ABSTRACT

AL-DAKHEELALLAH, ABDALLAH DAKHEEL. Evaluation of the Efficacy of a Commercial Heat Shock Method for Reduction of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in Gulf Coast Oysters. (Under the direction of Dr. Lee-Ann Jaykus).

Pathogenic *Vibrio* species (*V. vulnificus* and *V. parahaemolyticus*) are the most common cause of foodborne disease associated with the consumption of raw or undercooked seafood, especially molluscan shellfish. The failure to adequately control these pathogens prompted the U.S. Food and Drug Administration (FDA) in 2009 to propose that Gulf Coast oysters harvested in warm months and intended for the raw (half-shell) market be subjected to a post-harvest process (PHP). The National Shellfish Sanitation Program has proposed that, to be recognized as a valid PHP, the process must reduce the concentrations of *V. vulnificus* and/or *V. parahaemolyticus* to non-detectable levels, defined as <30 MPN/g and a minimum 3.52-$\log_{10}$ reduction. The purpose of this study was to evaluate a commonly used commercial heat-shock method used to facilitate oyster shucking to determine its efficacy in reducing pathogenic *Vibrio* levels to meet FDA requirements as a valid PHP method.

In the first phase of the work, naturally contaminated Gulf Coast oysters having levels of *V. vulnificus* and *V. parahaemolyticus* exceeding 1 x 10^4 MPN/g were subjected to a mild heat process under pilot-scale laboratory conditions. Oysters were submerged in a 200 liters stainless steel tank in which water was heated to a temperature of 60 ± 0.5°C by direct steam injection. After 2, 4, 6, 8, or 10 min hold times, the oysters were removed from the tank and submerged in ice until they reached a temperature of 10-13^{o}C. Control and treated oysters were quantitatively analyzed for surviving *V. vulnificus* and *V. parahaemolyticus* populations using the Most Probable Number (MPN) method. The pilot scale studies revealed that treatments of 8 or 10 min at 60^{o}C were promising in meeting the proposed PHP regulatory

requirements, producing $\log_{10}$ reductions of pathogenic *Vibrio* species ranging from 2.5 to 4.5 $\log_{10}$.

The pilot studies were followed by commercial trials designed in a similar manner except that the oysters were submerged in heat shock tanks containing approximately 400 L of water heated to a temperature of 50-75^{o}C with hold times of 6, 8, or 10 min. Three control oysters were up-fitted with thermocouples for continuous temperature monitoring during processing. For the 6 min treatment trials, maximum internal oyster temperature (IOT) averaged 50^{o}C ±6^{o}C. For the 8 min treatment, maximum IOT was 58^{o}C ±2.9^{o}C, while maximum IOT averaged 58±2.2^{o}C after 10 min in the heat shock tanks. In general, 8 and 10 min treatment times resulted in inactivation of the pathogens, which ranged from 3.8-4.6-$\log_{10}$ reductions.

F-values were calculated and the F-value/D_{ref} ratio was used to evaluate and compare process efficacy. Using the most conservative F-value obtained for each treatment trial, a $\log_{10}$ inactivation of 0.37 was projected for the 6 min treatment; 6.54 inactivation for the 8 min treatment; and 7.72 for the 10 min treatment. Microbiological testing revealed near complete elimination (up to 4.5 $\log_{10}$ MPN/g) of both *V. vulnificus* and *V. parahaemolyticus* after the 10 min treatment. These F-value calculations suggest that 8 to 10 min commercial treatment times is adequate to provide the targeted 3.52 $\log_{10}$ reduction in pathogenic *Vibrio* species. The commercial heat-shock method has the potential to be used by industry as a PHP method for control of *V. parahaemolyticus* and *V. vulnificus* in raw oysters once given approval by the Interstate Shellfish Sanitation Conference and the FDA.

Evaluation of the Efficacy of a Commercial Heat Shock Method for Reduction of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in Gulf Coast Oysters

by
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BIOGRAPHY

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CHAPTER 1

Literature Review

1.1 Introduction

According to the recent studies by Scallan et al. (2011a), every year in the United States there are 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths attributed to 31 known foodborne pathogens. Of these, 96 cases are caused by *Vibrio vulnificus*, 34,664 cases caused by *Vibrio parahaemolyticus* and 17,564 cases attributed to other *Vibrio* species. The hospitalization rate is 91.3% for *V. vulnificus,* 22.5% for *V. parahaemolyticus,* and 37.1% for other *Vibrio* species; mortality rates are 34.8%, 0.9%, and 3.7%, respectively. An additional 38.4 million illnesses, 258,003 hospitalizations, and 3,574 deaths each year are also caused by unknown or unspecified pathogens transmitted by foodborne routes (Scallan et al., 2011b).

