DETERMINING THERMAL LETHALITY TO REDUCE PRESENCES OF POTENTIAL PATHOGENIC *VIBRIO* SPP. IN OYSTERS, *CRASSOSTREA VIRGINICA*

By

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To my friends, brothers and sisters who always supported me through my endeavors

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LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
BAM	bacteriological Analytical Manual
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Unit
CL	Critical Limit
CT	Cycle Threshold
DI	Deionized Water
EPIPT	End-Point Internal Product Temperature
mm	Millimeter
FDA	Food and Drug Administration
g	Grams
HACCP	Hazardous Analysis and Critical Control Points
ISSC	Interstate Shellfish Sanitation Conference
LA	Luria Burtani Broth with NaCl Agar
LBN	Luria Burtani Broth with NaCl
LB	Luria Burtani Broth
mL	Milliliters
mCPC	Modified Cellobiose Polymyxin B Colistin
MPN	Most Probable Number
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
NMFS	National Marine Fisheries Service
PBS	Phosphate Buffer Saline
PHP	Post-Harvest Processing
qPCR	Quantitative Real Time Polymerase Chain Reaction

RPM	Rotations Per Minute
SCP	Safe Cooking Practices
TCBS	Thiosulfate Citrate Bile Salts Sucrose
TSA	Tryptic Soy Agar
V.	*Vibrio*
VC	*Vibrio cholera*
VP	*Vibrio parahaemolyticus*
VV	*Vibrio vulnificus*
vol	Volume
wt	Weight

Abstract of Thesis Presented to the Graduate School
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Controls are necessary to prevent illness associated with *Vibrio* spp. in oysters, *Crassostrea virginica*. The primary bacterial pathogens associated with human disease attributed to oysters are *Vibrio parahaemolyticus, V. vulnificus,* and *V. cholerae* serogroups. The most commonly recommended control has been cooking but descriptions of the specific application of this control are limited.

The following D and z-values were calculated based on thermal consequences in a phosphate buffered saline (PBS): *V. vulnificus* CMCP6 were as follows: D_{48}=2.24 min, D_{50}=2.05 min, D_{55}=0.50 min and z-value=10.19^{o}C, *V. cholera* 01 N16961: D_{48}=2.36 min, D_{50}=1.96 min, D_{55}=0.52 min, and a z-value=10.31^{o}C and *V. parahaemolyticus* TX2103 was the most heat stable with D_{48}=3.02 min, D_{50}=1.99 min, D_{55}=0.72 min with a z-value=11.3^{o}C.

Trials conducted at the same temperatures on whole oysters to demonstrate the protective effects of the food matrix and suggests that at lower temperatures the food matrix provided a protective effect, but at 55^{o}C internal temperatures the protective effect was diminished.

Further trials assessing the effectiveness of routine commercial cooking procedures and the Food and Drug Administration's (FDA) recommendations in the US Food Code (2009) was conducted on chargrilled half-shell shucked oysters containing *Vibrio* spp. Field trials confirmed routine chargrilling and frying exceed internal product temperatures of 200^{o}F (93.3^{o}C) and >145^{o}F for 15 seconds. Results indicate restaurant standard cooking practices proved effective in reducing or eliminating the potential *Vibrio* spp. pathogens. These results can be used to validate cooking controls in Hazardous Analysis and Critical Control Points (HACCP) plan's designed for restaurant applications.

CHAPTER 1
INTRODUCTION

Vibrio vulnificus V. cholerae and *V. parahaemolyticus* are naturally occurring potentially pathogenic bacteria that are commonly found in *Crassostrea virginica* (oysters) throughout the Gulf Coast (Blake *et al.,* 1979). These halophilic, gram-negative bacteria are the most common cause of seafood related bacterial illness in the United States but are still considered rare (CDC, 2009). They can be accompanied by additional *Vibrio* spp. that are more commonly associated with water contaminants of human origin, i.e. *Vibrio cholerae* and other *Vibrio* spp. depending on the species and serotype present, that can result in non-approved harvest conditions. Together these bacteria are commonly referred to as opportunistic pathogens because healthy persons do not get *V. vulnificus* fatal infections and *Vibrio* spp. infections that can be vectored to consumers through consumption of raw or improperly cooked oysters.

Vibrio spp. related disease has increased regulatory action for more appropriate controls to reduce or eliminate the presence of these particular *Vibrio* spp. Many coastal states are actively developing and implementing new recommendations for *Vibrio* spp. control plans through the Interstate Shellfish Sanitation Conference (ISSC). These plans specify controls for shellfish obtained from approved harvest waters through proper product identification (tagging), reducing product temperatures immediately after harvest and throughout processing and distribution, and a possible intervention with innovative post-harvest processing (PHP) methods designed to reduce or eliminate potential pathogens (ISSC, 2012). Although the effectiveness of these controls as single or combined procedures is debated, all regulatory and commercial interests agree that

cooking is the safest and most effective control. The ISSC recommends following the US Food Code (2009) for the control of molluscan shellfish (ISSC, 2012).

The Centers for Disease Control and Prevention (CDC) recognizes an urgent need for additional controls in the face of the trends for increasing oyster-borne *Vibrio* spp. illnesses and the increasing percentage of immunocompromised consumers. According to the CDC (2012) the reported incidences of *Vibrio* spp. illnesses had significantly increased in 2010 when compared to 2006-2008; with a rise of 39% within 24 months. This situation could be further complicated by environmental warming trends (Cox *et al.,* 2000) that could extend the more problematic summer seasons that favor the presence of *Vibrio* spp. and correlates with the period of higher occurrence of recorded oyster related illness (CDC, 2012). Serious infections from consumption of raw oysters harboring the *Vibrio* spp. most often occur within individuals that show some underlying condition that compromises the defenses of the host. Common underlying conditions include diabetes, liver disease, immuno-compromised, the elderly or very young, alcoholic cirrhosis or hemochromatosis (Blake *et al.,* 1979). These immune-compromised conditions appear to be increasing in our aging society (CDC, 2009) and the persistent occurrence of *Vibrio* spp. illness and environmental trends suggests a need for better controls to reduce or eliminate *Vibrio* spp. encounters by oyster consumers. In response, the ISSC and the CDC continues to recommend methods to minimize the risk through the use of controls or hurdles to prevent bacterial presence and growth. Their recommended options include: rapidly refrigerating oysters after harvest, treating the oysters at the processing plant with heat, freezing or high pressure, and finally by thorough cooking (CDC, 2012).

The use of cooking to control bacterial infections from seafood is not always apparent in terms of actual procedures. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) was asked by the Food and Drug Administration (FDA) and the National Marine Fisheries Service (NMFS) to provide advice on cooking seafood in order to provide consumer messages and directions necessary to ensure the safety of seafood (NACMCF, 2008). Several conclusions were drawn, but overall and most importantly for proper cooking of oysters, the committee stated that there is a lack of thermal inactivation data for relevant pathogens in appropriate seafood due, at least in part, to the wide variety of products available and the many methods of cooking that are commonly applied to these products. The committee agrees that the microbial safety of seafood is enhanced greatly when it is properly handled, cooked, served, and stored; however, the committee still recognizes the fact that some consumers prefer to eat certain seafood products raw or undercooked. The committee comments that cooking methods for seafood products differ and often are not necessarily based on scientific data. Although seafood cooking recommendations are widely available, there is no easy, practical measurement or indicator for the consumer to objectively determine sufficient cooking in order to ensure the safety of fishery products (NACMCF, 2008).

The inactivation of infectious non-spore-forming pathogens using a heat treatment is a critical control point in the safe preparation of many foods. Insufficient processing, cooking or reheating are often contributing factors in food-poisoning outbreaks (Roberts, 1991). Many food-processing systems contain a heating step to reduce the number of bacteria in a product to enhance food safety and increases overall shelf-life of the

product (Asselt and Zwietering, 2005). Many of these cooking parameters are based on challenge tests, legislation and overall common experience. Heat resistance and injury of food-borne pathogens such as *Salmonellae*, *Staphylococcus aureus,* and *Clostridia* have been studied extensively; yet, much less attention has been given to the thermal stability characteristics of *Vibrio* spp. (Beuchat and Worthington, 1976).

CHAPTER 2
LITERATURE REVIEW

Current thermal inactivation data involving *Vibrio* spp. is limited and shows substantial variability depending on the medium and species used in the trial studies. *Vibrio parahaemolyticus* is commonly referred to as one the most heat stable *Vibrio* spp., with *V. cholerae* and *V. vulnificus* being closely related in terms of thermal liability (Joseph and Colwell, 1982). Further studies need to be conducted because no common medium or standard procedure has been used for all three species that would allow a more comprehensive comparison between the species.

Hinton and Grodner (1985) purchased fresh shrimp from local seafood stores and created a shrimp homogenate using three parts peeled shrimp and one part distilled water. An addition of the *Vibrio cholera*e organism provided a final inoculated homogenate concentration of approximately 10^6 CFU/gram. Four grams of inoculated homogenate was then added to Pyrex test tubes fitted with a copper-constantan to monitor the heating rate and history. Fifty gram samples of the injected shrimp were then cooked using one of two methods: boiling water or steam (100^oC) for 10 min. Table 2-1 shows the thermal resistance of *V. cholera*e conducted by Hinton and Grodner (1985) using the Most Probable Number (MPN) technique based on the previously listed methods.

Table 2-1. Heat resistance of *V. cholerae* in shrimp homogenate conducted by Hinton and Grodner, 1985.

Temperature	D-value (min)
48.9	9.17
54.4	0.43
60	0.39
65.5	0.32
71.1	0.31
76.7	0.30
82.2	0.28

The data displays a drastic drop from 48.9^{o}C with a D-value of 9.17 min to only 0.43 min at 54.4^{o}C without the remaining data following thermal death trends. Also, these results do not coincide with a study conducted on *Vibrio cholera* in blue crab homogenates (Shultz *et al.,* 1984). Raw crabmeat was placed in a sterile Warring Blender in 150g quantities and three mL of a 1:3 dilution of the *V. cholerae* inoculums were added to achieve a final concentration of 10^{6} CFU/g; followed directly by homogenization for 2 min (Shultz *et al.,* 1984). Next, 4 g of homogenate was aseptically placed in glass tubes and sealed with a surface mix gas-oxygen torch. Fifteen total tubes, six of which were equipped with thermocouples, were totally submerged in a water bath for various time increments at 49, 54, 60, 66, and 71^{o}C. Upon removal, the inoculated tubes were cooled rapidly in a water bath for 30 sec and the contents were aseptically transferred to alkaline peptone water (APW). The APW tubes were then incubated at 35^{o}C for 6-8 hr and recovery techniques taken from the FDA's Bacteriological Analytical Manual (BAM) were used to determine the final bacteria counts. Table 2-2 below displays the results.

Table 2-2. Heat resistance of *V. cholerae* in crabmeat homogenate conducted by Shultz *et al.* (1984)

Temperature	D-Value
49	8.15
54	5.02
60	2.65
66	1.60
71	0.30

When comparing Hinton and Grodner's *V. cholerae* experiments with shrimp (1985) at similar temperatures to the aforementioned studies conducted by Shultz *et al.* (1984), it is noticeable that a margin of difference in the D-values. At 48.9^{o}C Hinton and Grodner (1985) found a D-value of 9.17 min, whereas at 49^{o}C Shultz *et al.* (1984) found the D-value to be 8.15 min. This is similar enough to be associated with the medium itself attributing to this difference, but when comparing the remaining values, the trend differs exponentially. At 54.4^{o}C the shrimp homogenate changes from 9.17 to 0.43 min (Hinton and Grodner, 1985); whereas, in the crabmeat homogenate, a D-value of 8.15 min at 49^{o}C only drops to 5.02 min at a similar 5^{o}C increase to 54^{o}C. Even with different media, the bacteria themselves should act similarly within one medium. As the temperature increases again the values differ even more drastically. Hinton and Grodner (1985) found that at a temperature of 60 and 71.1^{o}C the respective D-values were 0.39 and 0.31 min; while Shultz *et al.* (1984) reports a D-value at 60 and 71^{o}C to be 2.65 and 0.30 min. The values start similarly at lower temperatures, veer off correlation towards the middle temperature range, and then end nearly identically. This matter is not expected in normal D-value studies.

