excess gaping leads to economic loss as well as potential contamination from oyster drip (Kaysner et al. 1989). Other icing and refrigeration studies have also demonstrated that icing alone is not a viable means of reducing *V. vulnificus* and *V. parahaemolyticus* levels to non-detectable (Ruple & Cook 1992; Quevado et al. 2005). It is hypothesized that the temperature shock from the ice may be the reason for the increased gaping; yet, gaping percentages plateaued from day 7 to day 14 which indicates icing only had a significant effect on gaping during the first week. Although icing alone was shown not to be an effective treatment for *V. vulnificus* and *V. parahaemolyticus* reduction, it may have potential as a secondary treatment or in conjunction with other treatments, but further research outside the scope of this project is needed.

The high salinity exposure treatment showed excellent preliminary results (discussed in Chapter 3). After one week of high salinity exposure, both *V. vulnificus* and *V. parahaemolyticus* counts were reduced to the numbers comparable to initial levels when first harvested. These results are consistent with other studies that demonstrate higher salinities (>30 ppt) as having a negative effect on *V. vulnificus* and *V. parahaemolyticus* densities (Kelly & Stroh 1988; Kaspar & Tamplin 1993; Motes & DePaola 1996). As promising as the results were, the logistics of the project did not support its completion. Multiple equipment malfunctions and the oyster rack being tipped and buried in mud compromised the experiment and further results. The weight of the rack and oysters was too much for the winches on the boat and the project was

abandoned. Further trials outside of this thesis work will be conducted in the summer of 2008 to ascertain the effectiveness of high salinity exposure as a post-harvest treatment.

Despite the promising results, there are two concerns with the high salinity treatment. The condition index (CI) of the oysters was not measured on day 7, but the oysters appeared to contain less glycogen than when the project began. At the depth the oysters were placed, food availability may have been limited which would then limit glycogen stores, which are necessary to remain healthy, fight diseases, and for consumer appeal. If the high salinity treatment results in a significant loss of condition, then the product may not be as marketable as freshly harvested oysters. Secondly, *Perkinsus marinus* (Dermo) is a protist that was present in the sampled oysters, thrives at higher salinities, and can be easily spread between oysters in close proximity. Like CI, Dermo was not tested on day 7 so it cannot be stated if the disease was a factor in mortality, but it is likely that if Dermo intensified in the oysters then there could be increased mortality. The presence of Dermo and an apparent weakening over time could result in high product loss which would make high salinity exposure an economically unviable post-harvest treatment. Should high salinity exposure be shown to be effective in future studies, an offshore longline as discussed in Chapter 3 could be an ideal method for *Vibrio* reduction in Louisiana because most high salinity waters are found offshore.

Public safety and consumer protection are valid and appropriate concerns when it comes to raw oyster consumption and *V. vulnificus* and *V. parahaemolyticus* remediation. However, this research showed that icing is not an effective post-harvest treatment, and high salinity exposure has potential to reduce the bacteria to non-detectable numbers if the proper equipment is utilized. There is little research on either treatment on a commercial scale, so further research on both treatments is recommended.

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**APPENDIX A**

**2006 JUNE AND AUGUST TEMPERATURE DATA FROM ACR SMARTBUTTON DATA LOGGERS**

The presented data is for the minimum temperatures reached prior to the oysters being loaded onto the refrigerated truck, and the times to reach the minimum temperature, 10°C, and 7.2°C. Treatments included “on-board iced, wholesale storage” (OBW), “on-board iced, retail storage” (OBR), and “dockside iced” (DS). The “non-iced” oysters received no icing treatment and remained at ambient air temperatures.

The data for each replicate of each treatment is displayed.

Treatment	Replicate	Minimum Temp (°C)	Time to Reach (min):		
			Minimum Temp	10°C	7.2°C
June:					
OBW	1	2.32	355	186	256
	2	0.32	483	123	169
	3	0.83	430	86	123
OBR	1	0.37	538	86	146
	2	0.50	532	76	96
	3	1.98	538	197	270
DS	1	12.50	112	-	-
	2	8.92	111	102	-
	3	9.35	77	72	-
August:					
OBW	1	3.92	544	274	358
	2	1.44	440	103	148
	3	4.45	384	217	291
OBR	1	2.49	395	152	202
	2	2.99	384	137	191
	3	2.49	373	131	182
DS	1	12.36	88	-	-
	2	7.49	100	82	-
	3	11.92	98	-	-

**APPENDIX B**  
**PERMISSION FROM JOURNAL OF FOOD PROTECTION TO USE CHAPTER 2 IN THESIS**  
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## VITA

Kevin Patrick Melody was born in Cumberland, Maryland. His birth in Maryland was a cruel twist of fate for he is really a son of the Mountain State and was properly raised in Keyser, West Virginia. He graduated as co-valedictorian from Keyser High School in 2001 and immediately pursued a Bachelor of Science in biology from Wheeling Jesuit University in Wheeling, West Virginia. He graduated in the top ten of his class in the spring of 2005. During his tenure at Wheeling Jesuit University, he was active in many school organizations and even spent a summer at Texas A&M Corpus Christi as part of a National Science Foundation Summer Undergraduate Research Fellowship.

After graduating from Wheeling Jesuit University, he accepted a Graduate Research Assistantship with Dr. John Supan at Louisiana State University. His research focused on remediation of *Vibrio vulnificus* and *Vibrio parahaemolyticus* bacteria from shellstock oysters. On the other hand, his assistantship provided him with the opportunity to rebuild a hurricane shattered oyster hatchery. He is a member of the Louisiana Chapter of the American Fisheries Society and World Aquaculture Society, as well as a two-year Executive Board Member of Louisiana State University's Aquaculture and Fisheries Club. Post-graduation plans include a wedding with his long-time girlfriend, Lannette Booth, in the summer of 2008 and a hopeful career in the wide and wacky world of science.