2016

Rapid Cooling of Eastern Oysters (Crassostrea virginica) Using an On-Board Icing Unit to Slow the Growth of Vibrio vulnificus and Vibrio parahaemolyticus

Melody Amber Thomas
Louisiana State University and Agricultural and Mechanical College

RAPID COOLING OF EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) USING AN ON-BOARD ICING UNIT TO SLOW THE GROWTH OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS*

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Biological and Agricultural Engineering

by
Melody Amber Thomas
B.S., Louisiana State University, 2014
May 2016

ACKNOWLEDGEMENTS

I would like to thank my family and friends for their support throughout this project, including my parents who never gave up on me. I am grateful for the knowledge and guidance provided by my graduate committee; Dr. Steven Hall, Dr. David Constant, and Dr. John Supan. I would also like to thank the team at Dauphin Island particularly Dr. Jessica Jones for their help with the oysters and bacteria analysis. In addition, appreciation is also extended to Mr. Steve Bassett for his help with harvesting the oysters used in testing. I would also like to thank everyone in the Biological and Agricultural Department for their help with everything along the way, and Mr. Tom McClure who was always ready for any challenge I presented to him. Many people were instrumental in helping me see this project through to the end.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF TABLES v

LIST OF FIGURES vi

ABSTRACT viii

CHAPTER 1 : INTRODUCTION 1
- Literature Review 3
  - Oysters 3
  - Bacteria 4
  - Polymerase Chain Reaction (PCR) 7
  - On-board Icing 7
- Goals 7

CHAPTER 2 : ANALYSIS OF ON-BOARD CHILLING OF EASTERN OYSTERS 9
- Introduction 9
- Materials 9
  - Chilling and Washing Unit Design 9
- Methods 12
  - Temperature Tests 12
  - Harvest Oysters 14
  - Samples and Counts 15
  - Enriching Samples 15
  - Preparing for Vibrio Tests 17
  - Real-Time PCR Analysis 18
  - Streaking for Vibrio 20
  - Picking Colonies 21
  - Replicate Plating 22
  - Vibrio verification 23
- Results 23
- Discussion 29
- Conclusion and Future Work 29

CHAPTER 3 : THERMODYNAMIC PROPERTIES OF EASTERN OYSTERS 31
- Introduction 31
- Materials and Methods 31
- Results 33
- Discussion 34
- Conclusion and Future Work 35

CHAPTER 4 : CONCLUSION AND FUTURE WORK 36
Project Summary 36
Future Work 37

REFERENCES 38

APPENDIX A PC208W LOG PROGRAM 45

APPENDIX B SAS PROGRAM 47

VITA 49

LIST OF TABLES

Table 1 – All primers and probes used for *Vibrio* PCR. 18

Table 2 The pathogenic *Vibrio parahaemolyticus* results for the three trials run in May, July, and September. 26

LIST OF FIGURES

Figure 1 Anatomy of the eastern oyster (from Wheaton 2007) 3

Figure 2 Typical meat conditions of triploid (left) and diploid (right) oysters during spawning months. (Photo: J. Supan) 4

Figure 3 (a) The growth of *Vibrio parahaemolyticus* on TCBS and (b) *Vibrio vulnificus* on mCPC. 6

Figure 4 (a) Standard oyster washer typically used in Louisiana for on-shore shellfish washing. (b) The prototype on-board chilling unit. 10

Figure 5 Improvements to the lid. 11

Figure 6 Schematic of the lid closed and open used for calculations. 12

Figure 7 (a) A T-type thermocouple inserted into a live oyster and sealed with modeling clay. (b) Thermocouples secured with duct tape to ensure contact with the shell. (c) Smart Button attached to the outside of a live oyster using duct tape. 13

Figure 8 Google Earth images of oyster farm location. 15

Figure 9 Preparation of the MPN tubes. 16

Figure 10 (a) APW and PBS tubes before inoculation. (b) Opaque tube on the left indicates a positive APW test compared to a transparent negative tube. 17

Figure 11 (a) The results from PCR. (b) Table was used to estimate the *Vibrio* levels in each sample. 20

Figure 12 Streaked plate SSR4 -3C. S_1: September, S_2: Iced, R4: Refrigerated sample 4, -3C: 10^{-3} Dilution's 3rd tube (C). 21

Figure 13 Reference chart for the 96 well templates. 22

Figure 14 Completed *Vibrio* probes. 23

Figure 15 Total *Vibrio parahaemolyticus* throughout the three testing periods. 24

Figure 16 Total *Vibrio parahaemolyticus* for the months of May, July, and September. 24

Figure 17 Total *Vibrio vulnificus* results for the three trials run in May, July, and September 2015 25

Figure 18 Total *Vibrio vulnificus* for May, July, and September. 25

Figure 19 Oyster mortality in May 27

Figure 20 Oyster mortality in July 28
Figure 21 Oyster mortality in September 28
Figure 22 Center temperature history for a sphere (from Welty et al. 1976) 32
Figure 23 Chilling rate of oysters in static water 33
Figure 24 Chilling rate of oysters in circulating water 34

ABSTRACT

Oyster harvesting is a major industry along the Eastern and Gulf coasts of the United States. Eastern oysters (*Crassostrea virginica*) spawn when the waters start to warm between May and October in the Gulf of Mexico. The warmer temperature creates a problem with an increase of bacteria. The two major bacteria affecting the oyster industry are *Vibrio vulnificus* and *Vibrio parahaemolyticus*. These bacteria are the leading causes of seafood-borne illnesses in the United States. This increase of *V. vulnificus* and *V. parahaemolyticus* has led to stricter time/temperature requirements for harvesting oysters. The regulations are not plausible for smaller vessels too small to hold a refrigeration unit. Chilling the oysters to lower *Vibrio* levels using an on-board icing unit was proposed for further investigation. The oysters were tested for *V. vulnificus* and *V. parahaemolyticus* at each stage along with tested for gaping after 7 and 14 days in refrigerated storage. This experiment was conducted in May, July, and September 2015. The results will help advance the use of triploid oysters as viable options for the oyster industry during the spawning of diploid oysters.

The total *Vibrio parahaemolyticus* and *Vibrio vulnificus* results were $\log_{10}$ transformed to compare the treated and controlled data for all three months. The data was run through a mixed procedure in SAS 9.4. The *V. parahaemolyticus* showed a significant difference in the sampling months ($p<.001$) but not a difference in the treatments ($p=0.4715$). The differences in the *Vibrio vulnificus* sampling months were significant ($p<0.001$) but not the treatments ($p=0.2617$). Pathogenic *V. parahaemolyticus* was not flourishing in this area. A difference could not be found in the months or treatment because of the low amounts of bacteria. The gaping data was significantly different between the iced and controlled oyster sacks ($p<.001$) along with the sampling months ($p=.0014$). Further work is needed to improve this design and process.

CHAPTER 1 : INTRODUCTION

Oyster harvesting is a large business exceeding $100 million per year along the coastal regions, with extensive (on-bottom) and intensive oyster culture (off-bottom) continuously growing along the Eastern and Gulf coasts (ECSGA 2014; LODGA 2014). The eastern oyster (*Crassostrea virginica*) is a bivalve mollusk found in marine and estuarine environments along the east coast of Canada and the United States and into the Gulf of Mexico (Byrum 2014; FAO 2013; Carriker and Gaffney 1996). The eastern oyster has the ability to survive a variety of water conditions allowing the oysters to span the different environments along the Atlantic coast. The oysters can endure salinity ranges from 5 to 35 ppt, dissolved oxygen levels 2 to 3 ppm and temperature ranges from 0°C to 35°C with optimal conditions at 10 to 30 ppt, dissolved oxygen levels above 4 ppm and temperature ranges from 20°C to 30°C (Berrigan et al. 1991). A prolongation of conditions outside these ranges may cause mortality.

Spawning occurs yearly during the summer months of May-October in the Northern Hemisphere as water temperatures rise. The spawning puts a halt to public oyster harvesting to increase the spat survival rates. The National Oceanic and Atmospheric Administration (NOAA) funded this project in coordination with a multi-year research program funded by Sea Grant to develop the commercialization of triploid oysters in the region. The triploid oysters were created to capture the market during the spawning months since they have a highly reduced chance of spawning, therefore retaining the glycogen stores and continuing to grow. Triploid oysters can take over the market during the summer months to give the eastern oyster spat a better chance at survival. Gametes are produced using the glycogen stores of diploid oysters, while triploid oysters do not reproduce so the glycogen stores are retained and can continue to grow (Young 2010). A reliable method for producing triploid oysters is by spawning a tetraploid male with a

diploid female (Guo et al. 1996). Advantages of these oysters go beyond the faster growth rate due to sterilization; they also have a greater survival rate and better meat condition.

The triploid oysters can be grown using an intensive culturing method known as off-bottom cage culture to reduce predator related mortality, improve growth, and help control bio-fouling (Leonhardt 2013). This culture allows for an easier harvest of the oysters.

The warm waters during the spawning months bring along a new problem. The high temperatures in the warm months from May through October allow bacteria to flourish including *Vibrio vulnificus* and *Vibrio parahaemolyticus* (Rippley 1994; Hlady 1997; Cook et al. 2002). *Vibrio vulnificus* and *Vibrio parahaemolyticus* are naturally occurring rod-shaped, halophilic, gram-negative, motile bacteria (Oliver 2005). The bacteria accumulate in the oysters through their main source of food, plankton, which is consumed by filtering water through the gills (Chowdhury et al. 1990; Groubert and Oliver 1994; Kaneko and Colwell 1973; Kelly and Dinuzzo 1985). Regulations for time/temperature handling of oysters become more stringent during summer harvest to keep the growth of bacteria low and prevent health issues. The current regulation requires mandatory refrigeration within 1 hour of harvest for shellstock, which allows the internal meat temperature to reach 12ºC within 6 hours. For testing, the oysters were removed from the floating cages, separated into 100-count mesh sacks, placed in the on-board chilling unit until they reached 10ºC, kept on ice, and stored in a refrigeration unit once back on shore. Samples were taken at each transition point for lab testing. The samples were tested using polymerase chain reaction (PCR), which magnified the DNA strands to test for vibrio.

The data from the proposed method for on-board icing can encourage a change to the regulations by increasing the allowed time between harvest and mechanical cooling of oysters to allow smaller vessels to continue harvesting during warmer periods.

Literature Review

Oysters

The eastern oyster, *Crassostrea virginica,* is a bivalve mollusk found along the coast in the Gulf of Mexico and along the Atlantic coast of North America. Oysters feed by capturing particles in the gills as seen in Figure 1 and transporting the matter to the palps, mouth, and stomach (Ward et al. 1994). The growth of the oyster shell is from the inside edge of the shell by continuously adding new shell material (Kennedy et al. 1996). The water temperature can greatly affect the growth rate of oysters with the normal time an oyster reaching market size (75mm, 3 inches) taking 2 to 5 years, warmer water conditions can achieve this in 9 months (Menzel 1951; Shumway 1996).

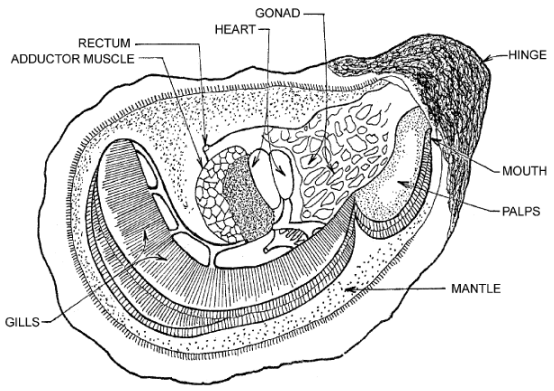


Figure 1 Anatomy of the eastern oyster (from Wheaton 2007)

The eastern oyster is a hermaphroditic organism with the ability to change sexes depending on the need (Thompson et al. 1996). The first year, oysters are usually male and can change depending on several environmental factors including water temperature and male to female ratio (Eble et al. 1996). The water temperatures also affect the spawning of the eastern oysters. Yearly, spawning occurs from May through October as the water temperatures rise in the Gulf of Mexico (EOBRT 2007). This release of the gametes decreases the meat yield for the

oyster market. The triploid oyster can capture the market during this time since the majority of triploids are sterile and retain their glycogen stores allowing them to grow faster than diploids, the difference can be seen in Figure 2.

Figure 2 Typical meat conditions of triploid (left) and diploid (right) oysters during spawning months. (Photo: J. Supan)

Triploid oysters are bred by spawning a tetraploid male with a diploid female (Guo et al. 1996). Tetraploids may be produced by spawning a triploid female with a diploid male then using cytochalasin B to inhibit polar body 1 (Guo et al. 1996; Young 2010). Triploid oysters were grown in off-bottom cages to better control the growth, health, and shape of the oysters. The better meat conditions make them optimal for summer harvest, however, the bacteria increase is a problem with warm water temperatures.

Bacteria

Harvesting during the summer months creates a public health concern because of the higher levels of vibrio bacteria present in the warmer water temperatures. As filter feeders, oysters can accumulate microbes present in the surrounding waters (Dombroski et al., 1999). The leading causes of seafood-borne illness in the United States are *Vibrio vulnificus* and *Vibrio parahaemolyticus* most commonly from the consumption of raw shellfish (Cook 1991; Drake et

al., 2007; Larsen 2012; Mead et al., 1999; Oliver et al., 1995). *V. vulnificus* and *V. parahaemolyticus* can cause primary septicemia which can be fatal to consumers with underlying medical conditions, and both bacteria can cause gastroenteritis (Daniels et al., 2000; Shapiro et al., 1998). Water temperatures have a direct relationship to the frequency of illnesses from *V. vulnificus* and *V. parahaemolyticus.* Therefore when the water temperature is high (>15°C) in the summer months the number of illnesses also increases (Duan and Su, 2005; Gooch et al., 2002; Kaneko and Colwell 1975; Kinsey et al., 2015; Murphy and Oliver, 1992; Newton et al. 2012). The frequency of infections of *V. parahaemolyticus* and *V. vulnificus* have steadily risen and for the United States it is estimated they account for 80 deaths annually along with 330 hospitalizations (Newton et al., 2012; Oliver 2012; Scallan et al., 2011).