Beuchat and Worthington (1976) conducted another study examining the thermal inactivation of *V. parahaemolyticus* where exponential phase cells, or bacterial cells in

the reproduction stage of life, were transferred to 190 mL of 0.1 M potassium phosphate containing 0.5%, 3.0% or 7.5% (wt/vol) NaCl at a pH of 7.2 with a concentration of approximately 10^7 CFU/mL for 3% NaCl-TSB and 10^7 CFU/mL for 0.5% and 7.5%. These mixtures were heated under constant agitation and samples were withdrawn at appropriate times, dispensed in a sterile chilled test tube and then serially diluted in 0.1 M potassium phosphate containing 3.0% NaCl. A tryptic soy agar (TSA) consisting of 1.2% agar and 3.0% NaCl was tempered at 42^{o}C and colonies were counted after 18-24 hr at 35^{o}C. Heat survivor curves of $\log_{10}$ viable number per milliliter versus time at 47^{o}C were plotted. With a growth temperature of 37^{o}C, 7.5% NaCl was inoculated and heated at 47^{o}C and produced a D-value of 65.1 min (Beuchat and Worthington, 1976). When compared to a study conducted in 1992 on *V. vulnificus* in a buffered saline solution at the same 47^{o}C, a D-value of 2.40 min was calculated (Cook and Ruple, 1992). This shows a difference of 62.7 min between similar mediums and subspecies that again shows a substantial variation between studies.

Thermal inactivation is not solely dependent upon the bacterial organism in question. Other factors for overall inactivation include: exposure time and temperature, pH, fatty acid composition, protein insulation and evaporative cooling. In addition bacterial concentration and location inside the specific mediums, water activity, rapid/slow cooling and heat penetration are also extremely important in the overall production of a safe product (Blackburn *et al.,* 1997). For example, the thermal inactivation of *Vibrio* spp. in PBS may vary slightly or drastically based upon the protective effects of the medium. PBS consists mostly of water, with low concentrations of phosphate and saline which should not play a major factor in the overall protection of

the bacteria from death, however, inside of an oyster exist large quantities of dispersed water, proteins and fats, as well as thicker portions of meats in which bacteria may be located that may inhibit heat penetration and ultimately shield and protect the *Vibrio* spp.

In the absence of adequate pathogen inactivation data for various seafood products, the current FDA recommendation for safe seafood is heating all parts of the food to 145^{o}F (63^{o}C) or above for 15 sec (FDA, 2009). These recommendations are listed in the US Food Code (2009), cooking section 3-401.11 for raw animal foods as compiled by the FDA working in company with the conference of Food Protection. The FDA base this decision on lethality data for *Salmonella*, a potential pathogenic bacterial contaminant commonly associated with seafood. The utility of these recommendations is not known relative to cooking oysters to eliminate *Vibrio* spp. but the expectation is that these recommendations will be sufficient in reducing potentially pathogenic *Vibrio* spp.

The second option offered in place of the monitoring of the internal time and temperature is mentioned in the Fish and Fishery Products Hazards and Controls Guidance for seafood processors. This is the nation's guidance document for compliance with the seafood HACCP regulations (21CFR Part 123). The HACCP manual provides options for simply monitoring End-Point Internal Product Temperatures (EPIPT) instead of continuous time and temperature monitoring during cooking or pasteurization. This is useful when reaching higher internal temperatures, mostly noted with deep frying, where shorter times are compensated by extremely high temperatures that bacteria cannot survive. Although this currently is offered as an option solely for

processors, restaurant settings may benefit from regulations that follow this manner as an alternative option to internal time and temperatures currently in place. The use of HACCP concepts in restaurant operations is encouraged in the US Food Code (2009) (FDA, 2009).

The Hazardous Analysis and Critical Control Points (HACCP) for Seafood recommends processes that create a 6D, or 6-log reduction, of potential pathogens for processors or distributors, but these do not apply to the restaurant level. Restaurants are not held to these standards because the food is cooked for immediate consumption. After processors reduce bacterial concentrations, the product may be transferred or stored for hours or days, allowing the surviving bacteria the time they need to regrow and increase concentrations, which is why the processors have such high D requirements. This is not the case with restaurant foods because they are consumed immediately after being cooked. This does not allow the time needed for any surviving bacteria to replicate again to the concentrations normally required for pathogenicity after cooking has occurred.

Current oyster cooking methods commonly used in restaurant industry range from chargrilling on an industrial gas grill, to steaming and deep frying; however, many restaurants now solely offer chargrilled and deep frying for several factors including ease and convenience. Also, average consumer demand is higher for these methods over steaming. Household consumers also grill and fry oysters, but most are limited on their ability to steam simply due to their lack of equipment.

Overall, mindful of prior restrictions and complications with confounding factors, additional research is necessary to better substantiate the recommendations and

dependence on appropriate cooking methods to control potential illness due to *Vibrio* spp. in raw oysters.

Determination of thermal death time (D-values) and corresponding z-values are appropriate methods to evaluate the performance of a thermal inactivation process. These concepts dictate required temperature and exposure time to effectively reduce or eliminate particular bacteria. These data can then be applied and altered to obtain thermal inactivation in different mediums in a laboratory or in food processing and handling situations. The same approach can be used to determine required cooking procedures for a retail or restaurant operation that prepares food for immediate consumption.

The D-value refers to the reduction time required at a certain temperature to kill 90%, or 1 log unit of the organism in question. Upon obtaining several D-values, a thermal destruction curve can be created by graphing the corresponding D-values to their appropriate times. The z-value is a temperature that is required for the thermal destruction curve to move 1 log cycle. Z-values can be calculated by taking the reciprocal of the slope resulting from the plot of the logarithm of the D-value versus the temperature at which the D-value was obtained. While the D-value gives the time needed to destroy organisms at a specific temperature, the z-value relates the resistance of an organism to differing temperatures (McLandsborough, 2004).

Mindful of the risk associated with potential pathogenic species of *Vibrio* bacteria vectored by oyster consumption, this study was conducted to provide more reliable measures for effective thermal treatments for cooking controls in restaurant and food service operations preparing oysters for immediate consumption. The scope of work

includes determinations of basic thermal parameters for bacterial lethality and comparisons against recommended FDA guidelines for general cooking of seafood and actual commercial (restaurant) practices.

CHAPTER 3
OBJECTIVES AND HYPOTHESIS

The main objective was to determine the thermal inactivation of pathogenic *Vibrio* spp. in order to obtain reliable and comprehensive pathogenic inactivation data to better direct appropriate cooking of oysters.

The hypotheses for this work included:

- *Vibrio vulnificus* CMCP6, *Vibrio parahaemolyticus* TX2103, and *Vibrio cholerae* N16961 have similar thermal-inactivation requirements.
- Protective food matrix effects may occur in whole oysters, but this will not be a sufficient enough barrier to prevent bacterial death during common industry cooking standards
- Cooking to an internal temperature of 145^{o}F for 15 sec as recorded in the US Food Code (2009) will sufficiently eliminate all three potentially pathogenic *Vibrio* spp. pathogenic threats in oysters, *Crassostrea virginica*.

CHAPTER 4
MATERIALS AND METHODS

The thermal parameters for bacterial lethality were first determined with media grown sources based on prior efforts to assure proper growth stages and conditions to monitor bacterial survival. The resulting thermal parameters were then compared with similar measures for thermal resistances during actual commercial operations to determine the resulting thermal parameters and the effectiveness of routine cooking procedures and recommended guidelines.

Bacterial Growth Curves

Growth curves are extremely important in determining the phase of bacterial growth. Many bacteria have different growth curves; however, nearly all follow the same pattern of growth: a lag, logarithmic, stationary and death phase. Practically, the mid-stationary phase is the most useful in these experiments because of the cells ability to withstand a greater range of stresses with a lower susceptibility to death. Most bacteria in foods tend to be in this phase. It is important to ensure that the bacteria used in the experiment is in a strong, mid-stationary phase for several reasons: it decreases the likelihood of variability during heat treatments, ensures the bacteria is metabolically sound with a majority of its energy going towards survival as opposed to reproduction, and finally to have a known quantity of bacterial concentration as a starting point. Triplicate experiments were conducted and the average $\log_{10}$ CFU/mL was plotted versus time to give the growth curve graph.

The selected bacterial strains included species previously associated with raw oysters. They include: *V. vulnificus* CMCP6, *V. parahaemolyticus* TX2103, and *Vibrio cholerae* N16961 and were obtained from the University of Florida Food Science and

Human Nutrition Department. Strains were stored as frozen stocks at -80^{o}C in Luria Burtani Broth with NaCl (LBN) and 50% glycerol with a pH of 7.5 and streaked onto LBN Agar (LA) for isolation and incubated at 37^{o}C overnight for each individual study. An isolated colony selected from the overnight LA was placed in 50mL of Luria Burtani Broth (LB) and incubated at 37^{o}C in a shaking incubator set to 90 rotations per minute (RPM) for 24 hr to ensure viable culture growth. Next, 1 mL of the incubated sample was inoculated into 50 mL of LB and placed into the shaking incubator with the same parameters. Every hr, including a time 0, serial dilutions of the sample were conducted in PBS test tubes in a ratio of 1:10 and 0.1 mL was aseptically spread plated onto LA in order to obtain an accurate measurement of the growth of the specific strains. Each sample was conducted with three replications to obtain the most solid statistical model of growth.

After each time point, the LA plates incubated at 37^{o}C for 24 hr to allow accurate growth of colonies. On the 24 hr mark, the plates were taken from the incubator and all individual viable colonies were counted and recorded for each time point at a dilution that met the requirements of being within 25-300 colony forming units (CFU) per plate. The recorded data were then converted into $\log_{10}$ CFU and graphed versus time to create a growth curve. These growth curves were used to determine the growth phase (e.g., lag, exponential, stationary and death phases) of the bacteria throughout their replication process.

For many human pathogens, the capacity to survive physical challenges during food processing is a critical step in their transmission to the host by the food-borne route (Rees *et al.,* 1995). Stationary phase cells are generally more resistant to a range of

stresses and inimical processes, environmental changes, temperature and pH alterations, and are known to be capable of surviving in conditions where logarithmic phase cells would tend to be more susceptible to death since a majority of their energy is put into reproduction as opposed to survival (Rees *et al.,* 1995). Therefore, cells from early stationary phase were used to determine bacterial survival in these studies.

Thermal Applications in Media

Following the substantiation for stationary growth, preliminary work was necessary to determine the conditions for thermal exposure of the *Vibrio* spp. in media. A come-up time, or the time required for the medium to reach the desired temperature, was determined for 5.0 mL of PBS in an identical test tube that was used during the thermo-tests for each temperature (48, 50, 55^{o}C). These heating temperatures were chosen based on previous studies that showed death occurring at these temperatures at a rate large enough to allow progressive measurements for change in bacterial levels. PBS (5.0 mL) at room temperature (21.2^{o}C ± 0.3) was dispensed into 16x125MM test tubes and the rate of temperature change in the tube of media. New, calibrated thermocouples were connected to an OCTTEMP 2000 and secured in the center of the test tube without contact to the side. A circulating water bath was filled with approximately 2.5-3.0 L of deionized water (DI water) and allowed to reach the respective temperature (48, 50, 55^{o}C). Fifteen test tubes filled with 5.0 mL of PBS at room temperature were placed in a test tube rack and two thermo-couples were placed in two test tubes at random to accurately create a thermo-profile and ensure no overloading of the water bath and its temperature would occur. A third thermocouple was placed inside the water bath to ensure the temperature remained constant.