1.1.1 Classification

The microbial family *Vibrionaceae* includes eight genera: *Vibrio, Enterovibrio, Salinivibrio, Allomonas, Catenococcus*, *Listonella, Grimontia,* and *Photobacterium* (Thompson and Swings, 2006). *Vibrio* species are Gram-negative, mesophilic, asporogenous, pleomorphic rods, usually motile by a single polar flagellum. There are 65 *Vibrio* species of which *V. vulnificus, V. parahaemolyticus*, and *V. cholera* are the most widely studied (Kaper et al., 1995; Kaysner and DePaola, 2004; McLaughlin et al., 1995; McPherson et al., 1991; Oliver 1989; Sakazaki et al., 1963; Thompson and Swings, 2006). Members of the *Vibrio*

genus are of the most significant pathogens causing foodborne illness associated with the consumption of raw or undercooked seafood (Rippey 1994; Kaysner and DePaola, 2004). Of these, *V. vulnificus* is the major cause of death associated with the consumption of raw or undercooked seafood, especially raw oysters originating from the Gulf of Mexico (Gulf Coast oysters) (Oliver 1989; Shapiro et al., 1998; Kaysner and DePaola, 2004). *Vibrio parahaemolyticus* is the most important agent of seafood-related gastroenteritis (Klontz et al., 1993; Rippey 1994; Kaysner and DePaola, 2004), and *Vibrio cholerae* is the cause of epidemic and pandemic cholera disease (Kaper et al., 1995; Faruque et al., 1998). *Vibrio mimicus* show phenotypic characteristics similar to *V. cholera* but unlike the latter, is capable of fermenting sucrose (Davis et al., 1981; Shandera et al., 1983; Spira et al., 1984; Kaysner and DePaola, 2004). Other *Vibrio* species that have on occasion been associated with disease include *V. fluvialis, V. metschnikovii, V. alginolyticus, V. hollisae, V. carchariae, V. damsela, V. cincinnatiensis, and V. furnissii* (Abbot and Janda 1994; Hanson et al., 1993; Rippey 1994; Kaysner and DePaola, 2004).

1.1.2 Habitat and Ecology

Vibrio species are indigenous to and ubiquitous in estuarine and marine environments worldwide (Blackwell and Oliver, 2008; Oliver 2006; Kaysner et al., 1987a; Tamplin et al., 1982). Their numbers peak during summer months but they can still be isolated from marine waters during the winter and from cold regions of the world. Vibrios have also been isolated from brackish to pelagic deep-sea waters and are considered part of the natural microbial biota of estuarine and marine animals. They can be isolated from the skin, gut, and other

digestive tissues of marine animals and crustaceans, and attach tenaciously to marine plankton. They are commonly isolated from bivalves (Drake 2004) and found at high levels in oysters and other molluscan shellfish (Linkous and Oliver 1999). Although Vibrios can also be found in marine sediments, they usually are not the dominating species (Urakawa and Rivera 2006).

In the U.S., *V. vulnificus* strains have been isolated as far north as the Great Bay estuary of New Hampshire and Maine (O'Neil et al., 1992; Drake 2004) and as far south as estuaries in Florida (Tamplin et al., 1982). These organisms are commonly found in waters in the Chesapeake Bay of Virginia (Wright et al., 1996), and occur in relatively high concentrations in the waters of the Gulf of Mexico during the warm summer months (Motes et al., 1998; Cook 1994; Vanoy et al., 1992). Like *V. vulnificus*, *V. parahaemolyticus* strains are common inhabitants of U.S. and Mexican coastal waters (Abbott et al., 1989; Kaysner et al., 1987b; DePaola et al., 2003), and have also been isolated from waters in the Pacific Northwest (Kaysner et al., 1994; CDC 1998), Long Island Sound (CDC 1999), and Alaska (McLaughlin et al., 2005). In a qualitative study performed by Kaysner et al. (1987a), in which 24 estuaries from Washington, Oregon, and California were screened for the presence of *V. cholerae*, serotype O1 (associated with epidemic cholera) was found only in Morro Bay, California, and while the non-O1 serotype was detected in 23 estuaries along the West Coast. Hood et al. (1983) tested Florida estuaries (Apalachicola and Tampa Bay) for the presence of *V. cholerae*, isolating the epidemic O1 strain in only one oyster sample, while non-O1 strains were abundant in water, sediments, oysters, and blue crab. These data support the fact that epidemic cholera is rare in the U.S. (CDC 2006b).