V. parahaemolyticus is a halophilic, Gram-negative bacterium naturally found in marine environments (DePaula et al., 1990; Kaneko and Colwell 1973; Kinsey et al., 2015). Not all *V. parahaemolyticus* are pathogenic and can contribute to the carbon cycle, they are also found in plants and can help with nutrient recycling (Cole et al., 2015; Johnson 2013; Souza et al., 2011). The pathogenic *V. parahaemolyticus* is associated with around 53% of all mollusk related illnesses and accounts for almost 40% of all *Vibrio* infections. The most frequent result of infections from *V. parahaemolyticus* is gastroenteritis (CDC, 2011; Daniels et al., 2000; Iwamoto et al., 2010; Levine and Griffin, 1993; Morris and Black, 1985). Thiosulfate-citrate-bile salts-sucrose agar (TCBS) shown in Figure 3, a has been used to target *V. parahaemolyticus* since it has provided better results at isolating this bacterium than other agars (Nicholls et al. 1976; Panicker et al., 2004).

V. vulnificus is also a halophilic, Gram-negative bacterium found in marine environments (Froelich and Oliver, 2013). The most severe infections, frequently resulting in septicemia, tend

to come from *V. vulnificus* (Blake et al., 1979; Newton et al., 2012). This bacterium is one of the most deadly food borne pathogens with a mortality rate of ~35% (Mead et al., 1999; Scallan et al., 2011). *V. vulnificus* is an opportunistic pathogen and patients with liver disease or other immune system deficiencies are at a greater risk when exposed to *V. vulnificus* since it can become particularly virulent in these cases (Hlady and Klontz, 1995; Levine and Griffin, 1993). Modified cellobiose-polymyxin B-colistin (mCPC) agar shown in Figure 3, b has been used to target *V. vulnificus* (Panicker et al., 2004).

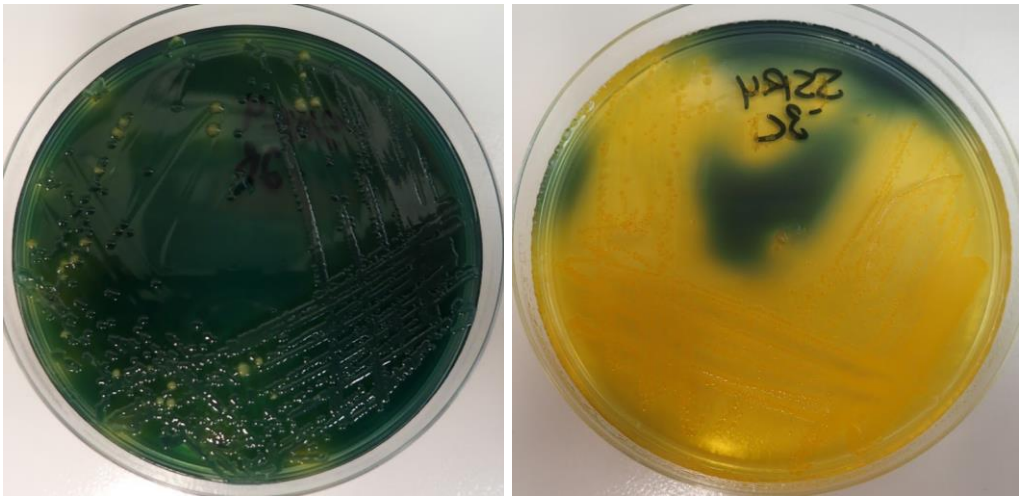


Figure 3 (a) The growth of *Vibrio parahaemolyticus* on TCBS and (b) *Vibrio vulnificus* on mCPC.

The many illnesses linked to these bacteria are cause for concern when the temperatures increase the bacteria flourish. This increase in bacteria has caused regulations to become much more severe for harvesting oysters with more stringent time/temperature requirements. The oysters must be refrigerated within 1 hour of harvest to obtain an internal meat temperature of 55ºF within 6 hours (LDHH 2014). This time requirement is not plausible for smaller vessels that are not large enough to hold a refrigeration unit.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technique developed by Kary Mullis in 1983 earning him a Nobel Prize in Chemistry in 1993 by synthesizing billions of copies of a samples specific region of DNA (Erlich 1989; Mullis 1990; Bartlett and Stirling 2003). The bacteria were analyzed using the Applied Biosystems 7500 Fast Real-Time PCR system (AB 7500; Life Technologies Waltham, MA). Real-time PCR runs a sample through cycles to collect fluorescent signals from polymerase chain reaction (Dorak 2006). To detect a fluorescent signal, the DNA target was amplified and a background end cycle was set to 10 to help identify when the PCR product is first detected (Bustin 2005; Kinsey 2015).

On-board Icing

Reducing the vibrio bacteria levels while keeping mortality of the oyster low will be the key to harvesting oysters on smaller vessels without an on board refrigeration unit. An on-board icing unit has been investigated to collaborate with previous research showing no significant increases in vibrio levels after dipping the oysters in an ice-slurry maintained at ≤4.5ºC for 15 minutes (Thomas et al. 2016). The ice-slurry can produce unexpected mortality in the oysters from gaping during cold storage. Melody et al. (2008) found after 7 and 14 days post harvest the oysters exposed to the ice had significant gaping. In accordance with the Nation Shellfish Sanitation Program (2013) all the sacks achieved the temperature reductions for the on-board icing side of the experiment. The high mortality rate is prompting further research for on-board icing.

Goals

The use of smaller vessels (e.g., skiffs) to harvest is desirable to reduce fuel costs and possible with intensive culture becoming more popular and eliminating the need and capital to

dredge for the oysters. The time/temperature requirements for summer harvest however limit the smaller vessels because the decks do not have space for on-board refrigeration.

The goal of this study was to design and test an on-board rapid chilling unit for small vessels to achieve time/temperature requirements for vibrio control of harvested triploid oysters during the warmer months while keeping oyster gaping to a minimum throughout cold storage.

The first objective was to determine if there was a significant difference in 7 and 14 day post-harvest oyster mortality (gaping) of iced vs. un-iced oysters while held in cold storage (H_0: $\mu_{\text{iced oysters}} = \mu_{\text{control}}$).

The second objective was to determine if there was a significant difference in levels of *V. vulnificus* and *V. parahaemolyticus* of treated and control oysters (H_0: $\mu_{\text{iced } Vp} = \mu_{\text{control } Vp}$ and H_0: $\mu_{\text{iced } Vv} = \mu_{\text{control } Vv}$).

The third objective was to determine if there was a significant difference in the heat transfer coefficient (h) of static vs. circulating water (H_0: $h_{\text{static}} = h_{\text{circulating}}$).

CHAPTER 2 : ANALYSIS OF ON-BOARD CHILLING OF EASTERN OYSTERS

Introduction

The high temperatures in the Gulf of Mexico's warm waters occurring from May through October allow bacteria to increase (Rippley 1994; Hlady 1997; Cook et al. 2002). Different methods of lowering bacterial counts in the eastern oyster *Crassostrea virginica* have been explored. Investigated methods include relaying, irradiation, mild heat treatments, and cooling. Relaying of oysters offshore in a high-salinity area reduced the *Vibrio* levels (Motes and DePaola 1996). The use of irradiation also lowers the amount of bacteria while also extending the shelf life; however unwanted gaping can occur (Dixon and Rodrick 1990; Colby et al. 1993; Kilgen 1994). Mild heat treatments were also used to reduce bacteria while maintaining a raw product (Goldmintz and Ernst 1979). These methods were successful at decreasing the amount of bacteria present in oysters. The fourth method of chilling the oysters to lower *Vibrio* levels was conducted using an on-board icing unit.

Materials

Chilling and Washing Unit Design

An on-board treatment prototype was conceived to match commercial oyster culture practice for shellstock cooling and storage using ice. The oysters were harvested and washed prior to marketing as 100 count and stored in small sacks that do not retain water. The washing is done prior to separation to remove silt and grit to justify high dockside pricing and so cold wash water will not be used nor rewarming of the oysters post-harvest.

The main features of the unit (Figure 4) included a commercially-built, rugged foam and fiberglass box (2.13 m x .91 m x .559 m) with two interior compartments, a large hinged lid, and a removable oyster washer. The two interior compartments consisted of a smaller "dipping

chamber" for rapid chilling of oysters while soaking in an ice-slurry of ambient seawater and a larger chamber for ice storage and chilled oysters. The lid was designed to extend past the gunwales of the boat to allow overboard drainage. The lid also served as a work platform to place harvested oysters for sorting, counting, and cleaning. The oyster washer (61 cm x 40 cm x 23 cm) was not used during testing but has interior seawater jets supplied by an onboard gasoline-powered pump; it was built as a spray hood typically used to wash shellstock at onshore facilities.

The design of the chilling and washing unit also includes a 90 cm x 60 cm plastic tray (not pictured) for placement of oysters to be washed by sliding beneath the washer hood, a plastic-mesh sacks (i.e., crawfish sacks) (not pictured) for placing 100 washed oysters for dipping and storage and, a sheet of coroplast (corrugated plastic) (not pictured) for placing bags of shellstock atop ice in the large chamber.

Figure 4 (a) Standard oyster washer typically used in Louisiana for on-shore shellfish washing. (b) The prototype on-board chilling unit.

Commercially available plastic mesh sacks (i.e., for crawfish handling) of 100-count oysters were tested three times on different days using the prototype cooling unit with a participating oyster farmer at the Grand Isle Oyster Farming Zone, Caminada Bay, LA.

A gas-charged lift support (MightyLift!, AutoZone, Memphis, TN) was added to each side of the oyster chilling box as seen in Figure 5 to offset the weight of the lid and improve the design.

Figure 5 Improvements to the lid.

The lifts were able to handle 500 N (112 lbs) each. The lifts had a range of 42.5 cm to 71 cm (16.75 in to 28 in). Lifting one side while standing on a scale and doubling the resulting number determined the weight of the lid. The weight was used to determine the size of the gas lifts needed to accommodate the force using the following formula:

$$F_y : F_{lift} \sin\theta - F_{lid} = 0$$

Additional support was needed to attach the lifts since the unit is made of foam and fiberglass. A 2 in x 4 in board was attached to either side using Liquid Nails (Liquid Nails Adhesive, Cranbery Township, PA). The placements of the lift supports were determined using the trigonometric equations for a right triangle (lid closed)

$$a^2 = b^2 + c^2$$

and a scalene triangle (lid open 60°) as shown in Figure 6.

$$a'^2 = b^2 + c^2 - 2bc \times \cos A$$

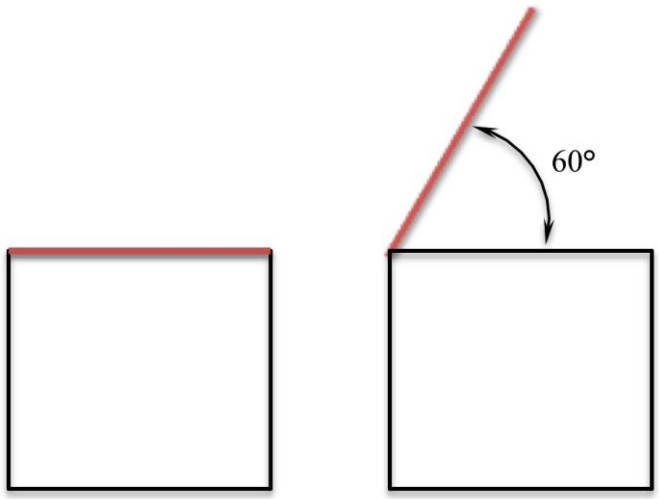


Figure 6 Schematic of the lid closed and open used for calculations.

The variables b and c are constants in both trigonometric equations. The variable a corresponds to the length of the lift with a closed lid larger than 42.5 cm. The variable a' references the length of the gas-lift with the lid open to 60° and cannot exceed 71 cm.

Methods

Temperature Tests

Two different methods for cooling eastern oysters were experimented with to determine the more efficient process. The methods compared dipping the oysters in static vs circulating ice-slurries maintained at ≤4.5°C to test the differences of thermal reduction of internal vs external oyster temperatures using thermocouples connected to a 21X Micrologger (Campbell Scientific, Logan, UT, United States) and Smart Button temperature loggers (ACR Systems Inc, Surrey, B.C, Canada). Static water uses conduction and natural convection to transfer heat to neighboring particles. By circulating the water, the tests were changing the second heat transfer method, forced convection, to accelerate the cooling of the oysters. The heat transfer of static versus circulating water are analyzed and compared in Chapter 3. An ice-slurry remaining static,

demonstrating natural convection was compared to an ice-slurry with water circulated by a pump with forced convection.

Smart Buttons are easily attached to the surface, can be left for a longer period of time, and do not have wires making them more ideal for fieldwork (Drake et. al, 2009). The Smart Buttons and thermocouples were tested for accuracy by being placed in different temperature environments. The two types of sensors were also compared to a mercury thermometer for verification.