Finally, an ice-slush was used for rapid the cooling and a fourth temperature probe was placed inside the ice-slush to verify the temperature remained at ~0^{o}C ± 0.4^{o}C. Once the circulating water bath reached the appropriate temperature, the test tube rack was placed in the center of the water bath and the timing began. The time was recorded for the PBS tubes to reach the desired temperature ± 0.4^{o}C, with three replications to accurately identify the come-up time at individual temperatures; once the last of the two test tubes containing the thermo-couple had reached the designated temperature the entire test tube rack was removed from the water bath and instantly put into the ice-slush. The time required to reduce the temperature of the PBS from the set point to 25^{o}C was recorded. Three replications were conducted, with two test tubes monitored per replication, giving a total of 6 temperature trials per study.

At 55^{o}C, the come up time was long enough to where the previous experiments (48 and 50^{o}C) provided data suggesting an elimination of a majority of the bacterial concentration before testing could begin. An additional study was conducted using an identical method to 48 and 50^{o}C with time points 0, 30 and 60 sec. The results proved that too large of a quantity of the *Vibrio* spp. were inactivated for this test to accurately define the lethality over the period of time required. Therefore, all studies conducted at 55^{o}C used pre-warmed PBS (4.5 mL) that were inoculated with 0.5 mL of bacteria was pipetted into each tube, individually, from a master mix. This ensured that testing could begin at a time zero, where no bacteria was lost in the come up and enough would remain to obtain a reliable death curve.

Determining D and z- Values in PBS Media

The first step in obtaining accurate D-values was to assure uniform methods across all experiments. Once the freezer stock bacteria was plated onto L-Agar and an

isolated colony was selected from the overnight growth, colonies were then allowed to culture overnight in L-Broth for 11-13 hr to ensure the bacteria had reached stationary phase as previously established in preliminary studies. After the bacteria reached mid-stationary phase, at approximately hr 16, it had the desired properties for overall strength and survival needed to conduct an accurate thermal-death matrix.

Freezer stock samples of each individual bacteria specimen were plated for isolation on LA and allowed to grow overnight at 37^{o}C in a standard incubator. An isolated colony of each was then inoculated into 50mL of LB for 16 hr, which was determined to be early stationary phase of the bacteria's growth based on prior analysis of the growth curves. This was performed to ensure all bacteria are in a strong metabolic, non-reproductive phase to guarantee the greatest heat resistance during trials and the least amount of variability possible between replications and experiments.

Next, 20mLs of the inoculated broth was put into a 50mL conical and spun in a centrifuge for 15 min at 3000 RPMs. The supernatant LB was then discarded, and the remaining specimen was re-suspended in 20 mL of PBS solution and vortexed until homogenized. The new solution was then used to create a master solution with a 1:10 dilution in PBS. Portions (5mL) of this solution was serologically pipetted into 5 sterile test tubes, after which the pipette tip was discarded and the master solution was re-vortexed in order to ensure even distribution of the sample. This procedure was repeated until the required amount of test tubes were filled.

Upon completion, the concentration of bacteria in the master sample was determined by serially diluting with a 1:10 ratio in PBS and spread plated onto LA before heat was administered. The same procedure was followed and plated on differential

media based on the subspecies: modified cellobiose polymyxin B colistin (mCPC) for *V. vulnificus* CMCP6, thiosulfate citrate bile salts sucrose (TCBS) for *V. cholerae* N16961, or CHROMagar for *V. parahaemolyticus* TX2103 and the results were compared to the counts on non-selective media to verify no contamination.

Once the overnight sample was plated, the circulating water bath was brought to the appropriate temperature, the ice-slush was prepared and the test tubes were racked in an identical setup to the preliminary come-up time procedure. The inoculated test tubes were then placed in the middle of the water bath and the time began.

After 2 min at 48^{o}C, a time 0 test tube was pulled to account for the initial death occurring during the initial heating. After achieving the desired temperature, every 4 minutes test tubes were pulled, cooled down in the ice-slush for 15 sec, and placed inside a new, dry test tube rack. The cool down period is used to immediately stop thermal related death and is as minimal as possible to prevent any death occurring from cold shock. After completion of all time intervals, test tubes were then serially diluted at a 1:10 ratio in PBS and 0.1mL of the heat-treated samples were plated onto LA and placed in a standard non-motion incubator over night at 37^{o}C. After 24 hr, the plates were counted in the standard range and the data was recorded. Finally, the colony numbers were converted into CFU/ml, then Log_{10} CFU/ml and graphed versus time. A linear trend of best fit was then applied to the graph to determine the D and z-values. The D-values were based on the thermal death that occurred at a specific temperature, whereas the z-value was determined based off of the results of all three D-value studies.

Identical methods were followed for preparation at 50^{o}C as 48^{o}C with the come up time varying slightly, from 2 min to 2.5 min, with the same 15 sec cool down period. The procedure for D and Z-values at 50^{o}C followed the methods at 48^{o}C with only the time interval altering from 4 min at 48^{o}C to 1 min intervals at 50^{o}C. This change allows for a more accurate death calculation with the increased death occurring from higher temperatures and energy in the system.

Due to the heat-sensitivity of *Vibrio* spp., the normal come-up time procedure was altered at this higher temperature in order to prevent high levels of death before reaching the desired time points. In order to prevent this, 4.5mL of PBS was pipetted into test tubes and the appropriate quantity of test tubes were then racked and placed in a circulating water bath set to 55^{o}C. Two temperature probes were inserted at random into two test tubes and the PBS was allowed to come up to 55^{o}C. Once the last monitored test tube reached 55^{o}C, an additional 5 min was given in order to prevent any tubes from hovering below the required temperature.

A portion (0.5mL) of the overnight sample, which was prepared and quantified identically to D48's procedure, was individually inoculated into the 4.5 mL heat-treated PBS producing a 1:10 dilution of the overnight sample. The time points were set to 30 sec intervals and upon reaching the time point, the test tube was pulled and instantly iced down in the same ice-slush for 15 sec in order to instantaneously end kill. The same quantification methods as the previous D48 and D50 were then followed to calculate the survival of the bacteria at this temperature.

Each sample was conducted in triplicate, and then the average CFU/mL was calculated as well as a standard deviation for each trial. The results were then plotted

on a time versus average $\log_{10}$CFU/mL. Once plotted, a linear regression for the average log was plotted and used to calculate the D-value by solving the equation with a 1 log unit reduction, or a reduction of the y-axis by 1 log unit.

Thermal Assessments with Oysters

Food products are commonly known to create a protective effect of bacteria lethality for many reasons including: evaporative cooling, protection from proteins, lipids and water in the systems, insulation from thicker or more dense portions of the product and the ability for heat to transfer to evenly throughout the system. Because of this phenomenon, trials were conducted in oysters at 48, 50 and 55^{o}C in order to evaluate the overall protective effect encountered in oysters at these lower-heat temperatures.

Preliminary tests were conducted on oyster homogenates (1:1 in PBS); however, protein separation, gelling and layering occurred. Further separation between the medium and the oysters as time and heat increased. Due to the non-uniformity of the homogenate after the heating process heating whole oysters as opposed to the homogenates were used in subsequent studies in order to provide greater accuracy of the thermal inactivation and the homogenate method was abandoned in favor of the use of whole oysters.

Live oyster samples were purchased from a local vendor in Apalachicola and levels of naturally occurring *Vibrio* spp. in oysters were increased by temperature abused using incubation at 26^{o}C for 24 hr. The oysters were subsequently stored at room temperature (ca. 21^{o}C). Initial *Vibrio* spp. concentration in oysters was determined from a standard weight of oysters (100g; ~10-12 oysters) that were shucked and placed into a Warring Blender and mixed with an equivalent amount of PBS to produce a 1:2 homogenate. The mixture (20 mL) was added to 80mL of PBS to form a 1:10 dilution of

oyster to PBS. Serial 10 fold dilutions were performed by taking 11 mL of sample to 99 mL of PBS (1:10) out to the 10^{-6} dilution. These dilutions were inoculated into APW with a pH of 8.5 ± 0.2 in triplicate, placed in an incubator set to 37^{o}C and allowed to grow overnight. All test tubes that were visually positive for growth in APW were recorded and processed through the DuPont Vibrio BAX system (Dupont, Wilmington, US) system for real time qPCR of *Vibrio* spp. All results were obtained using the qPCR were then manually analyzed and all positives with a cycle threshold (CT) value above 32 were excluded and considered a false positive. All positive APW test tubes were compared to confirmed *Vibrio* spp. positives from the BAX, and any qPCR *Vibrio* negatives based on the analyzed BAX results were removed in the calculation of MPN in order to obtain an overall death of *Vibrio* only and to eliminate any other bacteria that may be producing a positive in later dilutions that were not necessarily *Vibrio* spp. This was repeated in triplicate with four separate temperature abused samples in order to obtain the most likely amount of *Vibrio* bacteria in the samples.

A sample (100 g) of live oysters from the same temperature abused sampling were shucked and put into a 1 gallon size Ziploc® (Johnsons & Son, Racine, US) brand bag. The bags were fitted with three temperature probes attached to an OCTTEMP2000 (Thermoworks, Lindon, US), the temperature recorder. The probes were inserted into three random oysters and the bag was inserted into a circulating water bath set to 48^{o}C. Once the last probed oyster (considered the worst case scenario) reached an internal temperature of 48^{o}C, a timer set for 9 min and 4 sec began which was determined from a three log reduction based on the PBS D-values of *V. parahaemolyticus* conducted in previous experiments. This control point was chosen due to its heat stability over *V.*

vulnificus and *V. cholerae.* Once time had expired, the samples were immediately placed in an ice-slush to instantly stop death. The same MPN procedure as the overnight experiments was then followed, and false *Vibrio*-positives were removed.

The same procedure was conducted for the 50^{o}C heat treatment as the 48^{o}C with the exception of the water bath and internal temperature being set to 50^{o}C and the time point altered to 5 min and 58 sec based on the three log reduction D50 value conducted in PBS. The same MPN procedure was used in all heat treatment studies.

The identical procedure was conducted for the 55^{o}C heat treatment as the previous two heat treatments at 48 and 50^{o}C with the same alteration for water bath and internal temperature. The new set point for cooking was also changed based on a 3 log unit reduction in PBS from the D55 study from *V. parahaemolyticus* equating to a new time of 2 min 10 sec. The same MPN procedure as the previous two heat treatment studies was used to calculate the quantity of surviving bacteria.

Assessing Commercial Cooking Procedures

In order to compare lab-based results with actual commercial cooking procedures a series of trials were conducted to monitor thermal history of oysters when cooked by chargrilling or frying relative to bacterial reduction. The approached involved measuring thermal consequence in actual restaurant settings and using these results to confirm the bacterial consequences in controlled trials. The cooking method of choice was chargrilling. The chargrill method has recently become one of the most popular restaurant forms and appears to be replacing the traditional steamed form. Chargrilling in a restaurant setting involves shucking the oyster on the half-shell, then cooking the half-shell shucked oyster directly on a gas chargrill. Restaurants may flavor and butter the product differently; however, they all have the similar grills and cooking procedures.

Recording of the internal temperatures during routine commercial practices was conducted on site at reputable restaurants (Drago's Seafood Restaurant & Oyster Bar and Acme Oyster House, New Orleans) as the actual cooking procedures occurred to determine if common industry cooking practices meet, fall short of, or exceed lethal expectations and requirements based on the newly calculated D and z-values. The results from the on-site determinations were used to stage a series of similar trials measuring the consequence for bacterial loads in the same oysters, raw and after chargrilling.

Raw oysters were purchased directly from distributers in Apalachicola, Florida and were temperature abused at 26^{o}C overnight in an incubator. The next morning, bacterial concentrations were conducted by taking 100 g of shucked oyster, serially diluting out to 10^{-6} followed by spread plating on both selective media (mCPC and CHROMagar (CHROMagar, Paris, France)) as well as non-selective media (LA). CHROMagar was used to calculate the concentration of presumptive *V. parahaemolyticus* (identified by a mauve color), mCPC was used to calculate presumptive *V. vulnificus* (yellow colonies) and LA was used for total bacterial concentrations. Once spread plating was completed, the inoculated plates were stored overnight in an incubator set to 37^{o}C and the next morning the plates were counted and the CFU/mL was recorded. The assumption that there is no *V. cholerae* in natural oysters was applied and TCBS was not used to calculate *V. cholerae* concentrations in order to prevent false positives from *proteus/enterococci* on the media and skewing final results.