Despite the facts that both *V. vulnificus* and *V. parahaemolyticus* thrive in warm waters, they have been isolated during the winter and from cold regions of the world (Urakawa and Rivera 2006). Higher concentrations of these organisms are found in waters having elevated temperatures year round, such as occurs in the Gulf of Mexico, Chesapeake Bay, and in mid-Atlantic waters. Lower concentrations are isolated from cooler waters like those in the Canadian, Pacific, and North Atlantic regions (Drake et al., 2007). The optimal temperature for the growth of *V. vulnificus* is 37°C (Chase and Harwood, 2011) and it is frequently isolated in waters having temperatures between 19 and 32°C (Blackwell and Oliver, 2008), or generally when seawater temperature is above 20°C (Oliver 2005). The correlation between water temperature and concentration is similar for both *V. parahaemolyticus* and *V. vulnificus* (Blackwell and Oliver, 2008) and the former bacterium can be isolated when water temperature is above 17°C (Kelly and Stroh, 1988). In cold months, both *V. vulnificus* and *V. parahaemolyticus* are difficult to isolate. This phenomenon has been thought to be due to environmental stresses, mainly cold temperature, which induces physiological changes leading to transformation into the so-called viable but nonculturable (VBNC) state (Blackwell and Oliver, 2008; Drake et al., 2007; Wong and Wang, 2004; Bates and Oliver, 2004; Whitesides and Oliver, 1997; Oliver et al., 1995).

The effect of water temperature on the concentration of *V. vulnificus* and *V. parahaemolyticus* is also influenced by water salinity. The optimum salinity range for *V. vulnificus* falls between 5 and 25 ppt (Blackwell and Oliver, 2008; Oliver, 2005; Kaspar and Tamplin, 1993); higher salinity (30-38 ppt) can reduce densities of *V. vulnificus* by 50-90% (Kaspar and Tamplin, 1993). Kelly and Stroh (1988) found that *V. parahaemolyticus* could

not be isolated when water salinity was above 13 ppt, this finding could suggest that salinity above 13 ppt reduces densities of *V. parahaemolyticus.* Turbidity or cloudiness of seawater has also been shown to have an influence on levels of *V. vulnificus* and *V. parahaemolyticus*. Higher turbidity is associated with higher nutrient levels, promoting pathogen attachment to sediments and blocking of antimicrobial effects of solar radiation, ultimately promoting survival and perhaps proliferation of *Vibrio* spp. (Blackwell and Oliver, 2008; Parveen et al, 2008; Julie et al, 2010).

1.2 Virulence Factors

1.2.1 *Vibrio vulnificus*

Vibrio vulnificus has been shown to have several virulence factors, including (i) the presence of a capsular polysaccharide (CPS); (ii) the production of various exoproteins; (iii) the ability to acquire iron from iron transport proteins; and (iv) cellular endotoxins associated with the lipopolysaccharide (LPS) (Linkous and Oliver 1999; Drake 2004; Oliver 2006).

Differences in colony morphology of *V. vulnificus* strains is due to the capsular polysaccharide (CPS). Specifically, encapsulated strains appear as opaque colonies that are easily differentiated from the non-encapsulated translucent colonies by normal vision. All virulent strains of *V. vulnificus* have the ability to produce a capsular polysaccharide (CPS) (Linkous and Oliver 1999; Simpson et al., 1987); this molecule contributes to the ability of the bacterium to invade the host immune system and avoid the destructive effects of phagocytosis. This ability is reportedly due to an antiphagocytic surface antigen (Kreger et al., 1981; Linkous and Oliver, 1999).