The oysters were readied to test by being scrubbed and attaching sensors as seen in Figure 7. Three oysters had smart buttons attached using duct tape and three oysters were monitored using thermocouples. Two T-type thermocouples (copper-constantan) were attached to each of the three live oysters. Martin et al. 2007 found drilling a 6.35 mm (¼ in) hole into the oyster and inserting a thermocouple 2.5 cm into the meat is best to record the internal meat temperature. The hole was sealed using modeling clay. The second thermocouple was taped to the bottom of the shell and both thermocouples were secured with duct tape. The thermocouples were attached to Campbell Micrologger to record the temperatures using the code in APPENDIX A.

Figure 7 (a) A T-type thermocouple inserted into a live oyster and sealed with modeling clay. (b) Thermocouples secured with duct tape to ensure contact with the shell. (c) Smart Button attached to the outside of a live oyster using duct tape.

Each cooling run was timed for 20 minutes to ensure the oysters were thoroughly chilled to get a complete temperature profile. To monitor ambient temperature a thermocouple was placed in the sack with the oysters. The tests were replicated to verify, three tests for a static ice-slurry and three tests for a circulating ice-slurry were conducted. The data was collected from the smart buttons and thermocouples and analyzed to determine the amount of time needed for icing the oysters after harvest to achieve an internal meat temperature of 10°C. A 12V bilge pump was placed on the bottom of the slurry chamber to provide circulation with a discharge hose directed towards the surface. The testing temperature was maintained by monitoring and sustaining floating ice.

Harvest Oysters

The oysters were located in Portersville Bay (30º35’ 03.51”N: 88º19’32.93”W) (Figure 8) on a private oyster farm. Oysters were harvested, sorted into groups of 100, and stored in plastic mesh sacks that do not absorb water. Each sack was labeled corresponding to the treatment; six bags put individually in the ice-slurry for eight minutes and six bags left on the deck of the boat un-iced as the controls. The rapidly cooled oysters were placed in the second chamber of the icing box on top of spare bags of ice separated by a coroplast sheet. Each group of six consisted of three oyster sacks for sampling for vibrio analysis and three for gaping tests. To get a consistent sample, the Dauphin Island research team suggested collecting 15 oysters to ensure 12 live oysters for tests. Each testing period had 5 different points for vibrio testing: (1) at harvest (H); (2) after dipping in the ice-slurry (IS); (3) when placed in mechanical refrigeration storage (R0); (4) after 7 days in refrigeration (R7); and (5) after 14 days in refrigerated storage (R14).

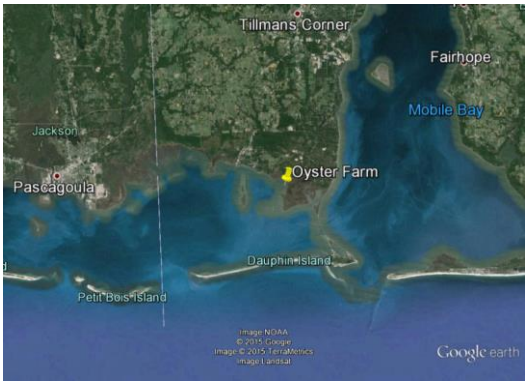


Figure 8 Google Earth images of oyster farm location.

Samples and Counts

The first day of the testing period consisted of harvesting the oysters, icing the appropriate samples and placing the oyster sacks in cold storage. Twelve samples were taken the first day. The three harvest samples were taken only from the three control sacks. The three samples after dipping in the ice-slurry were taken from the three iced sacks. Three samples were taken from each of the control and icing sacks before refrigeration. All samples were taken from the same six sacks labeled for sampling. Six samples were taken after 7 days and 14 days in refrigeration; three from the control and three from the iced oysters.

The other six sacks were used for the mortality counts. Each oyster was tested for mortality by taping on the shell to audibly detect gaping from a hollow sound (Melody et al, 2008). The hollow (dead) oysters were recorded and removed. The live oysters were replaced in the cold storage at R & A Oyster Company.

Enriching Samples

The samples were taken to the Food and Drug Administration Division of Seafood Science and Technology, Gulf Coast Seafood Laboratory in Dauphin Island, AL for processing and analysis. Each sample of 12 oysters was processed using sterile equipment: shucking knife, board, scrubber, and blender bottle. The process followed for each test began by scrubbing each

oyster to remove sediment, particularly around the hinge. The oyster knife was inserted in the hinge while the oyster was held upside down, pushed then twisted the knife to separate the shells. The abductor muscle was separated with the knife from the bottom shell (located on top at this point) to release the first piece of shell. The knife was then slid underneath the meat to separate the second shell. The entire oyster and liquid were put into the labeled blender bottle, weighed, and record. The oyster meat was homogenized in the blender on high for ~90s. The homogenized oyster meat was used for *Vibrio* analysis.

A three-tube most probable number (MPN) dilution series was used to analyze each sample per standard methods (Kaysner and DePaola, 2001; Kaysner and DePaola, 2004). To prepare for the MPN one rack was set up per sample of 12 oysters. Six vials filled with 9 mL phosphate-buffered saline (PBS; 7.65 g NaCl, 0.724 g Na_2HPO_4 [anhydrous], .21 g KH_2PO_4 in 1L distilled H_2O, pH7.4) and 21 vials filled with 9 mL of the growth media APW (Alkaline Peptone Water) per rack (**Figure 9**).

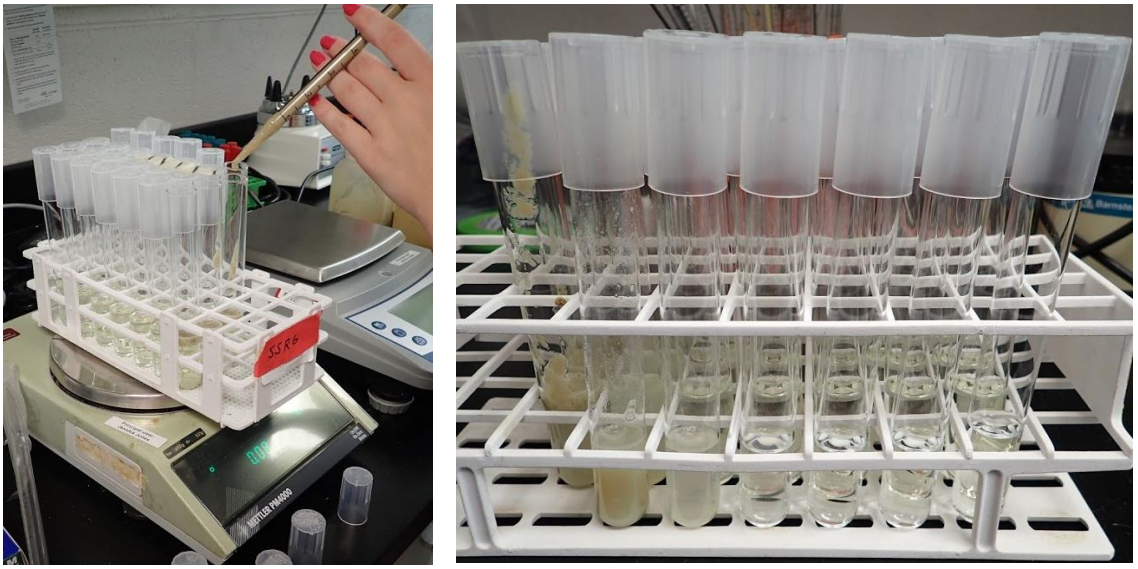


Figure 9 Preparation of the MPN tubes.

To create a 1:10 dilution, one gram of oyster homogenate was added to 9 mL of phosphate buffered saline (PBS) as seen in Figure 9. 10-fold dilutions were made from the first PBS tube through 10^{-6}. One gram of the oyster homogenate was also added to the first three vials filled with 9 mL of alkaline peptone water (APW; Bacto peptone 10g, NaCl 10g, dH_2O 1L). 1 mL was taken from each PBS tube and added to three tubes filled with 9 mL APW to complete the three-tube MPN. The tubes were incubated at 37 °C for 18 hours. All media volumes and contents were provided by Dauphin Island Gulf Coast Seafood Laboratory as used in Kinsey et al. 2015.

Preparing for Vibrio Tests

After the incubation period, the MPN tubes were ready to be processed. The tubes with visible growth were considered positive for bacteria seen in Figure 10. Each positive APW tube was marked in the appropriate column of the sheet. A 1 mL aliquot was transferred from each positive APW vial into an Ependorff 1.5 mL micro-centrifuge tube. The tubes were labeled according to the rack and location of the vial. The micro-centrifuge tubes were heated for 10 minutes at 100 °C then immediately placed on ice for 10 minutes. Micro-centrifuge tubes either continued on to vibrio testing using polymerase chain reaction (PCR) by being centrifuged for 2 minutes at 2000 rpm or were stored in the refrigerator.

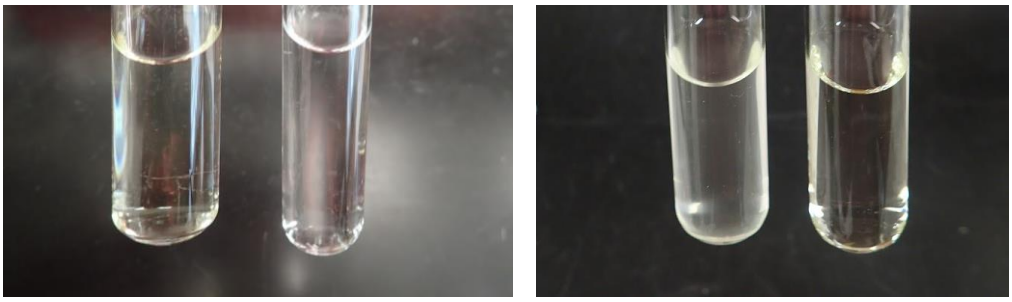


Figure 10 (a) APW and PBS tubes before inoculation. (b) Opaque tube on the left indicates a positive APW test compared to a transparent negative tube.

Real-Time PCR Analysis

The Applied Biosystems 7500 Fast Real-Time PCR system (AB 7500; Life Technologies Waltham, MA) was used to run three separate Rti-PCR assays for total *V. vulnificus* (*vvh*), total *V. parahaemolyticus* (*tlh*), and pathogenic *V. parahaemolyticus* (*tdh*+/*trh*+). 2µl of an Internal Amplification Control (IAC) DNA is used to eliminate false-negative reporting (Nordstrom et al., 2007). The concentrations for each assay are described in Table 1.

Table 1 – All primers and probes used for *Vibrio* PCR.

	V. vulnificus	*V. parahaemolyticus*	Pathogenic *Vp*
Component	Volume (µl)/RXN		
Target Template	2	2	2
PCR H_2O	12.22	12.77	11.87
Buffer	2.5	2.5	2.5
$MgCl_2$	2.5	2.5	2.5
dNTPs	0.75	0.75	0.75
vvhF	0.75		
vvhR	0.75		
IC 46F	0.188	0.188	0.063
IC 186R	0.188	0.188	0.063
vvh CY5	0.5		
IC JOE	0.375		
IAC DNA	2	2	
Platinum Taq	0.22	0.3	
ROX reference dye	0.06	0.06	0.06
tl 884F		0.5	
tl 1091R		0.5	
tl JOE 1043C		0.375	
IC CY5		0.375	0.375
trh 20F			0.75
trh 292R			0.75
tdh 89F			0.25
tdh 321R			0.25
trh VIC			0.188
tdh FAM			0.188

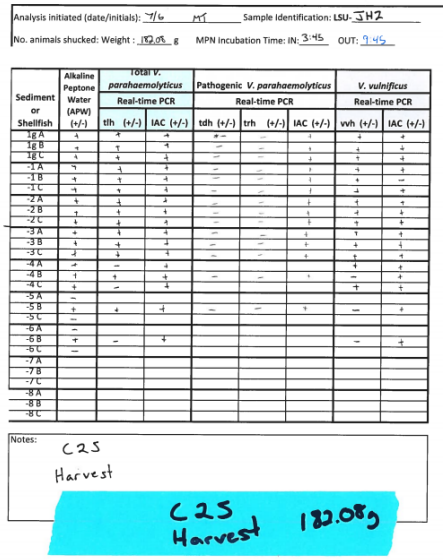
The final reaction volume for each template was 25 µl. The values in Table 1 were provided by the FDA lab in Dauphin Island, AL. The deoxynucleose triphoshpates (dNTPs) are used in all three Real-Time PCR assays. All components labelled F or R are a forward or reverse primer or probe. The IAC is an internal amplification control DNA used to identify negative PCR responses. CY5, JOE, ROX, VIC, and FAM are reference dyes that help mark positive samples.

The Real-Time PCR assay for *V. vulnificus* used *vvhA* primers and probes as published by Campbell, M. and A. Wright (2003). Modifications were needed for the reaction mixtures to run on the AB 7500 platform since the original mixtures were for a different machine. The cycling parameters for a 25 µl volume were 95°C for 60s for the initial denaturation, and 45 cycles of 95°C for 15s (denature), 57°C for 15s (anneal), and 72°C for 25s (extend). The threshold cycle was set to 0.02 and the background end cycle was set at 10.

The Real-Time PCR assay for *V. parahaemolyticus* used *tlh* primers and probes as described by Givens et al., 2014. Modifications were also required for this mixture to run on the AB 7500 platform. The cycling parameters for a 25 µl reaction volume were an initial denaturation of 95°C for 60s, and 45 cycles of 95°C for 5s (denature) and 59°C for 45s (anneal/extend). The threshold cycle was set to 0.02 and the background end cycle was set at 10.