An industrial gas grill was used to simulate the popular chargrilled method conducted onsite in many commercial settings. The center of the grill was recorded to

have temperatures exceeding 450^{o}F, while the outer skirts ranged from 420-445^{o}F, never dipping below 420^{o}F on any portion of the grills surface. The temperature-abused oysters were then shucked to the half shell and temperature probes were inserted into the oyster. Twelve oysters, three with temperature probes, were then placed (shell side down) on the grill near the center, identically to how it was observed inside a restaurant setting. The oysters were allowed to come to 200^{o}F, then were promptly removed from the grill and allowed to cool naturally to near room temperature. The oysters were then aseptically shucked into a sterile, stomacher bag and the contents were then blended in a Warring Blender. PBS dilutions and plating procedures were identical to those of the overnight temperature abused samples (mCPC, CHROMagar, and LA).

Assessing Regulatory Guidelines

The final examination was conducted on a process many commercial settings follow from the 2009 US Food Code: an internal temperature of 145^{o}F (62.78^{o}C) for 15 sec that is commonly based on the inactivation of *Salmonella* (FDA, 2009). Overnight temperature abused oysters were again shucked and left on the half shell. Again, twelve oysters, three with temperature probes, were placed shell side down on the grill near the center. The oysters were allowed to come up to 145^{o}F, and upon reaching that internal temperature a timer was started. After 15 sec, the oysters were removed from the grill and allowed to cool naturally. The identical steps to the 200^{o}F test above were conducted and the following morning, all selective and non-selective plates were counted to determine overall *Vibrio* spp. lethality as well as overall bacterial lethality. Three replications of each experiment, including the overnight, were conducted to increase overall reliability of the data as well as statistical analysis.

CHAPTER 5
VIBRIO SPP. GROWTH CURVES

Initial work determined the parameters for reaching a stationary phase prior to use in thermal trials. Figure 5-1 shows *V. cholerae* N16961 entering stationary phase after approximately 9 hr. The mid-stationary phase for this bacteria was determined to be between hr 11-13 with an approximate concentration of 10^{10} CFU/mL or very high 10^{9} CFU/mL. Figures 5-2 and 5-3 show *V. cholerae, V. vulnificus* CMCP6 and *V. parahaemolyticus* TX2103 with a final concentration of 10^{9} with the same 11-13 hr mid-stationary phase. All bacteria have extremely similar growth patterns, times and final concentrations based on these initial studies.

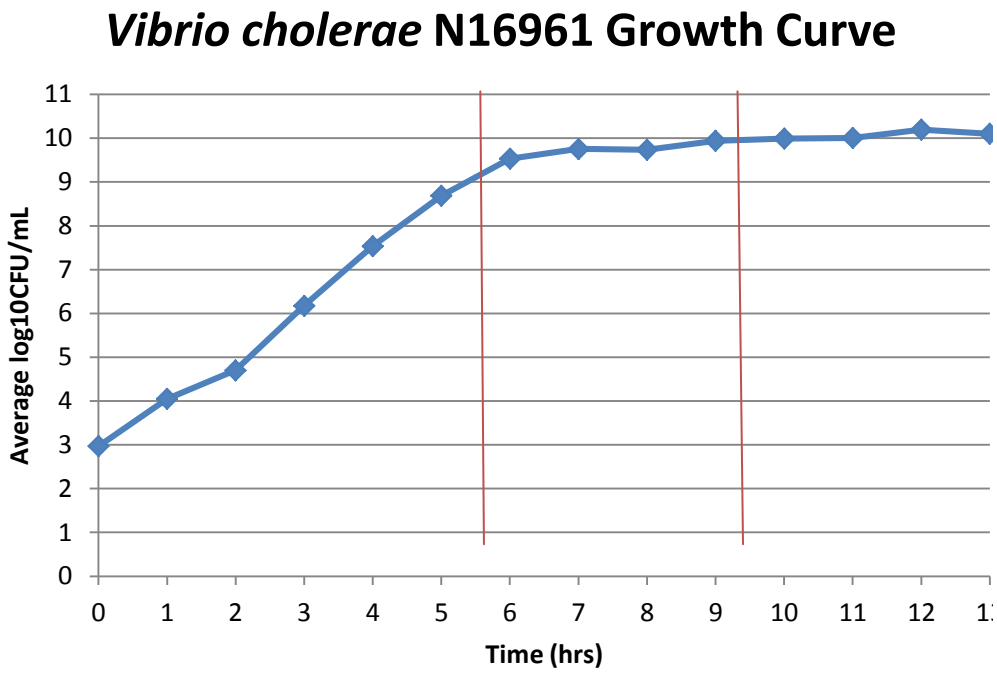

Figure 5-1. Growth curve of *Vibrio cholerae* N16961 in L-Broth. The line at 6 hr displays the end of exponential growth and the beginning of the stationary phase. The line at 10 hr displays the beginning of mid-stationary phase.

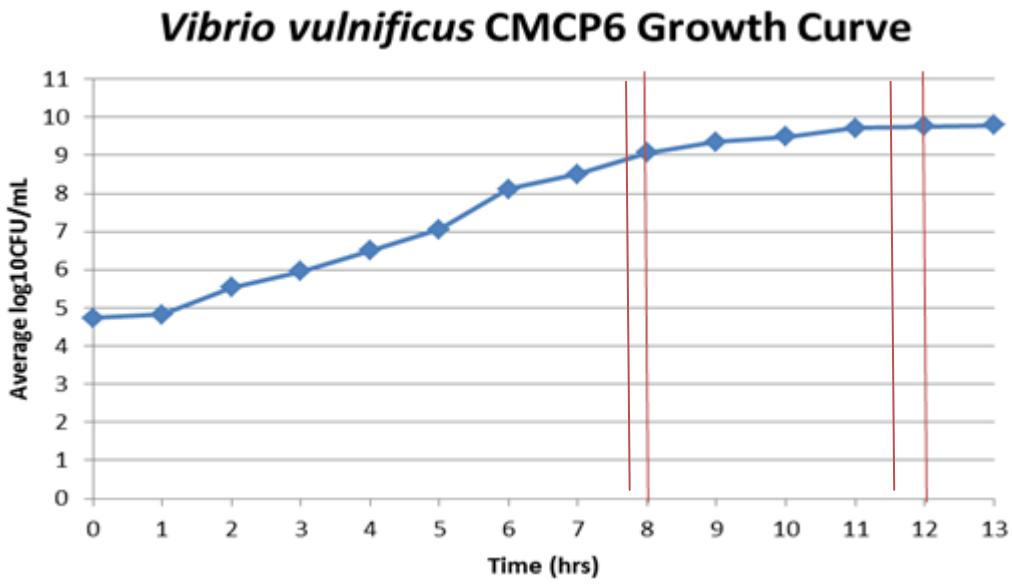

Figure 5-2. Growth curve of *Vibrio vulnificus* CMCP6 in L-Broth. The line at 8 hr displays the end of exponential growth and the beginning of the stationary phase. The line at 12 hr displays the beginning of mid-stationary phase.

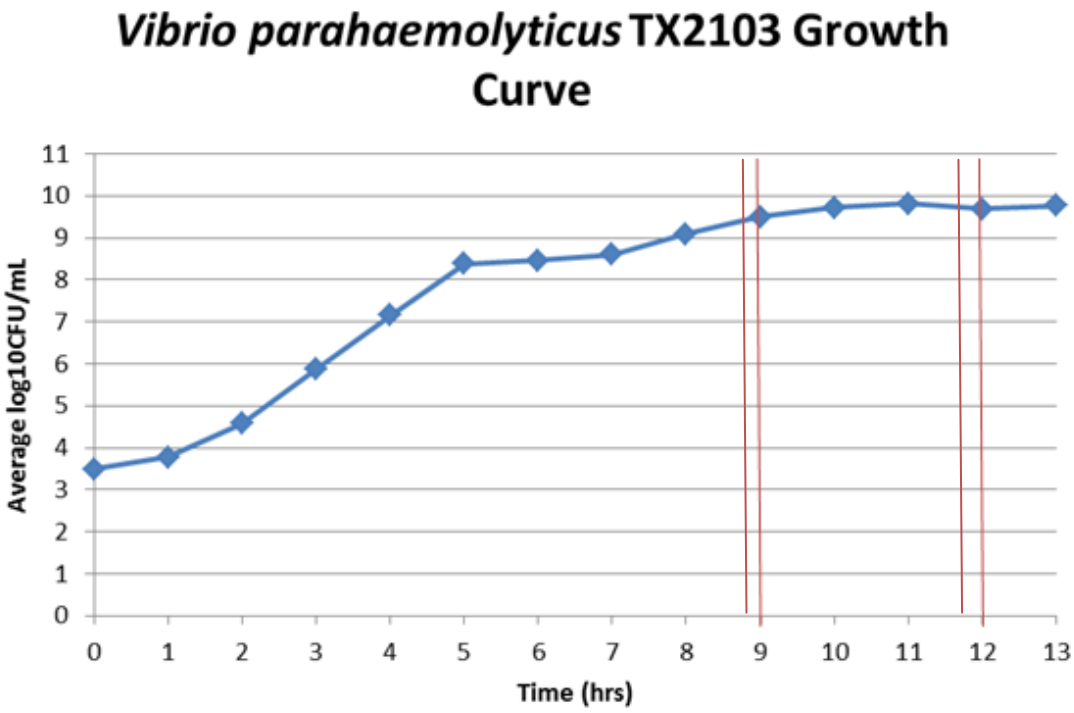

Figure 5-3. Growth curve of *Vibrio parahaemolyticus* TX2103 in L-Broth. The line at 9 hr displays the end of exponential growth and the beginning of the stationary phase. The line at 12 hr displays the beginning of mid-stationary phase.

CHAPTER 6
D- AND Z-VALUE ASSESMENTS

The D-values were determined using *Vibrio* spp. cultures in the mid-stationary growth phase. In trials at 48^{o}C using media, it was noted that *V. parahaemolyticus* had the longest survival with a D-value of 3.02 min; *V. vulnificus* and *V. cholerae* were equally heat liable at 2.36 min. Figure 6-1 through 6-3 display the average concentration of bacteria versus time.

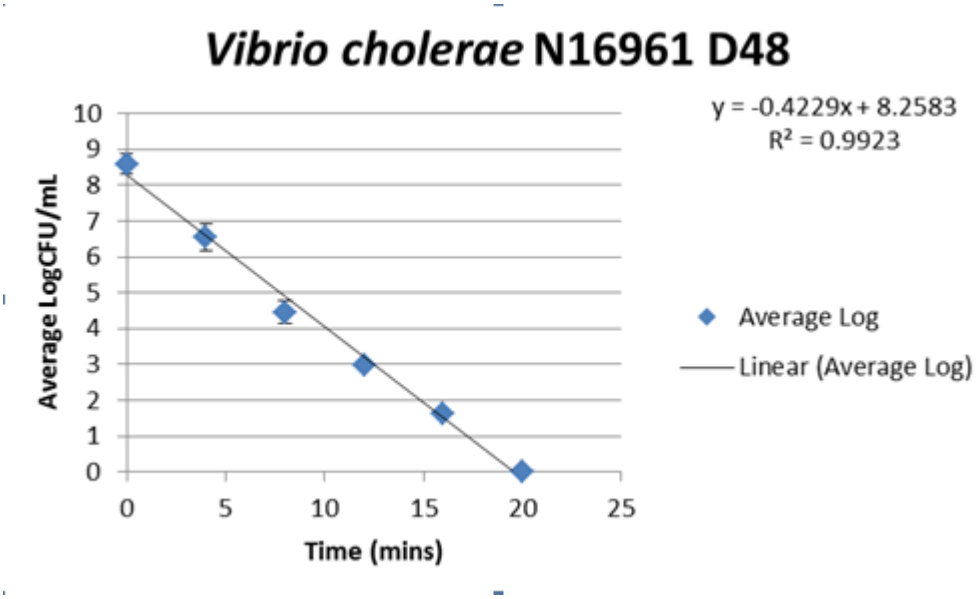

Figure 6-1. *Vibrio cholerae* N16961 D48 time versus average $\log_{10}$CFU/mL graph with standard deviation. R^2=0.9923

Figure 6-1 shows that from an initial concentration of 1.8x10^{8}, *V. cholerae* a 1 log unit reduction every 2.36 min at 48^{o}C occurred. After 20 min at this temperature, all *V. cholerae* in the sample had been eliminated. This is nearly identical to that for *V. vulnificus,* which had a starting concentration of 2.87x10^{8} CFU/mL, and also showed no survival after the 20 min mark and a normal 1 log unit reduction at 2.36 min.