Several studies have been done to identify which specific exoproteins produced by *V. vulnificus* contribute to its virulence. The extracellular enzyme cytolysin/hemolysin has been the most widely investigated exoprotein. It has an amino acid sequence with 65% similarity to the hemolysin of *V. cholerae* El Tor and 60% similarity to the *V. cholera* non-O1 cytolysin (Drake et al., 2007; Strom and Paranjpye, 2000; Wright and Morris, 1991; Yamamoto et al., 1990). This enzyme is heat-labile and able to lyse mammalian erythrocytes *in vitro* (Drake et al., 2007; Strom and Paranjpye, 2000; Gray and Kreger, 1985). The *vvh*A gene encodes the cytolysin/hemolysin exotoxin. In addition, a metalloprotease, encoded by the *vvp*E gene, is also believed to have a leading role in *V. vulnificus* virulence (e.g. Linkous and Oliver 1999; Miyoshi et al., 1995; Shinoda and Miyoshi 2000). Nonetheless, as described by Oliver (2006), the significance of these virulence factors is inconclusive, as several studies of *V. vulnificus* strains having mutations in the *vvh*A and *vvp*E genes (e.g. Fan et al., 2001; Wright and Morris, 1991) have shown no clear role for either the cytolysin/hemolysin or metalloprotease exoproteins in strain virulence. However, these studies were limited to evaluating the relationship between these putative virulence factors and the primary septicemia disease state. Their role in gastrointestinal disease or wound infections remains unknown.

The presence of available iron in the human host is an essential requirement for *V. vulnificus* virulence and lethality (Wright et al., 1981). Since iron is not normally freely available in humans, the organism acquires iron from hemoglobin and iron transport proteins such as transferrin, lactoferrin, and ferritin. Pathogenic *V. vulnificus* strains are capable of scavenging iron from hemoglobin and these transfer proteins via the production of iron

chelating phenolate and hydroxymate siderophores (Simpson and Oliver 1983; Strom and Paranjpye, 2000; Drake et al., 2007).

Male gender also appears to be a risk factor for more severe *V. vulnificus* disease. For example, approximately 85% of patients developing septic shock are males (Oliver 1989; Merkel et al., 2001). This interesting observation has been linked to the female hormone estrogen. Specifically, Merkel et al., (2001) showed that estrogen has a protective effect against *V. vulnificus* LPS-induced septic shock. Although the underlying mechanism is unknown, it has been shown that estrogen can have an effect on the cardiovascular system and immune cells. During septic shock, patients suffer hypotension of their cardiovascular system, and it may be that estrogen can reverse this effect, effectively circumventing septic shock.

1.2.2 *Vibrio parahaemolyticus*

Virulent strains of *V. parahaemolyticus* have traditionally been classified based on the Kanagawa Phenomenon (KP) on Wagatsuma (blood) agar (Sakazaki et al., 1968; Iida et al., 2006; Joseph et al., 1982; Miyamoto et al., 1969), which detects the β-hemolysis reaction. Strains displaying hemolytic activity are classified as Kanagawa positive (KP-positive); those with weak lysing ability are classified as KP-intermediate; and strains that are unable to lyse blood are non-hemolytic or KP-negative (Drake et al., 2007; Iida et al., 2006; Joseph et al., 1982; Miyamoto et al., 1969). It is now known that a heat-resistant protein designated the "thermostable direct hemolysin (TDH)" and encoded by the *tdh* gene, is the cause of the Kanagawa phenomenon. All KP-positive strains are also positive for the

tdh gene and it appears that all (or most) clinical isolates are also KP-positive. The TDH protein has direct hemolytic activity on erythrocytes (red blood cells) and the addition of lecithin has no effect on increasing or enhancing this hemolytic activity (Drake et al. 2007, Iida et al., 2006; Nishibuchi and Kaper, 1995; Honda and Iida, 1993). In some KP-negative *V. parahaemolyticus* strains, a TDH-related hemolysin (called the TRH protein) has been identified. Encoded by the *trh* gene and with approximately 67% similarity to the TDH toxin, this protein exhibits similar biological activity to the TDH virulence factor (Iida et al., 2006; Honda et al., 1988; Honda and Iida, 1993). These two toxins are currently thought to be the most important virulence factors for *V. parahaemolyticus* (Iida et al., 2006). This is supported by the fact that mutations in the *tdh* and *trh* genes results in partial or complete loss of hemolytic activity in strains having these genes (Nishibuchi et al., 1992; Xu et al., 1994; Park et al., 2004). Interestingly, many clinical isolates of *V. parahaemolyticus* are able to produce both the TDH and TRH proteins (Xu et al., 1994; Kaufman et al., 2002; DePaola et al., 2003). Nevertheless, it appears that these toxins alone are still not adequate to cause severe *V. parahaemolyticus* disease (Iida et al., 1998; Honda et al., 1990).