The Real-Time PCR assay for pathogenic *V. parahaemolyticus* used *tdh* and *trh* primers and probes as described by Jones et al., 2014. Modifications were also required for this mixture to run on the AB 7500 platform. The cycling parameters for a 25 µl reaction volume were the same as the assay for *tlh* with an initial denaturation of 95°C for 60s, and 45 cycles of 95°C for 5s (denature) and 59°C for 45s (anneal/extend). The threshold cycle was set to 0.02 and the background end cycle was set at 10.

The results seen in Figure 11 from the Real-Time PCR were used to estimate total *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* densities in each sample with standard MPN tables (Blodgett 2010).



Analysis initiated (date/initials): 7/6 MT Sample Identification: LSU- JH2

No. animals shucked: Weight : 122.08 g MPN Incubation Time: IN: 3:45 OUT: 9:45

Sediment or Shellfish	Alkaline Peptone Water (APW) (+/-)	Total *V. parahaemolyticus* Real-time PCR		Pathogenic *V. parahaemolyticus* Real-time PCR			*V. vulnificus* Real-time PCR	
		tlh (+/-)	IAC (+/-)	tdh (+/-)	trh (+/-)	IAC (+/-)	vvh (+/-)	IAC (+/-)
1g A	+	+	+	+	–	+	+	+
1g B	+	+	+	–	–	+	+	+
1g C	+	+	+	–	–	+	+	+
-1 A	+	+	+	–	–	+	+	+
-1 B	+	+	+	–	–	+	+	–
-1 C	+	+	+	–	–	+	+	+
-2 A	+	+	+	–	–	+	+	+
-2 B	+	+	+	–	–	+	+	+
-2 C	+	+	+	–	–	+	+	+
-3 A	+	+	+	–	–	+	+	+
-3 B	+	+	+	–	–	+	+	+
-3 C	+	+	+	–	–	+	+	+
-4 A	+	–	+				–	+
-4 B	+	+	+	–	–	+	–	+
-4 C	+	–	+				+	+
-5 A	–							
-5 B	+	+	+	–	–	+	–	+
-5 C	–							
-6 A	–							
-6 B	+	–	+				–	+
-6 C	–							
-7 A								
-7 B								
-7 C								
-8 A								
-8 B								
-8 C								

Notes: C25 Harvest

C25 Harvest 122.08g

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	--	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	--

Figure 11 (a) The results from PCR. (b) Table was used to estimate the *Vibrio* levels in each sample.

The data of total *V. vulnificus*, total *V. parahaemolyticus*, and pathogenic *V. parahaemolyticus* was $\log_{10}$ transformed and run through a mixed procedure in SAS 9.4.

Streaking for Vibrio

The positive MPN vials were also streaked onto selective agar for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Thiosulfate-citrate-bile salts-sucrose agar (TCBS) was used to target *V. parahaemolyticus* and modified cellobiose-polymyxin B-colistin (mCPC) agar was used to target *V. vulnificus* (Hoi 1998). The TCBS and mCPC plates were removed from refrigeration and placed in the incubator to prepare for streaking. Once warm, a TCBS and mCPC plate were labeled as seen in Figure 12 to correspond with each positive MPN vials for one rack of each treatment but not the replicates.

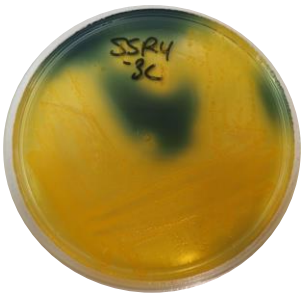


Figure 12 Streaked plate SSR4 -3C. S_1: September, S_2: Iced, R4: Refrigerated sample 4, -3C: 10^{-3} Dilution's 3rd tube (C).

Plates were streaked using 1 µL from the incubated vials and spread using an inoculating loop onto a quarter of the plate. The loop was cleaned by being repeatedly inserted into the agar around the edge of the plate. Using one side of the loop, one line was streaked through the previously section into a clean section of the agar and dragged back and forth in a zigzag motion into the second quarter of the plate. The loop was flipped to the opposite side and streaked one line through the previously section into the third quarter of the plate. The flat side of the loop was used to repeat the same process for the fourth quarter of the plate to isolate colonies. The same loop was used for the TCBS and mCPC plates of the same sample since the agars were sterile. The plates were incubated at 37°C overnight once all vials were streaked.

Picking Colonies

96 well templates were filled with 100 µL APW with separate templates for TCBS and APW. The TCBS agar targets *Vibrio parahaemolyticus* (green smooth colonies). Three colonies were picked from each plate using a toothpick and placed in three different wells labeled with the corresponding block on a reference sheet. The mCPC agar targets *Vibrio vulnificus* (flat yellow colonies with a transparent rim). Three colonies were also picked from each of the mCPC plates and put in the 96 well plates. The templates were incubated overnight.

Replicate Plating

The templates were removed from the incubated and stamped onto T_1N_3 (1% Trypton, 3% NaCl, 2% agar) plates using a metal 48-prong stamp, isopropanol, burner, and PBS. Label the T_1N_3 plates to match the corresponding template (Figure 13). The stamp was first sterilized by being dipped in the isopropanol and placed over the burner, do this twice the first time. The stamp was then placed in the PBS to cool the prongs before being set in half of the template to collect the colonies. Once in the template the stamp was removed and set on the corresponding T_1N_3 plate and gently wiggled to ensure the colonies were transferred. This process was repeated for each half and the other templates. The T_1N_3 plates were incubated overnight. The 96 well templates were stored in a -20°C freezer after adding 100 µL TSB + Glycerol to protect the colonies.

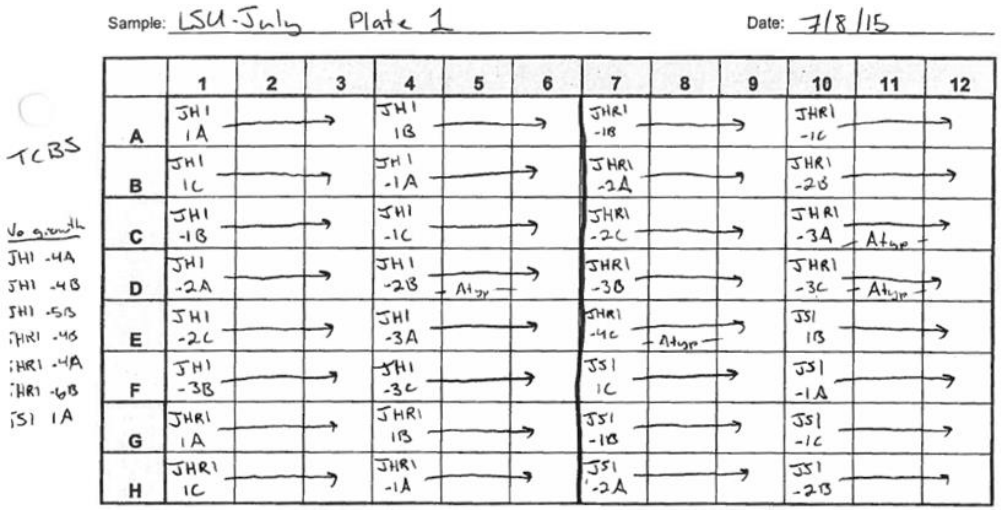


Figure 13 Reference chart for the 96 well templates.

TCBS and mCPC were stamped onto separate templates because of different probes used when confirming the bacteria.

Vibrio verification

The T_1N_3 plates were removed from the incubator and lift the colonies using filter paper. The filter paper is processed using the method laid out by Nordstrom and DePaola (2003) to probe and verify colonies. The processed filter (Figure 14) paper can verify if the bacteria from the oysters are *Vibrio parahaemolyticus* and *Vibrio vulnificus*.

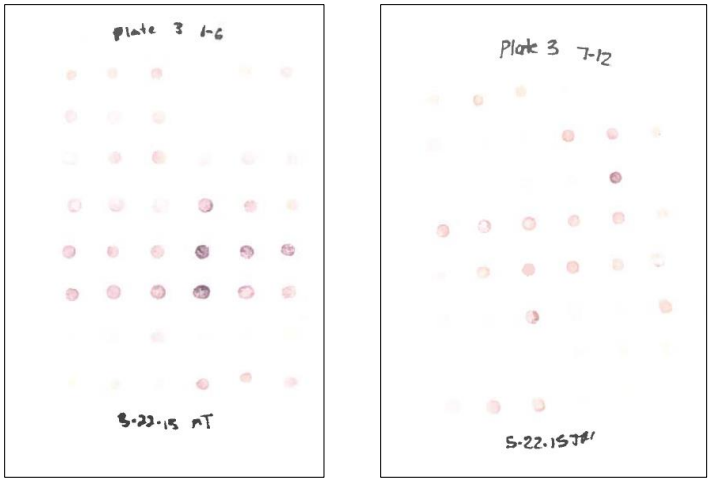


Figure 14 Completed *Vibrio* probes.

The purple colonies on each filter confirm the presence of *Vibrio parahaemolyticus*. This method takes longer to prepare and perform than Real-Time PCR.

Results

The total *Vibrio parahaemolyticus* results had a significant difference in the sampling months ($p<.001$) but not a significant difference in the treatments ($p=0.4715$). The amount of bacteria present in July was much higher than in May as shown in Figure 15. The graphs of the individual months (Figure 16) display how closely the bacteria numbers were for treated compared to the controls.

The total *Vibrio vulnificus* data followed a similar pattern as total *V. parahaemolyticus*. The sampling months were significantly different ($p<0.001$) and the treatments were not

significantly different (p=0.2617). Figure 17 demonstrates how the bacteria levels were much lower in September than in May or July.

Pathogenic *Vibrio parahaemolyticus* were low so no significant differences were found for sampling month (p=0.3716) or treatment (p=0.7885). The data is shown in Figure 15-Figure 18 and Table 2.

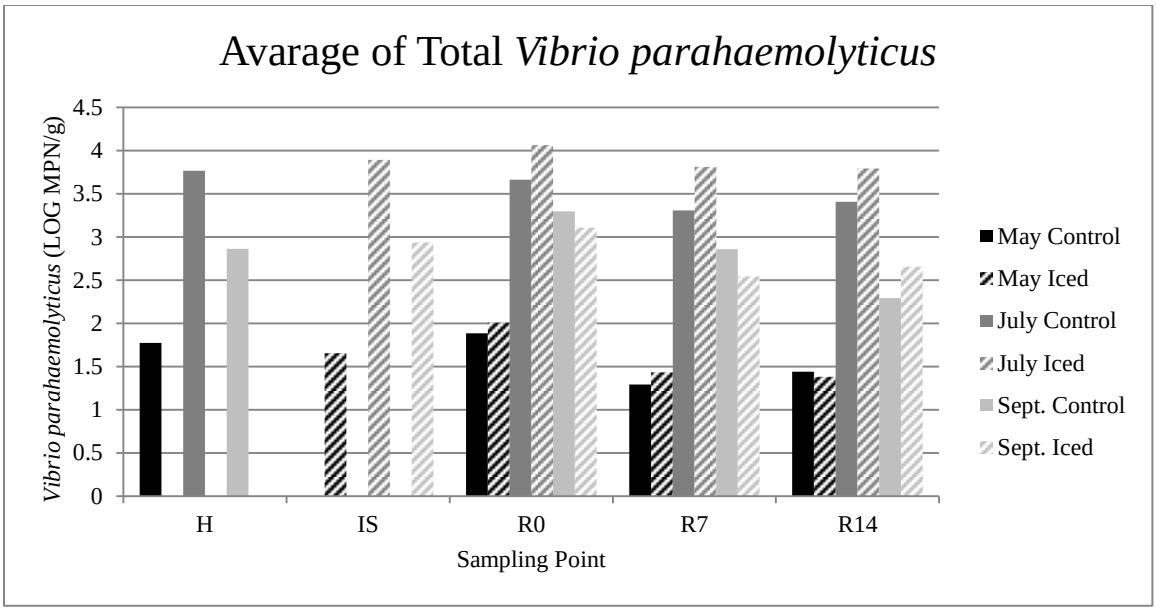


Figure 15 Total *Vibrio parahaemolyticus* throughout the three testing periods.

There was a significant difference between the sampling months (p<.001) showing the vibrio levels greatly increase during the warmer summer months as seen in Figure 15. However, there was no significant difference between the oysters that were iced and the control.

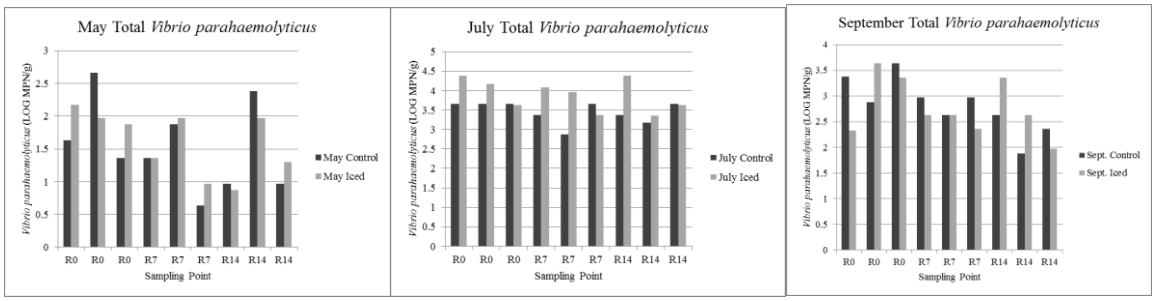


Figure 16 Total *Vibrio parahaemolyticus* for the months of May, July, and September.