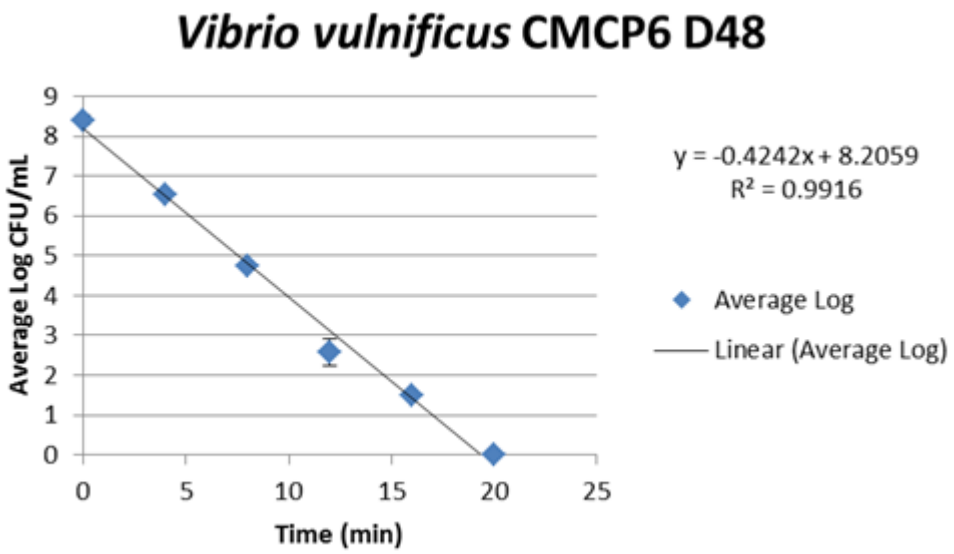

Figure 6-2. *Vibrio vulnificus* CMCP6 D48 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9916

Figure 6-3 shows the slight heat-stable advantage *V. parahaemolyticus* has over the other two subspecies. At this temperature, *V. parahaemolyticus* displayed a greater thermal resistance compared to the other species with a D-value of 3.02. This is roughly 3.02 min versus *V. vulnificus* and *V. cholerae* having approximately 2.37 min, respectively.

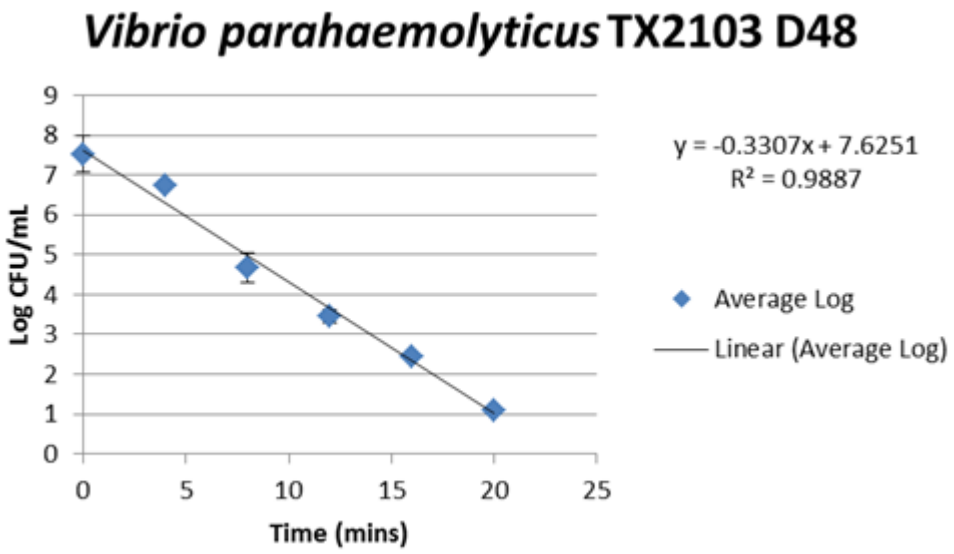

Figure 6-3. *Vibrio parahaemolyticus* TX2103 D48 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9887.

All of these experiments were extremely robust as interpreted from the small standard deviations of the repetitions. The line of best fit produced by all three graphs was also accurate, with the lowest being 0.9887 for *V. parahaemolyticus* and even higher for *V. cholerae* and *V. vulnificus* producing R^2 values greater than 0.99.

Similar results occurred between the thermal inactivation at 48^{o}C and 50^{o}C (Figure 6-4 through Figure 6-6). All three *Vibrio* spp. had a relatively similar thermal resistance at this temperature, varying only slightly. *Vibrio vulnificus* was the most heat stable at this temperature displaying a thermal resistance of 2.05 min per log unit reduction, with *V. parahaemolyticus* at 1.99 min and *V. cholerae* with 1.96 min. Figures 6-4, 6-5 and 6-6 display the time versus average log for *V. cholerae*, *V. vulnificus,* and *V. parahaemolyticus,* respectively.

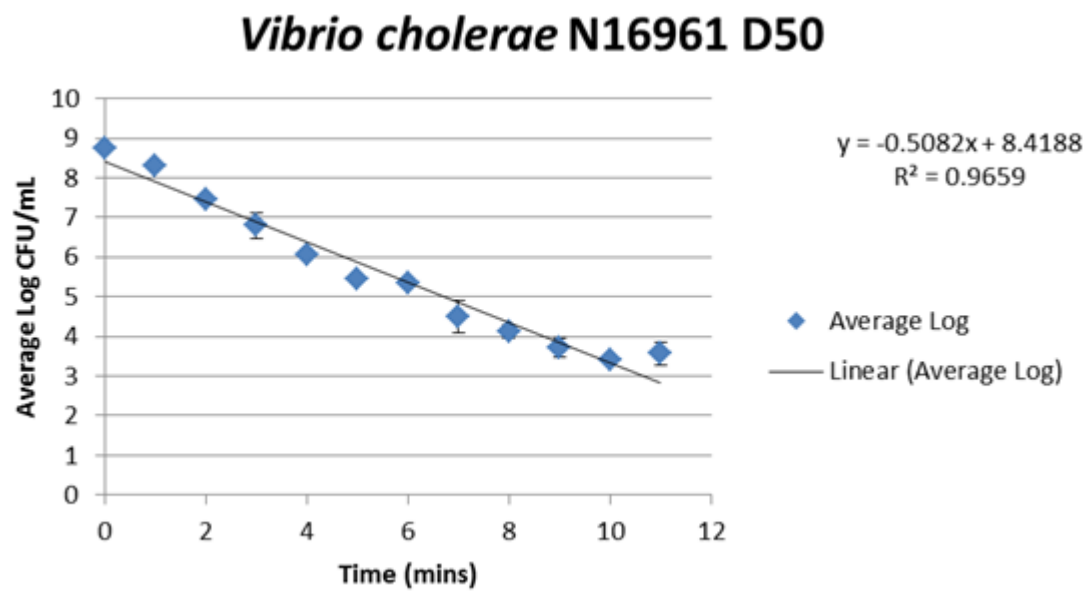

Figure 6-4. *Vibrio cholerae* N16961 D50 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9659.

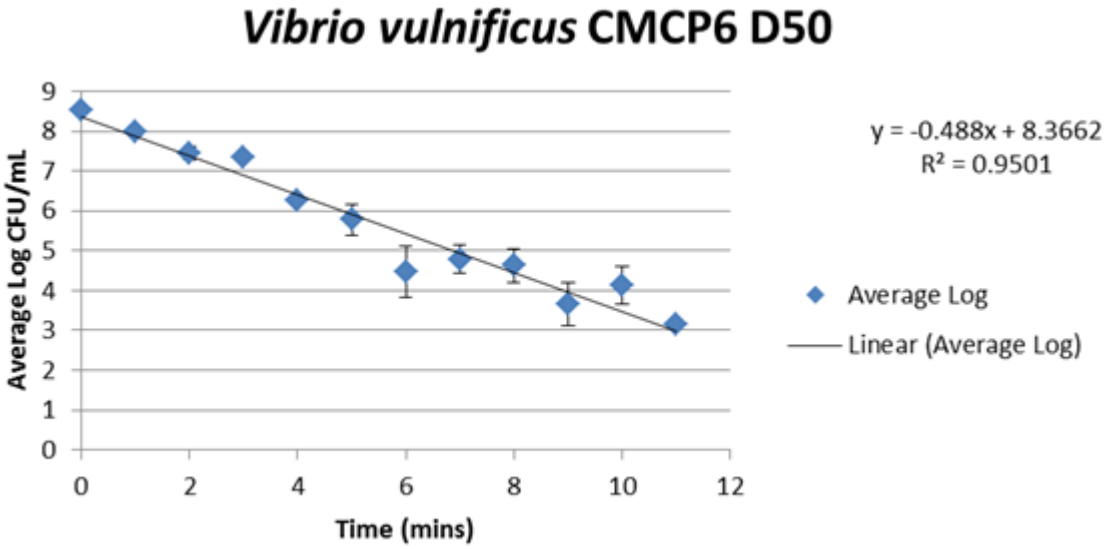

Figure 6-5. *Vibrio vulnificus* CMCP6 D50 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9501.

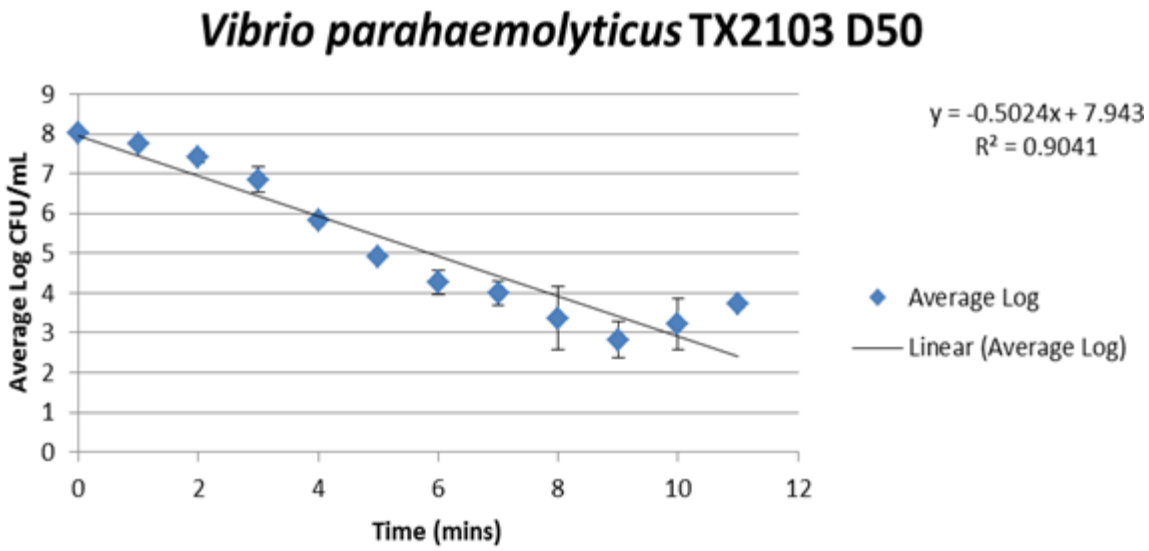

Figure 6-6. *Vibrio parahaemolyticus* TX2103 D50 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9041.