The treated and controlled vibrio levels stayed consistent for each month. The bacteria were more abundant in July and September as seen in Figure 16. A significant difference is seen in the sampling months ($p<.001$) but there was not a significant difference in the treatments ($p=0.4715$).

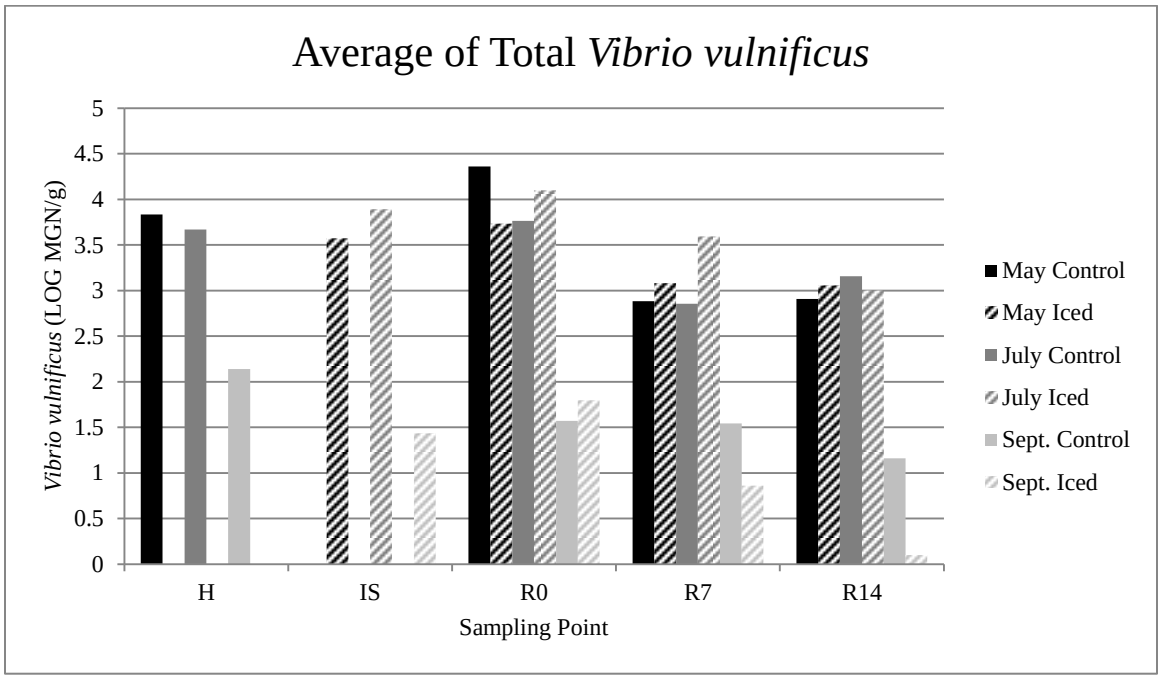


Figure 17 Total *Vibrio vulnificus* results for the three trials run in May, July, and September 2015.

There was a significant difference in the sampling months ($p<.001$) with a higher *V. vulnificus* count in May and July. The treatment was not statistically significant ($p=0.2617$) showing the treated and control bacterial counts were similar for each month.

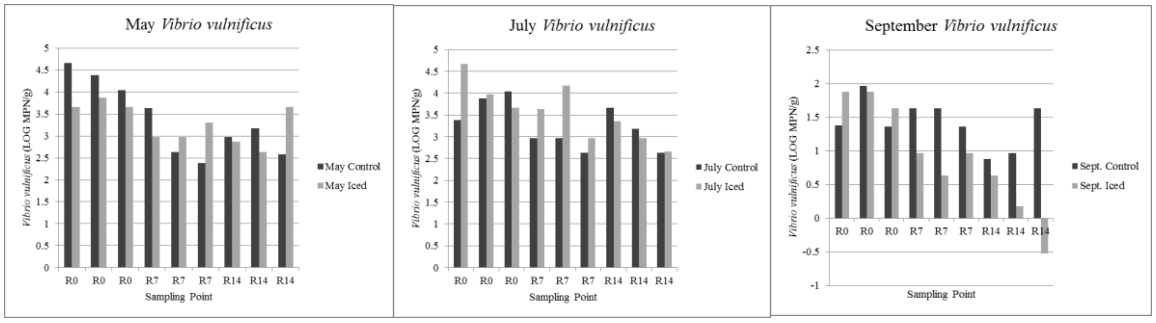


Figure 18 Total *Vibrio vulnificus* for May, July, and September.

The treated and controlled vibrio levels alternate having higher levels at different sampling points, giving overall similar results. There is no significant difference between the treated and untreated oysters for *Vibrio vulnificus* ($p=0.2617$). However, there was a significant difference between the sampling months ($p<0.001$) with much lower bacteria levels in September than in May or July.

Table 2 The pathogenic *Vibrio parahaemolyticus* results for the three trials run in May, July, and September.

Pathogenic *Vibrio parahaemolyticus* (MPN/g)						
MH1	0		JH1	0.3		SH1	0.92
MH2	0.36		JH2	0		SH2	0
MH3	0		JH3	0		SH3	0.36
MHR1	0		JHR1	0		SHR1	0
MHR2	0		JHR2	0		SHR2	0
MHR3	0.36		JHR3	0		SHR3	0.36
MHR4	0		JHR4	0.3		SHR4	0
MHR5	0.3		JHR5	0		SHR5	0
MHR6	0		JHR6	0		SHR6	0
MHR7	0		JHR7	0		SHR7	0
MHR8	0		JHR8	0		SHR8	0
MHR9	0		JHR9	0		SHR9	0
MS1	0		JS1	0		SS1	0
MS2	0		JS2	0		SS2	0
MS3	0		JS3	0.36		SS3	0
MSR1	0		JSR1	0		SSR1	0.36
MSR2	0		JSR2	0		SSR2	0
MSR3	0		JSR3	0		SSR3	0
MSR4	0		JSR4	0.92		SSR4	0.3
MSR5	0		JSR5	0.61		SSR5	0
MSR6	0		JSR6	0		SSR6	0.3
MSR7	0		JSR7	0		SSR7	0
MSR8	0		JSR8	0		SSR8	0
MSR9	0		JSR9	0		SSR9	0

The numbers of pathogenic vibrio in the oysters were very low and icing the oysters did not make a difference. The analysis did not show a statistical significance between the two

treatments (p=.7885). The labels for each sample had to be condensed and a shorthand was created where the first letter depicted which month the sample was collected (M=May, J=July, S=September). The second letter groups the samples into iced or un-iced categories (H=Control, S=Iced). Samples with R1-3 were taken as soon as the oysters were put in refrigeration. The labels R4-6 and R7-9 corresponded with the 7 and 14 day sampling respectively.

Results from the oyster mortality tests showed a higher level of oyster gaping for iced oysters as shown in Figure 19-Figure 21. The iced oysters mortality must be ≤15% compared to the controls for this process to be successful. The rate of oyster mortality can potentially be altered with lessening the stress on the oysters by slowing the cooling rate and not circulating the water. The oyster mortality can also be affected by the oysters remaining moist for extended periods of time after the harvest. The mortality results for the iced oysters in May, July, and September were higher than the 15% allowed limit above the controls.

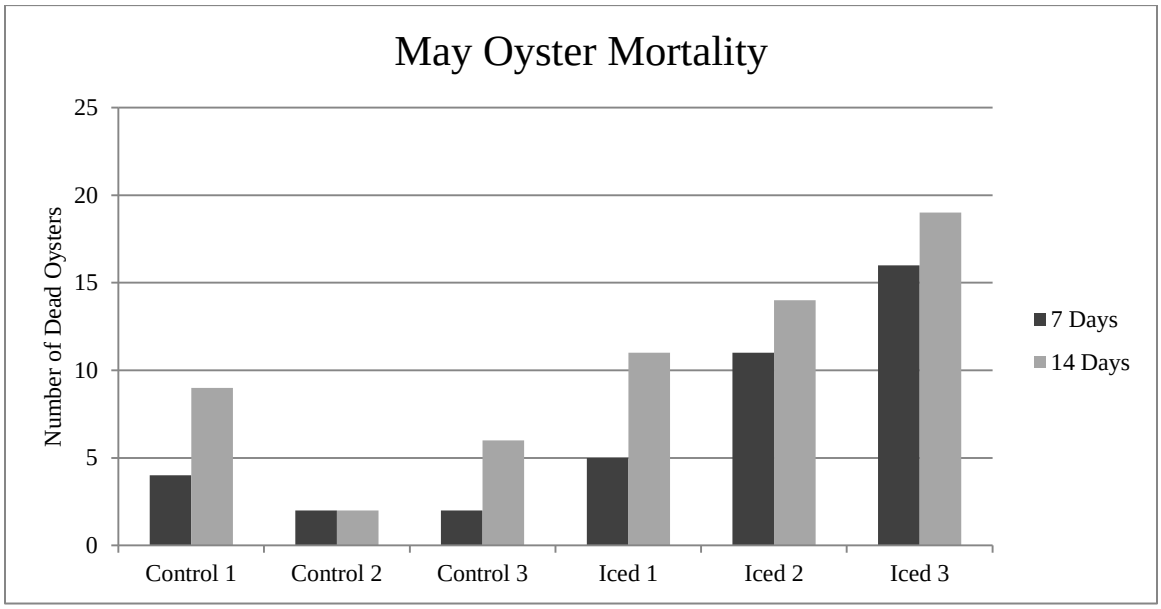


Figure 19 Oyster mortality in May

The oyster mortality for May showed the number of oysters that were gaping (open or hollow sounding) after 7 and 14 days in refrigeration. The results showed a 159% increase in mortality of treated versus control.

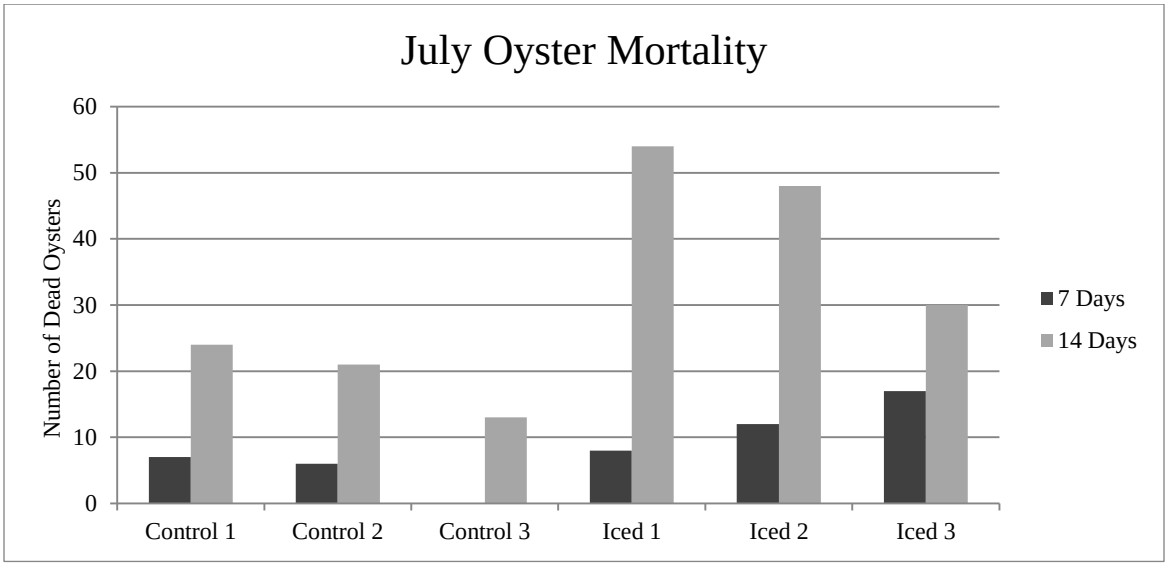


Figure 20 Oyster mortality in July

The oyster mortality for the second trial in July greatly increased from May. The results showed a 127% increase in mortality of treated versus control seen in Figure 20.

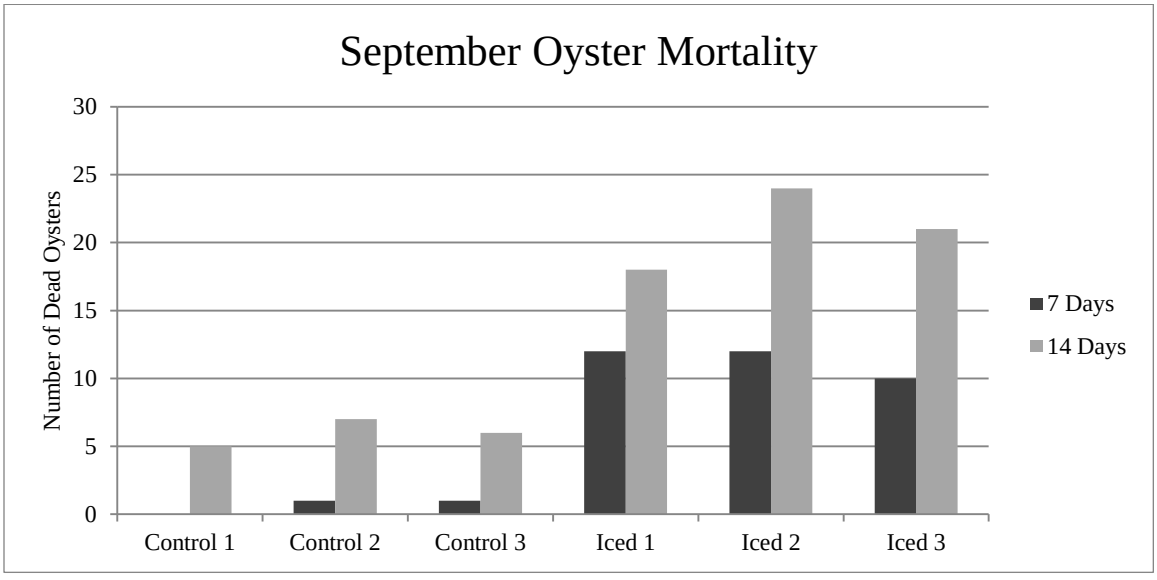


Figure 21 Oyster mortality in September

The oyster mortality in the third trial showed the control sacks with a 250% increase in mortality of treated versus controls.

Discussion

Total *Vibrio parahaemolyticus* results showed the amount of bacteria present in July was much higher than in May as shown in Figure 15. The graphs of the individual months display how closely the bacteria numbers were for treated compared to the controls.

The *Vibrio vulnificus* Figure 17 demonstrates how the bacteria levels were much lower in September than in May or July.

Pathogenic *Vibrio parahaemolyticus* was not flourishing in this area. The data presented was raw since the levels were too low to transform. With the minimal bacteria, there was not a significant difference in the months or treatment.

The analysis of the gaping data showed a statistical significance between the iced and controlled oyster sacks ($p<.001$). There was also a significant difference between the sampling months ($p=.0014$). Several factors could have influenced these results including salinity of the water at harvest, the oysters remaining wet from being dunked in the ice-slurry, and shock from being chilled so quickly. Further work is needed to improve this design and process.

Conclusion and Future Work

The first objective was to find a difference between mortality of iced vs. un-iced oysters while held in cold storage for 7 and 14 days. The data showed an increase of gaping for iced oysters over the controls rejecting the null hypothesis that the two would be equal. The second objective was to decrease the *Vibrio* levels by rapidly cooling the oysters immediately after harvesting. The bacteria levels were not significantly different so we fail to reject the null hypothesis.

The mortality rates were not acceptable for this test. Design improvements need to be made to try to improve these numbers. Possible reasons for these results include the oysters not being dry after going through the ice-slurry, salinity of the ocean when harvesting being low because of a storm the previous day, and shock from extreme change in temperature in a short time. A new design for a chilling unit could include a system to dry the oysters after the ice-slurry to avoid gaping from moisture. The new system would also be enclosed to prevent the cool air from escaping while handling the oysters between the ice-slurry and storage.

The insulation of the current design was more than sufficient for the tested use. The ice did not have to be added to the ice-slurry during any of the harvests. However, since this unit is designed for smaller boats, the new system would need to be lighter. The weight of the lid would be a problem for repeated use since lifting 450+N is not ideal for everyday use. Ideally the storage should also be protected from temperature change by having a separate lid so cool air is not lost every time the icing chamber is opened for a new batch of oysters. The design still has a few problems that can be improved on in the future.

CHAPTER 3 : THERMODYNAMIC PROPERTIES OF EASTERN OYSTERS

Introduction

A large problem with the consumption of oysters is the possibility of food poisoning from eating un-cooked oyster meat (Su and Liu 2007). The oysters must be cooled while whole to preserve the shelf life and ensure a live oyster when sold in-the-shell (Wheaton 2007). The cooling of Eastern Oysters (*Crassostrea virginica*) depended greatly on the thermal properties of the shell.

Two methods were investigated to rapidly cool oysters; a static ice-slurry and a circulating ice-slurry. The ice water was circulated using a submersible sump pump with a hose attached. This method added forced convection by circulating the water and transferring the heat by fluidic motion (Bejan 2013). The forced convection changed the rate of cooling and the total time to cool the oysters. The third objective focused on finding the difference in the heat transfer of an oyster in static vs. circulating water.

Materials and Methods

The oysters were connected to thermocouples as described in Chapter 2, one inserted into the oyster and one connected to the outside of the shell. The oysters were placed in a plastic mesh sack similar to the ones used during the actual testing/harvest and submerged into an ice-slurry for 20 minutes to obtain a full temperature profile.

The width of the oyster shell was used instead of the length to get a more accurate thermodynamic profile. An average width of the shell was taken from Shays and Wheaton (1980). The heat transfer coefficients for an oyster in static and circulating water were calculated using Figure 22 for a sphere since an oyster is an irregular shape.

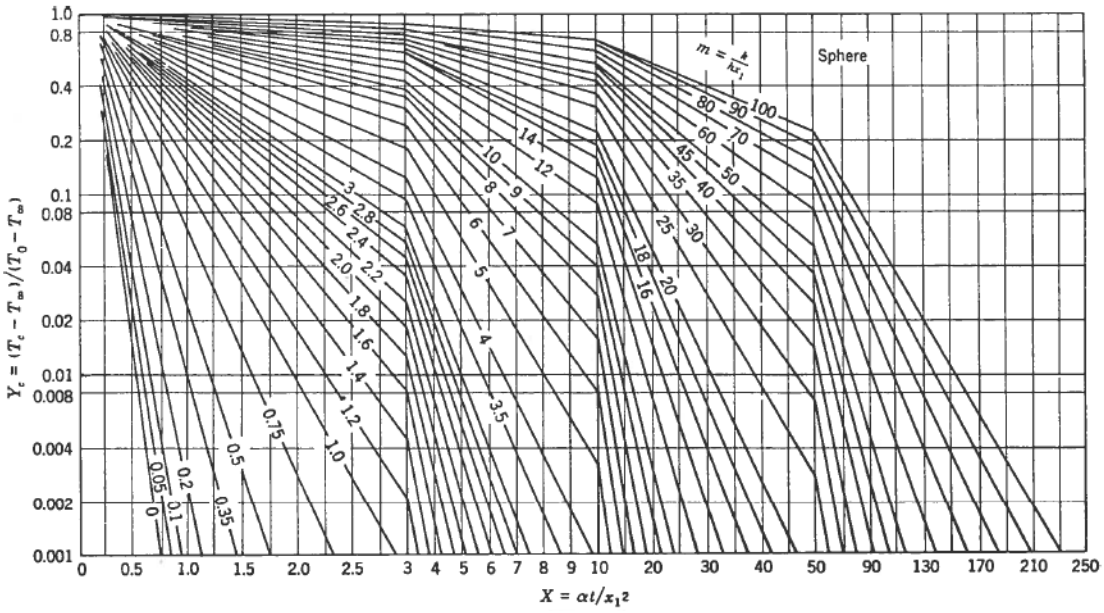


Figure 22 Center temperature history for a sphere (from Welty et al. 1976)

The thermal diffusivity and thermal conductivity of Eastern Oysters were provided by Gomez-Martinez et al. (2002) and used with the following equations to determine the X and Y axis of Figure 22 to determine the relative resistance.

$$Y_c = \frac{T_C - T_\infty}{T_0 - T_\infty}$$

Where, T_c = oyster temperature at a relative time,
T_∞ = water temperature, T_0 = initial oyster temperature.

$$X = \frac{\propto t}{{x_1}^2}$$

Where, α = thermal diffusivity, t = time,
x_1 = radius of the oyster.

$$m = \frac{k}{hx_1}$$

Where, m = relative resistance, k = thermal conductivity,
h = heat transfer coefficient, x_1 = radius of the oyster.

The temperatures were determined from the time-temperature trials using the thermocouple inserted into the oysters. The relative resistance was found with the center temperature history for a sphere table and used to determine the heat transfer coefficients for static and circulating water.

Results

The addition of forced convection by circulating the water increased the rate of cooling and lowered the total time to cool the oysters as shown in Figure 23 and Figure 24. Using the data collected by the thermocouples using the Campbell Scientific Micrologger and the code in APPENDIX A, a table of temperatures at specific times provided the data required to calculate the thermal properties of the oysters. The circulating water had a higher rate of heat transfer than the static water (h_{static} = 141.21 W/m^2 K, $h_{circulating}$ = 247.116 W/m^2 K.

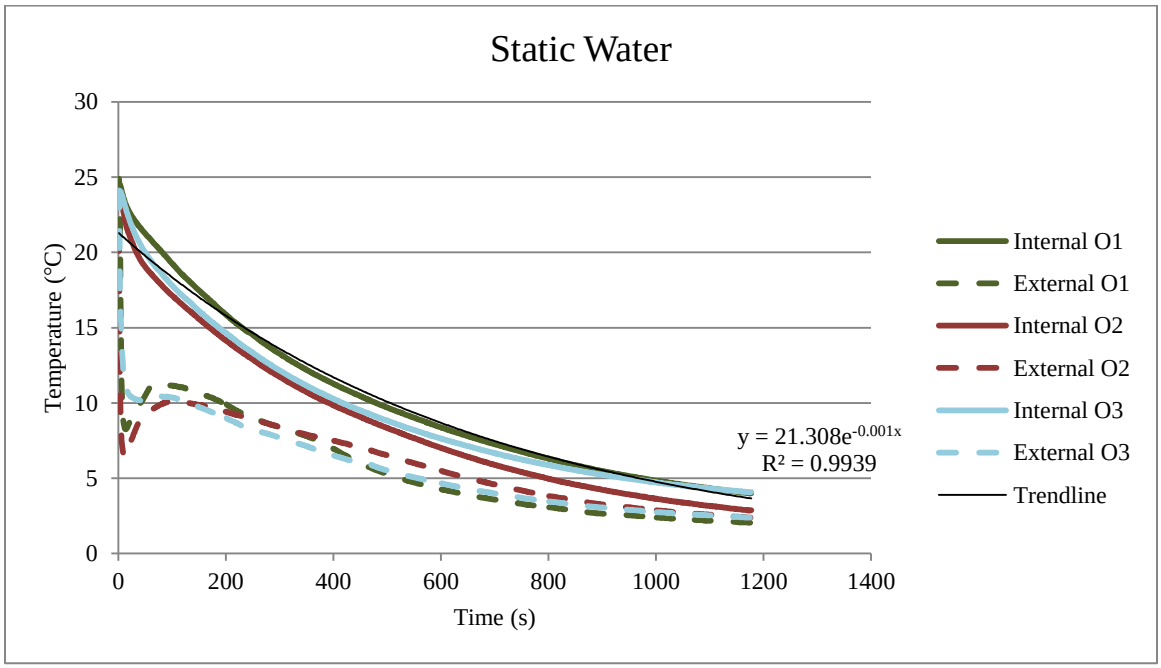


Figure 23 Chilling rate of oysters in static water

The thermocouples were inserted into the oysters shown in Figure 23 were submerged into a static ice-slurry for 20 minutes to develop a complete temperature profile of the cooling oysters.

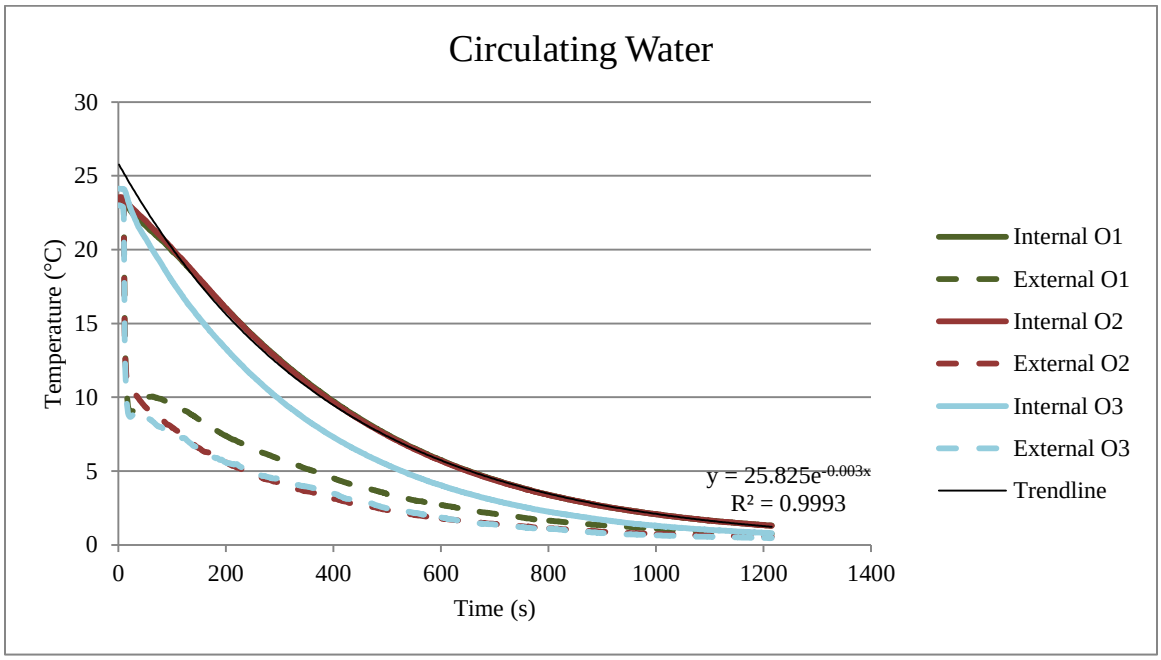


Figure 24 Chilling rate of oysters in circulating water

The process was repeated with a circulating ice-slurry with the results shown in Figure 24.

Discussion

The time for the oysters to reach thermal equilibrium with the water was less for the circulating ice-slurry than the static. This is confirmed with the heat transfer data finding $h_{static} < h_{circulating}$. Circulating the water and adding a second mode of heat transfer was beneficial to the time constraint and helped the efficiency of the process. The coefficient for heat transfer with natural convection was $h_{static} = 141.21$ W/m^2 K and forced convection brought the coefficient up to $h_{circulating} = 247.116$ W/m^2 K.