The R-values displays minimal variability in these trials; however, the standard deviations throughout the experiments display the capability of these procedures to be replicated with accuracy. The overall low variation of the standard deviation of these experiments coupled with the R-values give support to the accuracy of the experiments conducted.

As the temperature increases, it is commonly expected to have an exponential reduction in bacterial counts over the same time period. Because of this, the normal D-value procedure was altered from having a come up time with the lower temperatures, to being inoculated directly into the already heated tubes. Due to the exponential increase in death over a shorter period of time, the time points were decreased from 4 min per time at 48^{o}C point to only 30 sec at 55^{o}C, and after only three min nearly all bacteria were destroyed. Figure 6-7, 6-8 and 6-9 display the death versus time graph of *V. cholerae, V. vulnificus,* and *V. parahaemolyticus,* respectively.

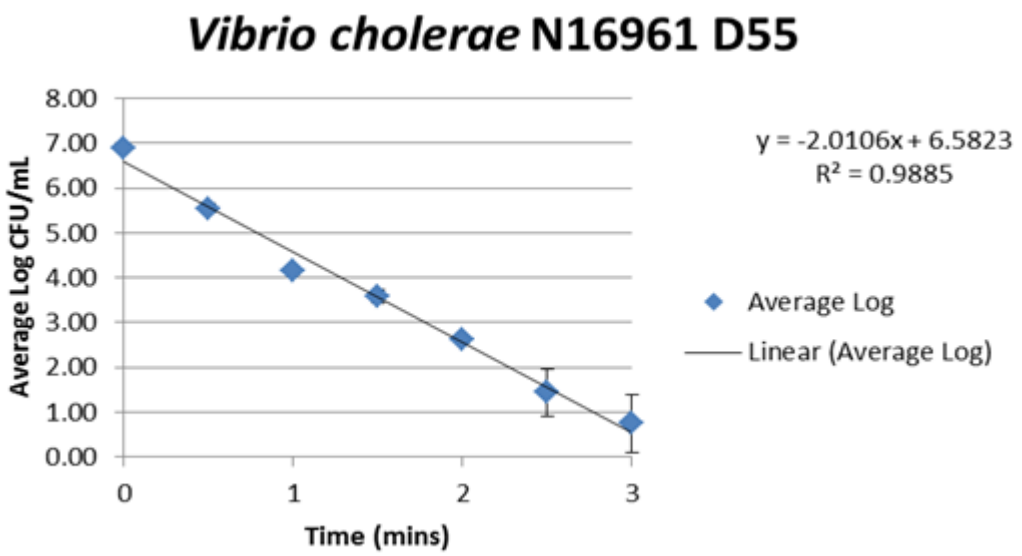

Figure 6-7. *Vibrio cholerae* N16961 D55 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9885.

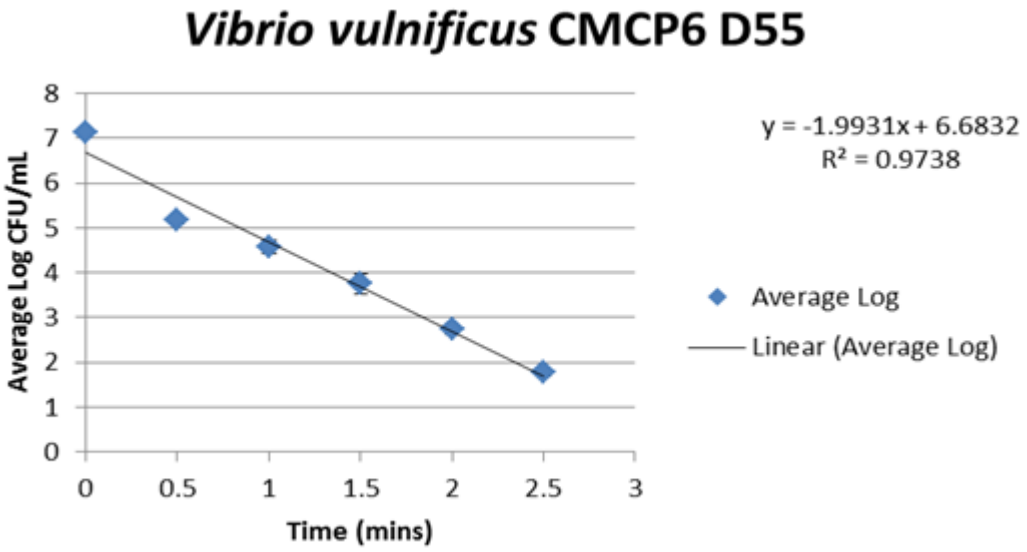

Figure 6-8. *Vibrio vulnificus* CMCP6 D55 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9738.

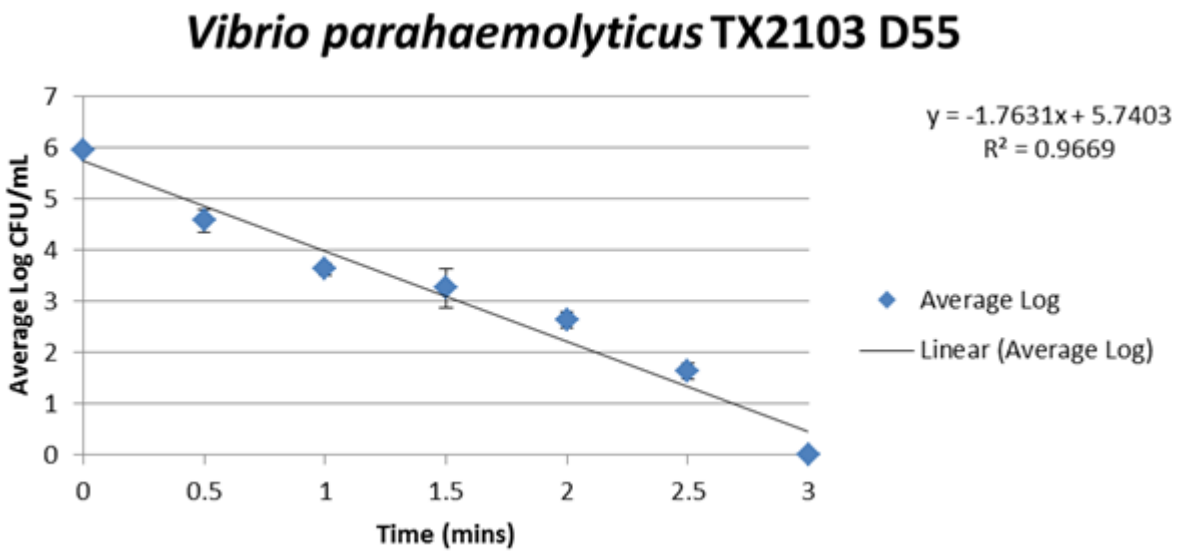

Figure 6-9. *Vibrio parahaemolyticus* TX2103 D55 time versus average $\log_{10}$CFU/mL with standard deviation. R^2=0.9885.

The low variability of the standard deviations coupled with the high R^2 values show that these experiments were reproducible as well as follow an accurate linear path that is expected in all the thermal-death studies that were conducted. The pathogens followed similar suit to previous expectations with *V. vulnificus* and *V. cholerae* having very similar D-values, with *V. parahaemolyticus* being the most heat stable with 0.72 min compared to *V. vulnificus* at 0.50 and *cholerae* at 0.53.

Prior research has shown that at ~48^{o}C ± 1^{o}C, *Vibrio* spp. can have a D-value of 8.15 min in crabmeat (Shultz *et al*., 1984) for *V. cholerae*, 0.82 min for *V. parahaemolyticus* in a clam homogenate (Delmore and Chrisley, 1979), or even as high as 65.1 min in 7.5% NaCl (Beuchat and Worthington, 1976). No prior research conducted had the ability to compare the thermal resistance of the *Vibrio* spp. simply because no prior research had conducted the same methods or the same media across all three pathogens in question. Using the same methodology and medium between *V. vulnificus, V. parahaemolyticus,* and *V. cholerae* allows for a better overall

understanding of the pathogens as well as the ability to cross reference and compare the three species.

After obtaining a set of three reliable D-values per organism, the z-value was calculated by taking the reciprocal of the slope resulting from the plot of the logarithm of the D-value versus the temperature at which the D-value was obtained. This is equivalent to the temperature required for a 1 log unit reduction in the D-value. Table 6-1 displays the calculated z-values per organism based on the obtained D-values.

Table 6-1. D and z-values for *V. vulnificus, V. parahaemolyticus,* and *V. cholerae*. Letters are used to denote statistical differences with a p≤0.05 of the mean.

Species	D48 (min)	D50 (min)	D55 (min)	z-value (^{o}C)
V. vulnificus CMCP6	2.24 B	2.05 CD	0.50 E	10.19
V. parahaemolyticus TX2103	3.02 A	1.99 CD	0.72 E	11.3
V. cholerae N16961	2.36 BC	1.96 D	0.52 E	10.31

Once the D-values were calculated, a one-way ANOVA analysis was carried out and Fisher's LSD was used for multiple mean comparisons using statistical analysis system software, version 9.1.3 (SAS Institute Inc., Cary, NC). Differences are noted by different letters and were determined by a statistical difference with a p≤0.05 of the mean.

Based on these trials, *V. parahaemolyticus* is significantly different from both *V. vulnificus* and *V. cholerae* at 48^{o}C. As the temperature increases to 50^{o}C, *V. cholerae* is significantly different from *V. parahaemolyticus* as well as *V. vulnificus*. However, once the temperature reaches 55^{o}C, all three bacteria show no significant difference. With all three bacteria acting in this manner, *V. parahaemolyticus* could be considered the target organism due to its heat stability over *V. vulnificus* and *V. cholerae* at lower temperatures and no difference at higher temperatures. All *Vibrio* spp. experimented on displayed minimal thermal survival capacities that will directly result in their ability to be lowered or eliminated during standard cooking procedures.

CHAPTER 7
THERMAL RECOVERY STUDY

Thermal abuse (26^{o}C for 24 hr) elevated the level of *Vibrio* spp. for heating trials using whole oysters (Table 7-1). The overnight bacterial concentration was conducted by MPN and verified by the BAX *Vibrio* spp. system. Each individual *Vibrio* spp. concentration was calculated, however, no *V. cholerae* cultures were found in any samples at any dilution. The MPN calculations for *V. vulnificus*, *V. parahaemolyticus*, and overall MPN for total aerobic bacterial counts (bacteria that are capable of growth in APW at 37^{o}C) are probable in raw oysters harvested from approved waters for commerce (ISSC, 2012).

Table 7-1. Overnight Temperature Abused Oyster MPN Calculations for *V. vulnificus, V. parahaemolyticus* and total bacteria in MPN/mL.

Sample	*V. vulnificus*	*V. parahaemolyticus*	Total Aerobic Plate Count*
1	$7.3x10^{1}$	$2.4x10^{4}$	$>2.4x10^{5}$
2	$2.3x10^{1}$	$2.4x10^{4}$	$>2.4x10^{5}$
3	$4.3x10^{1}$	$9.3x10^{3}$	$>2.4x10^{5}$
4	$2.3x10^{1}$	$2.4x10^{4}$	$>2.4x10^{5}$
Mean	$4.0x10^{1} \pm 2.0x10^{1}$	$2.0x10^{4} \pm 6.4x10^{3}$	$>2.4x10^{5} \pm 0.0$

*Total aerobic plate counts were calculated by bacteria that are capable of growth in APW at 37^{o}C incubation overnight.