Conclusion and Future Work

The third objective was to determine if there was a difference in the heat transfer coefficient of an oyster in static vs. circulating water (H_0: $h_{static} = h_{circulating}$). The null hypothesis is rejected with the static ice-slurry having a lower heat transfer than the circulating ice-slurry. The added circulation of the water was more efficient at cooling the oysters.

The data from this test is helpful for providing a more thorough understanding of thermal oyster properties. However, oysters are a biological organism and abrupt changes in environment can affect them negatively. A continuation of this research could determine the stress an oyster goes under when rapidly cooled to determine the best process for handling oysters that will limit vibrio growth, keep the oysters healthy, and simplify the procedure.

CHAPTER 4 : CONCLUSION AND FUTURE WORK

Project Summary

The goal of this study was to test an on-board rapid chilling unit designed for small vessels to reduce *Vibrio parahaemolyticus* and *Vibrio vulnificus* in eastern oysters (*Crassostrea virginica*) during summer harvest while keeping oyster mortality to a minimum throughout the project. The reduced bacteria levels would create a market for triploid oysters during the spike in water temperature. This unit would also allow smaller vessels unable to fit a mechanical refrigeration unit on-board to continue harvesting when the time/temperature requirements become more stringent. The objectives were to determine if dipping the oysters in an ice-slurry affected the *V. parahaemolyticus* and *V. vulnificus* levels, the mortality after cold storage, and the thermal difference on cooling oysters of static and circulating water.

The second chapter investigated the bacteria levels and mortality in iced and controlled oysters. The tests showed an increase of gaping for iced oysters over the controls. The difference was significant and on several occasions failed to meet the required 80% survival rate. The total *V. parahaemolyticus* and *V. vulnificus* levels were not significantly different between the iced and un-iced oysters. However, not all *V. parahaemolyticus* are pathogenic and the levels of pathogenic *V. parahaemolyticus* were found to be extremely low in this area. Pathogenic *V. parahaemolyticus* was also tested and since the levels were almost non-existent no significance could be found between the iced and un-iced oysters. Along with testing for bacteria, this study also examined the heat transfer of an oyster in static and circulating ice water.

The third chapter explored the difference in the heat transfer coefficient of an oyster in static and circulating ice-slurries. The circulating ice-slurry was more efficient at cooling the

oysters since it had the second heat transfer method of forced convection to the already present conduction.

The data from this test is helpful for providing a more thorough understanding of oysters and their reaction to ice-slurry dips. This work and future endeavors are could provide an efficient process for safely harvesting oysters in the summer for smaller vessels.

Future Work

Modifications need to be made to try to improve oyster survival and bacterial levels. Possible reasons for these results include the oysters not being dry after going through the ice-slurry, salinity of the ocean when harvesting being low, and shock from extreme change in temperature in a short time. A new design for the chilling unit would include a drying system for the oysters after the ice-slurry dip to avoid gaping from continued exposure to moisture. The new system would also prevent cool air from escaping when the lid was opened while handling the oysters between the ice-slurry and storage by being enclosed.

The insulation of the current design was more than sufficient for the tested use. However, this unit was designed for smaller vessels and the weight was an issue. The unit was designed for insulation and to withstand rugged use, which resulted in the lid being extremely heavy and not ideal for everyday use. The new unit should have enough insulation to keep the oysters cool during the harvest and be designed with repeated use in mind, with lighter material to decrease the total weight.

Further investigations could also include more research into the effects icing has on oysters such as the stress an oyster goes through when rapidly cooled. Oysters are biological organisms and abrupt changes in the environment can affect them negatively.

REFERENCES

Bartlett, J. M., and D. Stirling. 2003. A short history of the polymerase chain reaction PCR protocols. Humana Press. Pp. 3-6.

Bejan, A. 2013. Convection Heat Transfer. John Wiley and Sons Inc.

Berrigan M, T. Candies, J. Cirino, R. Dugas, C. Dyer, J. Gray, T. Herrington, W. Keithly, R. Leard, J. R. Nelson, and M. V. Hoose. 1991. The oyster fishery of the Gulf of Mexico, United States: a regional plan management. Gulf States Marine Fisheries Commission, Ocean Springs, Mississippi. Number 24.

Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein. 1979. Disease caused by a marine vibrio: Clinical characteristics and epidemiology. New England Journal of Medicine. 300:1-5.

Blodgett, R. 2010. Most probable number determination from serial dilutions. Bacteriological Analytical Method. 9.

Bustin, Stephen. 2005 . Real-Time PCR. Encyclopedia of diagnostic genomics and proteomics. 10:1117-1125.

Byrum, M. C. 2014. Optimizing bioengineered coastal materials. Master thesis. Louisiana State University, Baton Rouge, Louisiana, USA.

Campbell, M. S., and A. C. Wright. 2003. Real-Time PCR analysis of *Vibrio vulnificus* from oysters. Applied Environmental Microbiology. 69:7137-7144.

Carriker M. R. and P. M. Gaffney. 1996. A catalogue of selected species of living oyster (*Ostreacea*) of the world in Kennedy VS, Newell RIE, and Eble AF. The Eastern Oyster *Crassostrea virginica.* Maryland Sea Grant College, University of Maryland, College Park, Maryland. 1-18.

Cholerae and other vibrio illness surveillance overview. 2011. Centers for Disease Control and Prevention (CDC). US Department of Health and Human Services, Atlanta, GA. http://http://www.cdc.gov/ncezid/dfwed/pdfs/nat-covis-surv-overview-508c.pdf.

Chowdhury, M. A. R., H. Yamanaka, S. Miyoshi, and S. Shinoda. 1990. Ecology and seasonal distribution of *Vibrio parahaemolyticus* in aquatic environments of a temperate region. FEMS Microbiology Ecology. 74: 1-10.

Colby, J. W., L. G. Enriquez-Ibarra and G. J. Flick. 1993. Shelf Life of Fish and Shellfish. Shelf Life Studies of Foods and Beverages. Chemical, Biological, Physical and Nutritional Aspects. G. Charalambous. New York, Elsevier, New York, NY. 33: 85-143.

Cole, K. M., J. Supan, A. Ramirez, and C. N. Johnson. 2015. Suspension of oysters reduces the populations of *Vibrio parahaemolyticus* and Vibrio vulnficius. Letters in Applied Microbiology. 61:209-213.

Cook, D. W. 1991. Microbiology of bivalve molluscan shellfish. D.R. Ward, and C. Hackney (ed.), Microbology of Marine Food Products. P. 19-39.

Cook, D. W., P. O'Leary, J. C. Hunsucker, E. M. Sloan, J. C. Bowers, R. J. Blodgett, and A. DePaola. 2002. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: A national survey from June 1998 to July 1999. Journal of Food Protection. 65: 79-87.

Daniels, N. A., L. MacKinnon, R. Bishop, S. Altekruse, B. Ray, R. M. Hammond, S. Thompson, S. Wilson, N. H. Bean, P. M. Griffin, and L. Slutsker. 2000. *Vibrio parahaemolyticus* infections in the United States, 1973–1998. Journal of Infectious Diseases 181:1661–1666.

Dixon, D. W. and G. E. Rodrick. 1990. Comparative Effects of Ionizing Radiation and High Energy Electron Beams on Molluscan Shellfish. Annual Conference: Tropical and Subtropical Fisheries Technological Conference of the Americas, in Joint Meeting with Atlantic Fisheries Technology Conference, Orlando, FL, Florida Sea Grant Coll. Program, Gainesville, FL.

Dorak, Tevfik. 2006. Real-time PCR. New York: Taylor & Francis Group.

Dombroski C. S., L.-A. Jaykus, and D.P. Green. 1999. Occurrence and Control of *Vibrio vulnificus* in Shellfish. Journal of Aquatic Food Product Technology 8(4):11-25.

Drake, S. L., R. Beverely, A. Chawla, M. Janes, J. Supan, J. Bell, J. F. Levine, and L. A. Jaykus, 2009.Comparison of traditional thermocouples and data loggers for simplified temperature monitoring using shellstock oysters as a model. Food Prot. Trend. 29:268-271.

Drake, S. L., A. DePaola, and L.-A. Jaykus. 2007 An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Comprehensive Reviews in Food Science and Food Safety. 6:120-144.

Duan, J. and Y.-C. Su. 2005. Occurrence of *Vibrio parahaemolyticus* in two Oregon oyster growing bays. Journal of Food Science. 70:M58-M63.

East Coast Shellfish Growers Association (ECSGA), 2014. http://www.ecsga.org. Accessed: December 4, 2015.

Eble, A. F. and R. Scro. 1996. General Anatomy. The Eastern Oyster *Crassostrea virginica*. V.S. Kennedy, R.I.E. Newell and A.F. Eble. College Park, Maryland, Maryland Sea Grant College, University of Maryland. 19-74.

Erlich, H. A. 1989. Polymerase chain reaction. Journal of Clinical Immunology. 9(6): 437-447.

Food and Agricultural Organization of the United Nations (FAO). Fisheries and Aquaculture Dept. *Crassostrea virginica* Gmelin. 1791. http://www.fao.org/fishery/culturedspecies/Crassostrea_virginica/en. Accessed: December 8, 2015.

Froelich, B. and J. D. Oliver. 2013. The interactions of *Vibrio vulnificus* and the oyster *Crassostrea virginica*. Microbial Ecology. 65:807-816.

Givens, C. E., J. C. Bowers, A. DePaola, J. T. Hollibaugh, and J. L. Jones. 2014. Occurrence and distribution of *Vibrio vulnificus* and *Vibrio parahaemolyticus* – potential roles for fish, oyster, sediment, and water. Letters in Applied Microbiolgy.

Goldmintz, D. and R. Ernst. 1979. "Pasteurization of Oysters." Proceedings of the National Shellfish Association 69: 200.

Gomez-Martinez, O., M. Zambrano-Arjona, and J. J. Alvarado-Gil. 2002. Imaging of subsurface defects in bivalve shells by photothermal techniques. Materials Research Society. In: Material Research Society Symposium Proceedings. Vol. 711, Merida, Mexico.

Gooch, J. A., A. DePaola, J. Bowers, and D. I. Marshall. 2002. Growth and survival of *Vibrio parahaemolyticus* in postharvest American Oysters. Journal of Food Protection. 65:970-974.

Groubert, T. N. and J. D. Oliver. 1994. Interaction of *Vibrio vulnificus* and the Eastern oyster, *Crassostrea virginica*. Journal of Food Protection. 57:224-228.

Guo, X., G. A. DeBrosse, and S. K. Allen Jr. 1996. All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture. 142: 149-161.

Hlady, W. G. 1997.*Vibrio* infections associated with raw oyster consumption in Florida, 1981-1994. Journal of Food Protection. 60: 353-357.

Hlady, W. G. and K. C. Klontz. 1995. The epidemiology of Vibrio infections in Florida, 1981-1993. Journal of Infections Diseases. 173:1176-1183.

Hoi, Lise, I. Dalsgaard, and A. Dalsgaard. 1998. Improved Isolation of *Vibrio vulnificus* from Seawater and Sediment with Cellobiose-Colistin Agar. Applied and Environmental Microbiology. Vol. 64, No. 5 p. 1721-1724.

Iwamoto, M., T. Ayers, B. E. Mahon, and D. E. Swedlow. 2010. Epidemiology of seafood-associated infections in the United States. Clinical Microbiology Reviews. 23:399-411.

Johnson, C. N. 2013. Fitness factors in Vibrios: a mini-review. Microbial Ecology. 65:825-851.

Jones, J. L., C. H. M. Luedeke, J. Bowers, K. DeRosia-Banick, D. H. Carey, and W. Hastback. 2014. Vibrio cholera, V. vulnificus, and V. parahaemolyticus abundance in oysters (*Crassostrea virginica*) and clams (Mercenaria mercenaria) from Long Island Sound. Applied Environmental Microbiolgy. 24: 7667-7672.

Kaneko, T., and R. R. Colwell. 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Journal of Bacteriology. 113:24-32.

Kaneko, T., and R. R. Colwell. 1975. Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Applied and Environmental Microbiology. 30:251-257.

Kaysner, C. A. and A. DePaola. 2001. Vibrio. In: F.P. Downes and k. Ito (eds.), Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington, DC. Pp. 405-420.

Kaysner, C. A. and A. DePaola. 2004. Vibrio. Bacteriological Analytical Method. O. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/default.htm.

Kelly, M. T. and A. Dinuzzo. 1985. Uptake and clearance of *Vibrio vulnificus* from Gulf Coast oysters (*Crassostrea virginica*). Applied and Environmental Microbiology. 50:1548-1549.

Kennedy, V. S., R. I. E. Newell, and A. F. Eble. 1996. The eastern oyster *Crassostrea virginica*. College Park, Maryland, Maryland Sea Grant College, University of Maryland. pp. 734.

Kilgen, M. 1994. Other Gulf Coast oyster research, p. 111–116. In W. Watkins and S. McCarthy (ed.), Proceedings of the 1994 Vibrio vulnificus Workshop. Office of Seafood, Washington, D.C.

Kinsey, Thomas, K. Lydon, J. Bowers, and J. Jones. 2015. Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (*tdh+/trh+*) *Vibrio parahaemolyticus* Levels. Journal of Food Protection. No. 8 pp. 1428-1617.

Larsen A, Rikard S, Walton W and Arias CR. 2012. Effective reduction of *Vibrio vulnificus* in the Eastern oyster (*Crassostrea virginica*) using high salinity depuration. Food Microbiology.

Leonhardt, J. M. 2013. An evaluation of oyster stocks, grow-out conditions, and off-bottom culture methods for increasing commercial production of eastern oysters (*Crassostrea virginica*) in the Northern Gulf of Mexico. Masters Thesis. Louisiana State University, Baton Rouge, Louisiana, USA.

Levine, W. C., and P. M. Griffin. 1993. Vibrio infections on the Gulf coast: results of first year of regional surveillance. Journal of Infectious Diseases.167:479-483.

Louisiana Department of Health and Hospitals (LDHH), 2014. Shellfish Sanitation Program, Refrigeration requirements; http://new.dhh.louisiana.gov/index.cfm/page/629; Accessed: December 4, 2015.

Louisiana Oyster Dealers and Growers Association (LODGA), 2014. http://www.louisianaoystersdealersandgrowersassn.org. Accessed: December 4, 2015.

Martin, D., Supan J., Theriot J., and Hall, S., 2007. Development and Testing of a Heat-Cool Methodology to Automate Oyster Shucking. Aquacultural Engineering 37 (2007), 53-60.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5:607–625.

Melody, K., R. Senevirathne, M. Janes, L. A. Jaykus and J. Supan. 2008. Effectiveness of icing as a post-harvest treatment control of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in the Eastern oyster (*Crassostrea virginica*). Journal of Food Protection 71(7): 1475-1480.

Menzel, R. W. 1951. Early Sexual Development and Growth of the American Oyster in Louisiana Waters. Science. pp 719-721.

Morris, J. G. and R. E. Black. 1985. Cholera and other vibrioses in the United States. New England Journal of Medicine. 314:678-681.

Motes M. L. and A. De Paola. 1996. Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*), Appl. Environ. Microbiol. 62: 3875.

Mullis, K. B. 1990. Target amplification for DNA analysis by the polymerase chain reaction. Annales de Biologie Clinique. 48(8):579-582.

Murphy, S. K., and J. D. Oliver. 1992. Effects of temperature abuse on survival of *Vibrio vulnificus* in oysters. Applied and Environmental Microbiology. 58:2771-2775.

National Oceanic and Atmospheric Association (NOAA) Eastern Oyster Biological Review Team (EOBRT). 2007. Status review of the eastern oyster (*Crassostrea virginica*). Report to the National Marine Fisheries Service, Northeast Regional Office. February 16, 2007. pp 105.

National Shellfish Sanitation Program (NSSP). Guide for the control of molluscan shellfish 2013 Revision. 2013. U.S. Food and Drug Administration. http://www.fda.gov/Food/GuidanceRegulation/FederalStateFoodPrograms/ucm2006754.htm.

Newton, A., M. Kendall, D. J. Vugia, O. L. Henao, and B. E. Mahon. 2012. Increasing rates of vibriosis in the United States, 1996-20010: Review of surveillance data from 2 systems. Clinical Infectious Diseases. 54 Suppl 5:S391-5.

Nicholls, K. M., J. V. Lee, and T. J. Donovan. 1976. An evaluation of commercial thiosulfate citrate bile salts sucrose agar (TCBS). Journal of Applied Microbiology. 41(2):265-269.

Nordstrom, J. L. and A. DePaola. 2003. Improved recover of pathogenic *Vibrio parahaemolyticus* from oysters using colony hybridization following enrichment. Journal of Microbiological Methods. 52(2):273-277.

Nordstrom, J. L., M. C. L. Vickery, G. M. Blackstone, S. L. Murray, and A. DePaola. 2007. Development of a multiplex Real-Time PCR assay with an Internal Amplification Control for the detection of total and pathogenic *Vibrio parahaemolyticus* in oysters. Applied Environmental Microbiology. 73:5840-5847.

Oliver, J. D. 2005. *Vibrio vulnificus.* In: Oceans and Health: Pathogens in the Marine Environment, Chap 10. S. Belking & R. R. Colwell (ed). Springer, New York. 253-276.

Oliver, J.D. 2012. *Vibrio vulnificus*: Death on the half shell, a personal journey with the pathogen and its ecology. Microbial Ecology. 65:793-799.

Oliver, J. D. and R. Bockian. 1995. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Applied Environmental Microbiology 61(7):2620-23.

Pankicker, G., D. R. Call, M. J. Krug, and A. K. Bej. 2004. Detection of pathogenic vibrio spp. in shellfish by using multiplex PCR and DNA microarrays. Applied and Environmental Microbiology. 70(12): 7436-7444.

Rippley, S. R. 1994. Infectious diseases associated with molluscan shellfish consumption. Clinical Microbiology Reviews. 10: 419-425.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the Unites States-major pathogens. Emerging Infectious Disease. 17:7-15.

Shapiro, R. L., S. Altekruse, L. Hutwagner, R. Bishop, R. Hammond, S. Wilson, B. Ray, S. Thompson, R. V. Tauxe, and the Vibrio Working Group. 1998. The role of Gulf Coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988–1996. Journal of Infectious Diseases. 178:752–759.

Shays, D. R. and F. W. Wheaton. 1980. Biological Properties of Shellfish Shells. Paper No. 80-5048. American Society of Agricultural Engineers, St. Joseph, Michigan.

Shumway, S.E. 1996. Natural and Environmental Factors. The Eastern Oyster Crassostrea viriginica. V.S. Kennedy, R.I.E. Newell and A.F. Eble. College Park, Maryland, Maryland Sea Grant College, University of Maryland. pp 467-514.

Souza, C. P., B. C. Almeida, R. R. Colwell, and I. N. G. Rivera. 2011. The importance of chitin in the marine environment. Marine Biotechnology. 13:823-830.

Su, Y. C. and C. Liu. 2007. *Vibrio parahaemolyticus*: A concern of seafood safety. Food Microbiology. Vol 26(6): 549-558.

Thomas, M. A., S. G. Hall, and J. Supan. Effects of rapid cooling of eastern oysters *Crassostrea virginica* using an onboard chilling unit. Poster presentation at the World Aquaculture Society meeting, Las Vegas, NV, February 22-26, 2016.

Thompson, R. J., R. I. E. Newell, V. S. Kennedy, and R. Mann. 1996. Reproductive processes and early development. In: V. S. Kennedy, R. I. E. Newell and A. F. Eble, editors. The Eastern Oyster *Crassostrea virginica*. Maryland Sea Grant College, University of Maryland, College Park, Maryland. pp. 335-370.

Ward, J. E., R. I. E. Newell, R. J. Thompson, and B. A. Macdonald. 1994. In vivo studies of suspension-feeding processes in the eastern oyster, *Crassostrea virginica* (Gmelin). The Biological Bulletin. 186: 221-240.

Welty, J. R., C. E. Wicks and R. E. Wilson. 1976. Fundamentals of Momentum, Heat, and Mass Transfer 2. John Wiley and Sons Inc.

Wheaton, F. 2007. Review of the properties of eastern oysters, *Crassostrea virginica* Part 1. Physical properties. Aquacultural Engineering. 37:3-13.

Wheaton, F. 2007. Review of oyster shell properties Part II. Thermal Properties. Aquacultural Engineering. 37: 14-23.

Young, E. F. 2010. Reproduction in female diploid and triploid eastern oysters (*Crassostrea virginica*): Comparison of gonadal changes correlated with prostaglandin E_2. Masters Thesis. Louisiana State University, Baton Rouge, Louisiana, USA.

APPENDIX A : PC208W LOG PROGRAM

```
;{21X}
;
*Table 1 Program
  01: 1.0000    Execution Interval (seconds)

1:  Batt Voltage (P10)
 1: 1       Loc [ BatVolt    ]

2:  Internal Temperature (P17)
 1: 2       Loc [ RefTemp    ]

3:  Thermocouple Temp (DIFF) (P14)
 1: 8       Reps
 2: 1       5 mV Slow Range
 3: 1       DIFF Channel
 4: 1       Type T (Copper-Constantan)
 5: 2       Ref Temp (Deg. C) Loc [ RefTemp    ]
 6: 3       Loc [ RefTemp_2 ]
 7: 1.0     Mult
 8: 0.0     Offset

4:  Do (P86)
 1: 10      Set Output Flag High

5:  Sample (P70)
 1: 11      Reps
 2: 1       Loc [ BatVolt    ]

*Table 2 Program
  02: 0.0000    Execution Interval (seconds)

*Table 3 Subroutines

End Program

-Input Locations-
1 BatVolt   5 1 1
2 RefTemp   9 2 1
3 RefTemp_2 13 1 1
4 RefTemp_3 9 1 1
5 RefTemp_3 9 1 1
6 RefTemp_4 9 1 1
7 RefTemp_5 9 1 1
8 RefTemp_6 9 1 1
```

9 RefTemp_7 9 1 1
10 RefTemp_8 25 1 1
11 RefTemp_9 9 0 1
12 RefTem_10 17 0 1
13 _____ 0 0 0
14 _____ 0 0 0
15 _____ 0 0 0
16 _____ 0 0 0
17 _____ 0 0 0
18 _____ 0 0 0
19 _____ 0 0 0
20 _____ 0 0 0
21 _____ 0 0 0
22 _____ 0 0 0
23 _____ 0 0 0
24 _____ 0 0 0
25 _____ 0 0 0
26 _____ 0 0 0
27 _____ 0 0 0
28 _____ 0 0 0
-Program Security-
0
0000
0000

Appendix B : SAS Program

```
DATA data5;
INPUT SM $ TRT $ SACK $ RT $ Y1 Y2;
DATALINES;
1 1 1 1 1.361727836 3.968482949
1 1 1 2 2.176091259 3.662757832
1 1 1 3 1.361727836 2.968482949
1 1 1 4 0.875061263 2.875061263
1 1 2 1 1.968482949 3.361727836
1 1 2 2 1.968482949 3.875061263
1 1 2 3 1.968482949 2.968482949
1 1 2 4 1.968482949 2.633468456
1 1 3 1 1.633468456 3.380211242
1 1 3 2 1.875061263 3.662757832
1 1 3 3 0.968482949 3.301029996
1 1 3 4 1.301029996 3.662757832
1 2 4 1 1.579783597 3.662757832
1 2 4 2 1.633468456 4.662757832
1 2 4 3 1.361727836 3.633468456
1 2 4 4 0.968482949 2.968482949
1 2 5 1 2.380211242 4.176091259
1 2 5 2 2.662757832 4.380211242
1 2 5 3 1.875061263 2.633468456
1 2 5 4 2.380211242 3.176091259
1 2 6 1 1.361727836 3.662757832
1 2 6 2 1.361727836 4.041392685
1 2 6 3 0.633468456 2.380211242
1 2 6 4 0.968482949 2.579783597
2 1 1 1 3.968482949 3.968482949
2 1 1 2 4.380211242 4.662757832
2 1 1 3 4.079181246 3.633468456
2 1 1 4 4.380211242 3.361727836
2 1 2 1 3.662757832 3.662757832
2 1 2 2 4.176091259 3.968482949
2 1 2 3 3.968482949 4.176091259
2 1 2 4 3.361727836 2.968482949
2 1 3 1 4.041392685 4.041392685
2 1 3 2 3.633468456 3.662757832
2 1 3 3 3.380211242 2.968482949
2 1 3 4 3.633468456 2.662757832
2 2 4 1 3.380211242 3.380211242
2 2 4 2 3.662757832 3.380211242
2 2 4 3 3.380211242 2.968482949
2 2 4 4 3.380211242 3.662757832
2 2 5 1 3.875061263 3.968482949
2 2 5 2 3.662757832 3.875061263
2 2 5 3 2.875061263 2.968482949
2 2 5 4 3.176091259 3.176091259
2 2 6 1 4.041392685 3.662757832
2 2 6 2 3.662757832 4.041392685
2 2 6 3 3.662757832 2.633468456
2 2 6 4 3.662757832 2.633468456
3 1 1 1 2.968482949 1.875061263
3 1 1 2 2.322219295 1.875061263
```

3	1	1	3	2.633468456	0.968482949
3	1	1	4	3.361727836	0.633468456
3	1	2	1	2.968482949	1.968482949
3	1	2	2	3.633468456	1.875061263
3	1	2	3	2.633468456	0.633468456
3	1	2	4	2.633468456	0.176091259
3	1	3	1	2.875061263	0.462397998
3	1	3	2	3.361727836	1.633468456
3	1	3	3	2.361727836	0.968482949
3	1	3	4	1.968482949	-0.522878745
3	2	4	1	2.579783597	1.857332496
3	2	4	2	3.380211242	1.380211242
3	2	4	3	2.968482949	1.633468456
3	2	4	4	2.633468456	0.875061263
3	2	5	1	2.968482949	2.380211242
3	2	5	2	2.875061263	1.968482949
3	2	5	3	2.633468456	1.633468456
3	2	5	4	1.875061263	0.968482949
3	2	6	1	3.041392685	2.176091259
3	2	6	2	3.633468456	1.361727836
3	2	6	3	2.968482949	1.361727836
3	2	6	4	2.361727836	1.633468456

```
Run;

Proc Mixed Data=data5;
Class SM TRT SACK RT;
Model Y1=SM|TRT|RT/ddfm=KR;
Random SACK(SM*TRT);
LSmeans SM|TRT|RT;
Run;
Quit;
```

VITA

Melody Thomas was born in Tifton, Georgia. She moved to Baton Rouge, Louisiana as a teenager and later enrolled in Louisiana State University earning a Bachelor of Science in Biological Engineering in 2014. She continued at Louisiana State University working towards a degree of Master of Science in Biological and Agricultural Engineering.