After determining the initial concentration, the overall reduction after the heating process was calculated to determine if there was any protective effect from the oyster on the survival of the *Vibrio* spp*.* A lower final concentration of *V. vulnificus* was expected due in part to *V. vulnificus'* decreased heat tolerance as well as its lower starting concentration versus *V. parahaemolyticus.* The oysters that were heat treated at 48^{o}C showed a complete reduction of *V. vulnificus*; however, *V. parahaemolyticus* had survivors. This could be due to a lower starting concentration of *V. vulnificus* versus *V. parahaemolyticus* as well as the more heat stable *V. parahaemolyticus*. This

experiment was arranged to ensure at least a 3 log unit reduction in the bacteria. Although this shows a nearly complete inactivation of *V. vulnificus,* it is apparent that there is little increase in the inactivation of *V. parahaemolyticus* or total bacteria counts. This suggests that there are significant protective effects from the oyster at this temperature, which was fully expected.

Table 7-2. Log unit reduction of Heat Treated Temperature Abused Oysters for *V. vulnificus, V. parahaemolyticus* and total bacteria in MPN/mL.

Sample	*V. vulnificus*	*V. parahaemolyticus*	Total Aerobic Plate Count*
Overnight Mean	$4.0x10^1 \pm 2.0x10^1$	$2.0x10^4 \pm 6.4x10^3$	$>2.4x10^5 \pm 0.0$
48°C Mean Reduction	1.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.6
50°C Mean Reduction	1.0 ± 0.0	1.3 ± 0.6	2.0 ± 1.0
55°C Mean Reduction	1.0 ± 0.0	2.7 ± 0.6	2.7 ± 0.6

*Total aerobic plate counts were calculated by bacteria that are capable of growth in APW at 37°C incubation overnight.

Table 7-2 show the log unit reductions of *V. parahaemolyticus, V. vulnificus* and total bacteria after the 50 and 55°C treatment respectively. At the 50°C treatment, again, all the *V. vulnificus* was completely eliminated, however, an average of a 1.3 log unit reduction was observed for *V. parahaemolyticus.* Again, with the expectation of a minimum of a 3 log unit reduction, we can conclude that the protective effect at this temperature results in a reduction in the overall inactivation of *V. parahaemolyticus* and thus not safe for human consumption. It is important to note that the protective effect at 50°C was less than at the previous 48°C temperature, which could suggest that as the temperature increases, the ability for the oyster to provide a protective effect may be minimized.

At 55°C, however, the protective effect becomes even less apparent than in the previous two cases. Using the mean of the overnight sample's *V. parahaemolyticus* concentrations ($2.0x10^4$), a 3 log unit reduction in trials 1 and 2 and a 2 log unit in trial 3

is found. The mean shows a 2.7 ± 0.6 log unit reduction, displaying that the protective effect at this temperature has been reduced. Again, *V. vulnificus* was completely eliminated, most probably related to the low initial starting concentration. This temperature not only significantly reduced the *Vibrio* spp. but also reduced the total bacterial count by 2.7 ± 0.6 log units. A pattern can be noticed that as the temperature increases, the protective effect appears to be minimized, thus suggesting the higher the temperature the less likely protective effects influence the survival of these potential pathogens.

Overall, the study concludes that cooking to a 3 log unit reduction at lower temperatures is not sufficient in eliminating the test organisms due to the protective effect of the oyster. As the temperature increased, it was observed that protection from the food system was reduced and a safer product is the end result. Because of this issue, safe cooking parameters were not recommended on these temperatures, and other studies were conducted to allow safe cooking practices based on higher temperatures.

CHAPTER 8
ASSESSING COMMERICAL COOKING PROCEDURES

With the results from the lower temperatures providing evidence of a protective effect, the next step was determining if the standard cooking methods inside of restaurants were sufficient in the overall thermal inactivation of potential pathogens.

All the temperatures for commercial chargrilling reached an end point internal temperature of at least 200^{o}F (93.3^{o}C) while during frying all internal temperatures reached a minimum of 340^{o}F (171.1^{o}C). Figures 8-1 and 8-2 display the internal temperatures of chargrilling and deep frying, respectively.

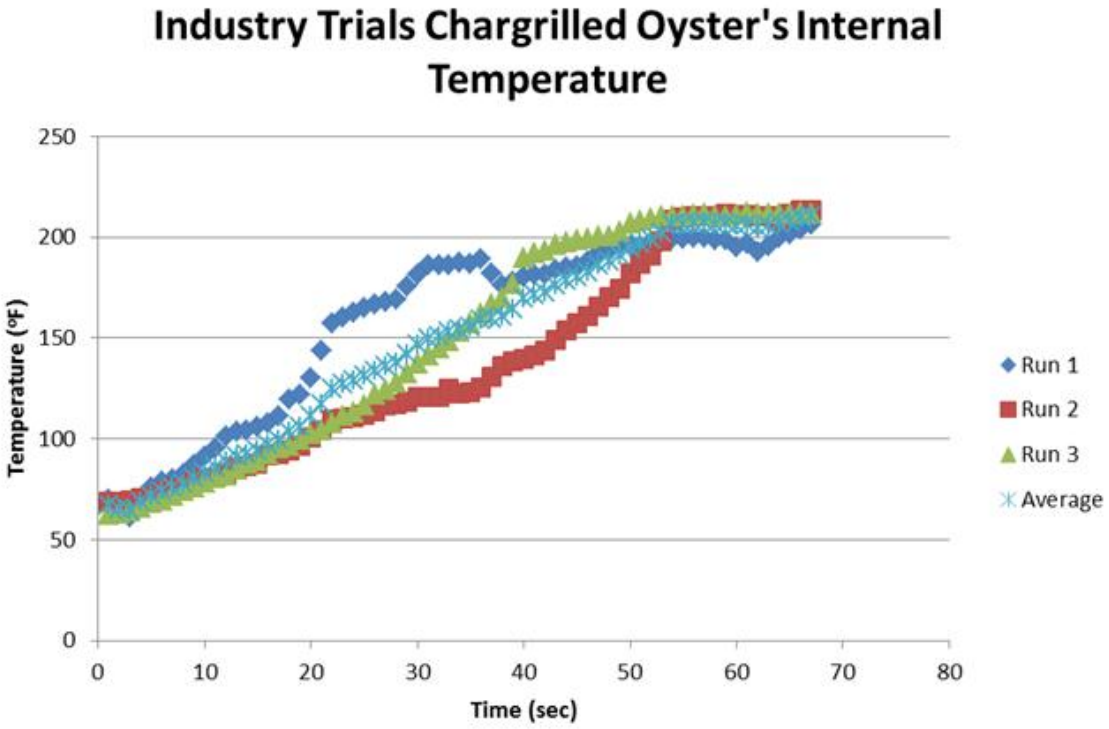

Figure 8-1. Internal temperature of half-shucked oyster on an open chargrill gas grill conducted on site in a commercial environment. Each trial was conducted with a minimum of 12 oysters with three temperature probes per run. Each run is the average of the three temperature probes per run with the final average calculated and added to the graph.

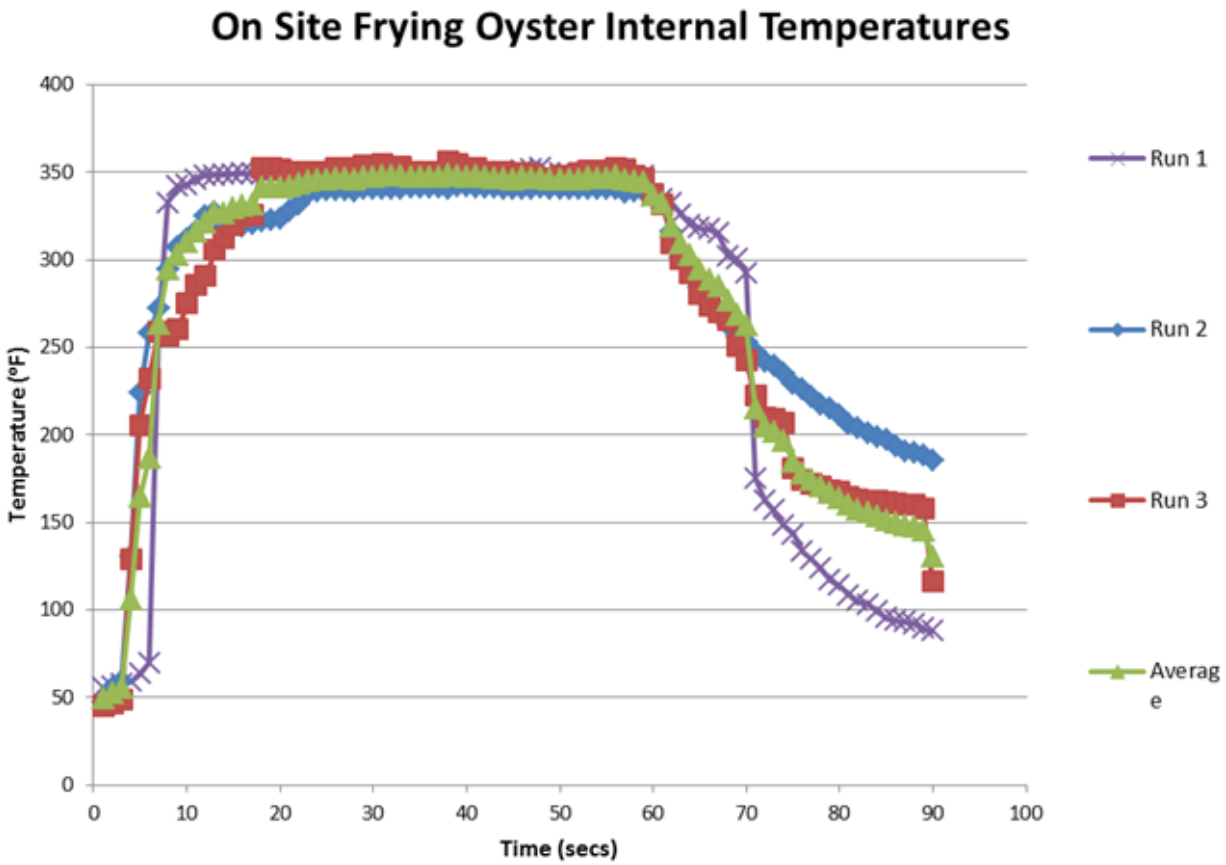

Figure 8-2. Internal Oyster Temperatures during on site trials conducted on the restaurant level. Twelve oysters were dropped per frying batch with each run having three temperature probes per 12 oysters. Each run is the average of those 3 trials and then the average of those trials was averaged.

These results should differ from the lower temperature if following the assumption that as the temperature increases the overall protective effects of the oyster will decrease as noted in previous experiments. Using the D and z-values determined in a PBS solution, it was hypothesized that if mimicking the lowest internal temperature recorded in commercial restaurant operations (200^{o}F), enough energy should enter the system to sufficiently eliminate potential *Vibrio* spp. pathogens. The staged trials used whole shucked oysters with elevated levels of *V. vulnificus* and *V. parahaemolyticus* prepared using identical temperature abuse procedures as described for the thermal

assessment at internal temperatures of 48, 50 and 55^{o}C (Table 8-1). The resulting levels exceeded 10^3 CFU/mL *V. vulnificus* and *V. parahaemolyticus.* The staged chargrill system provided direct heating between 420-450^{o}F. The oysters all reached an internal temperature of 200^{o}F before removal from the grill, and they were allowed to cool (for handling) as would customarily occur in a restaurant setting. Total bacteria were calculated by bacteria that are capable of growth in APW at 37^{o}C incubation overnight.

It is important to note that the *V. vulnificus* concentrations in these trials were much higher than in initial thermal recovery study (10^3 CFU/mL versus 10^1 CFU/mL). This helped to also determine the overall reduction of *V. vulnificus* as well as *V. parahaemolyticus* where the prior study could only hypothesize that if *V. parahaemolyticus* was reduced, *V. vulnificus* should be reduced at least to an equivalent rate

Table 8-1. Initial concentrations and Log unit Reduction of Temperature Abused Oyster *V. vulnificus, V. parahaemolyticus* and total bacteria after a 200^{o}F heat treatment in CFU/mL.

Sample	*V. vulnificus*	*V. parahaemolyticus*	Total Aerobic Plate Count*
Initial concentration	$6.0x10^3$	$4.0x10^3$	$7.3x10^5$
Mean log unit reduction at 200^{o}F	3 ± 0.0	3 ± 0.0	4.7 ± 0.6
Mean log unit reduction at 145^{o}F	3 ± 0.0	3 ± 0.0	1.7 ± 0.6

*Total aerobic plate counts were calculated by bacteria that are capable of growth in APW at 37^{o}C incubation overnight.

The results suggests that when reaching an internal temperature of 200^{o}F in a standard cooking practice as followed by many reputable restaurants, not only are the potential *Vibrio* spp. pathogens eliminated, so are nearly all other bacterial counts (Table 8-1).

Mindful of the FDA US Food Code (2009) recommendations to cook seafood to 145^{o}F internal for 15 sec, the final trial intended to demonstrate the bacterial influence of this procedure.

The approached used the same batch of overnight temperature abused oysters that were shucked on the half shell and placed on the grill. However, the product was only allowed to reach and internal temperature of 145^{o}F. After 15 sec, the product was removed from the grill (temperatures reached greater than 145^{o}F internally from the extra 15 sec on the grill) and the product was then allowed to cool to room temperature identically as used in the 200^{o}F trials.

Table 8-1 displays the temperature abused oyster's mean aerobic plate counts for *V. vulnificus, V. parahaemolyticus* and total bacteria after a 145^{o}F for 15 sec heat treatment in CFU/mL as well as the total log unit reduction after heat treatment. *Total bacteria were calculated by bacteria that are capable of growth in APW at 37^{o}C incubation overnight.

Heating to an internal temperature of 145^{o}F for 15 sec with a starting concentration of ~10^{3} CFU/mL will eliminate potential *Vibrio* spp. pathogens (Table 8-1). Interestingly, although there were no *Vibrio* spp. survivors that grew on the selective media, some bacteria did survive and grew on standard non-selective media APC at 37^{o}C. With this data, it can be conclusively stated that with an internal temperature of 200^{o}F or 145^{o}F for 15 sec, a passive cool down, and a common 3 log unit initial bacterial count for *Vibrio* spp. will produce a safe product for human consumption.

Another important note about these trials is the amount of energy entered into the 200^{o}F system is drastically greater in terms of reducing *Vibrio* spp. than in the 145^{o}F.

Since both reduced the *Vibrio* spp. concentrations by at least 3 log units, it is reasonable to assume that cooking to 200^{o}F will eliminate even greater than 3 log units simply because of the time and energy in the system after the 145^{o}F temperature has been reached.

CHAPTER 9
DISCUSSION

Vibrio vulnificus, V. parahaemolyticus and serogroup 01 *V. cholerae* are halophilic, potential pathogenic mesophiles commonly found inside of *Crassostrea virginica*. Although consumption of this product includes raw to steamed, fried and chargrilled products, little has been documented on how to create safe cooking parameters to eliminate potential pathogenic threats. This study provides detailed information about D and z-values in a common PBS medium and relates that with the actual food commonly associated with human infections.

The findings determined in this study are similar to some previous studies (Tables 9-1, 9-2 and 9-3).

Table 9-1. D-values for *V. cholerae*

Temperature		D-Value	Medium	Reference
(^{o}C)	(^{o}F)	(min)		
48	118.4	2.36	PBS Solution	Hanna and Otwell, unpublished
48.9	120	9.17	Shrimp Homogenate	Hinton and Grodner, 1985.
49	120.2	8.15	Crabmeat	Shultz *et al*., 1984
50	122	1.96	PBS Solution	Hanna and Otwell, unpublished
54	129.2	5.02	Crabmeat	Shultz *et al*., 1984
54.4	129.9	0.43	Shrimp Homogenate	Hinton and Grodner, 1985
55	131	0.52	PBS Solution	Hanna and Otwell, unpublished
60	140	2.65	Crabmeat	Shultz *et al*., 1984
60	140	0.39	Shrimp Homogenate	Hinton and Grodner, 1985
65.5	149.9	0.32	Shrimp Homogenate	Hinton and Grodner, 1985
66	150.8	1.60	Crabmeat	Shultz *et al*., 1984
66	150.8	1.22	Crayfish Homogenate	Grodner and Hinton, 1985
71	159.8	0.30	Crabmeat	Shultz *et al*., 1984
71	159.8	0.30	Crayfish Homogenate	Grodner and Hinton, 1985
71.1	160	0.31	Shrimp Homogenate	Hinton and Grodner, 1985
76.7	170.1	0.30	Shrimp Homogenate	Hinton and Grodner, 1985
77	170.6	0.27	Crayfish Homogenate	Grodner and Hinton, 1985
82	179.6	0.27	Crayfish Homogenate	Grodner and Hinton, 1985
82.2	180	0.28	Shrimp Homogenate	Hinton and Grodner, 1985

Table 9-2. D-values for *V. parahaemolyticus.*

Temperature		D-Value	Medium	Reference
(^{o}C)	(^{o}F)	(min)		
47	116.6	65.1	7.5% NaCl	Beuchat and Worthington, 1976
48	118.4	3.02	PBS Solution	Hanna and Otwell, unpublished
49	120.0	0.82	Clam Homogenate	Delmore and Chrisley, 1979
50	122	1.99	PBS Solution	Hanna and Otwell, unpublished
51	123.8	0.66	Clam Homogenate	Delmore and Chrisley, 1979
53	127.4	0.40	Clam Homogenate	Delmore and Chrisley, 1979
55	131	0.29	Clam Homogenate	Delmore and Chrisley, 1979
55	131	0.72	PBS Solution	Hanna and Otwell, unpublished

Table 9-3. D-values for *V. vulnificus.*

Temperature		D-Value	Medium	Reference
(^{o}C)	(^{o}F)	(min)		
47	116.6	2.4	Buffered Saline	Cook and Ruple, 1992
48	118.4	2.24	PBS Solution	Hanna and Otwell, unpublished
50	122	1.15	Buffered Saline	Cook and Ruple, 1992
50	122	2.05	PBS Solution	Hanna and Otwell, unpublished
55	131	0.50	PBS Solution	Hanna and Otwell, unpublished

The lack of literature on D-values for *V. cholerae's* based on media as opposed to a food matrix makes such comparisons difficult. An apparent food protective effect is noticed in the shrimp homogenate at 48.9^{o}C when compared directly to this study's value at 48^{o}C (9.17 min in the shrimp homogenate versus 2.36 min in PBS). This is expected as protective barriers found in foods can vary based on food product type, density, shape and related composition.

Similar discrepancies were noticed with *V. parahaemolyticus* (Table 9-2). A difference was noted when heating *Vibrio* spp. at 48^{o}C in PBS (Table 9-2) versus Beuchat and Worthington's (1976) trials in 7.5% NaCl at 47^{o}C. Beuchat and Worthington (1976) recorded a 65.1 min D-value at this temperature, whereas this study resulted in a 3.02 min D-value in PBS, a similar media.

At certain temperatures, the values in this study correspond closely to values in previous literature for *V. vulnificus* (Table 9-3). Cook and Ruple (1992) noted a 2.4 min D-value in a buffered saline at 47^{o}C, whereas this study noted a 2.24 min D-value at 48^{o}C. As the temperatures increased to 50^{o}C, Cook and Ruple (1992) noted a 1.15 min (in a buffered saline) whereas this study recorded a 2.05 min D-value (in PBS).

Because of the significant role of the food matrix in the protection of the bacteria during cooking processes, the thermal recovery studies were essential to determining how the PBS D-values would compare to trials conducted in oysters. The thermal recovery studies show that at lower temperatures (48 and 50^{o}C), protective effects from the oyster are greater than protective effects at higher temperatures (55^{o}C+). This justifies cooking to higher temperatures in order to produce a product safe for human consumption. These studies also provide directions to develop cooking control programs in actual restaurant operations that can support HACCP-based concepts. Every trial conducted on site at multiple locations had an internal temperature reaching a minimum of 200^{o}F. Using this as a standard, this study proves that not only were all potential *Vibrio* spp. pathogenic threats reduced from concentrations commonly found in oyster products (~10^{3} CFU/mL), but other bacterial counts were reduced as well.

Another extremely beneficial aspect of this study is the validation of the US Food Code's (2009) internal temperature of 145^{o}F for 15 sec as a safe guard for reducing pathogenic threats. Results from this study conclude that following the US Food Code's (2009) recommendation eliminates all potential *Vibrio* spp. pathogenic threats to a threshold of a minimum of 3 log units, which is a normal concentration of *Vibrio* spp. commonly associated with oysters.

This research demonstrated that following the parameters of cooking to an internal temperature of either 145^{o}F for 15 sec or 200^{o}F will sufficiently eliminate potential pathogenic threats of *Vibrio* spp. Because of these data it can be stated that using oyster products with natural *Vibrio* spp. loads (~10^{3} CFU/mL) can be cooked to a point of safe for human consumption with little risk of infection. With this knowledge, a HACCP plan for the restaurant level can be recommended and standard cooking procedures can be applied and validated. Restaurants following these validated methods can assume that their product is safe for human consumption as long as it follows the criteria in the validated methods.

Restaurants can rely on these science-based results as a validation of proper cooking procedures. For example, one well established famous oyster restaurant in New Orleans has traditionally followed in-house protocols or recipes to cook their most popular oyster dish, chargrilled oysters. They train their staff on very specific cooking instructions with visual aids to help ensure their methods are followed. They recommend placing the required amount of oysters onto a preheated grill and allowing the oysters to heat to the point that they begin to expel some of their water, using the visual aid of the separation and expansion of the edges of the oyster to help determine when this point is reached. Next, they tip and remove the liquor (commonly called the liquid) of the oyster, and continue to cook for approximately 6 min. After applying proprietary seasonings and butter sauces, they allow to cook for an additional 1 min and 30 sec and then remove from the heat. They state that at this point the oyster should be the color of a brown paper bag and are cooked to an internal temperature of >200^{o}F at this point.

The results from this thesis can be integrated with traditional recipes to build an applicable HACCP program (Figure 9-1). The program would have a validated critical limit (CL) of an internal temperature of >145^{o}F for 15 sec. This CL is consistent with existing 2009 US Food Code regulations (FDA, 2009). This thesis provides the validation for compliance monitoring and it would generate necessary records to provide evidence of compliance. This approach is innovative and new for the oyster industry.

		CCP 1
Critical Control Point (CCP) (1)		Cooking
Significant Hazard (2)		*V. vulnificus, V. parahaemolyticus, V. cholerae*
Critical Limits (3)		145^{o}F for 15 sec + cool down
Monitoring	What (4)	Internal Product Temperature
	How (5)	Internal Temperature Probes
	When (6)	3 trials daily
	Who (7)	Cook manager
Corrective Actions (8)		If internal product temperature is not reached, continue cooking until reaching 145^{o}F for 15 sec.
Records (9)		Daily cooking log Supervisor training records
Verification (10)		Validation study on file

Figure 9-1. Illustration of a possible HACCP plan for cooking oysters in a commercial restaurant operation.

Additional recommended research based on this study should include D and z-values conducted on *Vibrio* spp. inside of a whole oyster product.

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BIOGRAPHICAL SKETCH

Christopher W. Hanna attended the University of Florida from 2005-2009 for his Bachelor of Science in food science and human nutrition. He completed courses ranging from chemistry 1- organic 2, biology 1 and 2, microbiology, biochemistry, physics 1-2, nutrition courses, and general electives. He then took a year off and applied to the food science graduate program at the University of Florida. During his graduate degree he received all A-marks in his core classes: advanced food chemistry, advanced food microbiology, advanced food processing, product development, and sensory analysis. Chris completed this course work under Dr. Wade Yang initially, and transferred to the aquatics seafood department and began his thesis with Dr. Steve Otwell as his chair. His appointed committee members include Dr. Keith Schneider, Dr. Anita Wright, and Dr. Chuck Adams.

Chris eventually desires to pursue a career as a technical sales associate, however, he hope to one day end up managing and becoming the vice president of sales at a large company.