The urease structural gene (*ure*) is another putative *V. parahaemolyticus* virulence factor with an apparent link to the *trh* gene (Iida et al, 1997; Iida et al., 1998). Specifically, many but not all *tdh*-positive strains are also *ure*-positive (Kaysner et al., 1994; Drake et al., 2007). However, the significance of this is currently unknown. Other suspected virulence factors for *V. parahaemolyticus* include lipase activity and the presence of factors that promote adherence of the organism to mucosal cells of the small intestine. Adhesiveness differs between KP-negative and KP-positive strains, with the latter observed being highly

adhesive while the former show relatively weak adherence (Hackney et al., 1980; Yamamoto and Yokota, 1989; Baffone and others, 2001; Drake et al., 2007).

1.3 Epidemiology

Each year in the U.S. there are about 100 cases of illness caused by *V. vulnificus*, over 30,000 caused by *V. parahaemolyticus*, and over 17,000 caused by other *Vibrio* species. Of these, *V. vulnificus* has a particularly high mortality rate (Scallan et al., 2011a). Disease caused by different *Vibrio* spp. can vary from quite mild to extremely severe and generally fall into three categories: wound infections, primary septicemia, and gastroenteritis.

1.3.1 Wound Infections

Both *V. vulnificus* and *V. parahaemolyticus* can cause wound infections (Hlady and Klontz, 1996; Yeung and Boor, 2004). Wound infections are described as "those cases where a patient incurred a wound before or during exposure to seawater, seafood drippings, or punctures from fish spines or bones, and from which *V. vulnificus* or *V. parahaemolyticus* was subsequently cultured from that wound, blood, or an otherwise normally sterile site" (Drake et al., 2007). Around 69% of all wound infections present in fishermen and oyster shuckers as a consequence of work exposures (Strom and Paranjpye, 2000). Such infections cause ulceration of skin and surrounding tissue necrosis and often amputation or debridement of infected tissue is required. Wound infections can occasionally progress to septic disease (Faruque and Nair, 2006). The mortality rate associated with wound infections caused by *V. vulnificus* is about 25% (Blackwell and Oliver, 2008).

Vibrio vulnificus wound infections usually occur sporadically, but an unprecedented outbreak occurred in 2004 in Port O'Connor, Texas. Six male patients between the ages of 44-65 years fell ill, and all presented as either infection of pre-existing wounds or as a consequence of acquiring new wounds while working in the same location. One patient, who incurred a minor cut on his leg after reportedly having slipped on a dock was, hospitalized for 26 days, had to have both his legs amputated, and subsequently died as a consequence of the disease (Oliver, 2005). Even though the majority of wound infections are caused by *V. vulnificus*, it should be noted that *V. parahaemolyticus* can be responsible for as many as 18-33% of these cases. Other *Vibrio* species, like *V. alginolyticus* and *V. cholerae* non-01, can also cause wound infections (Oliver, 2005).

1.3.2 Primary Septicemia:

Strom and Paranjpye (2000) define primary septicemia as "A systemic illness characterized as fever and shock where *V. vulnificus* was isolated from blood or other sterile site, and with a history of raw shellfish consumption but no wound infection preceding Illness." This is typically a disease manifestation of *V. vulnificus,* although it can also be caused by *V. parahaemolyticus* and *V. cholerae* non-O1 (Hlady and Klontz, 1996). Septic disease affects individuals having specific risk factors, including underlying liver disease caused by chronic active hepatitis or cirrhosis, resulting in increased serum iron levels and hemochromatosis (Faruque and Nair, 2006). Sepsis occurs sporadically, has a mortality rate of 50% (Blackwell and Oliver, 2008; Kumamoto and Vukich, 1998), and is responsible for 95% of all deaths related to seafood consumption (Blackwell and Oliver, 2008). The

hypothesized infectious dose of *V. vulnificus* for healthy humans is currently unknown; but for individuals with underlying disease risk factors, septicemia can result from exposure to as few as 100 infectious bacteria (FDA, 2011a). It is particularly associated with the consumption of raw shellfish, especially oysters harvested from the Gulf of Mexico in warm summer months (Strom and Paranjpye, 2000; Shen et al., 2009). In the United States, approximately 100 cases of primary septicemia occur each year (Mead et al., 1999). In Taiwan, the numbers of *V. vulnificus* infection have been increasing, which may in part be due to the high hepatitis B and C disease prevalence in this area of the world (Hsueh et al., 2004; Faruque and Nair, 2006).

1.3.3 Gastroenteritis:

Gastrointestinal disease is the most common manifestation of infection with *V. parahaemolyticus*. Other pathogenic *Vibrio* spp. such as *V. mimicus* and *V. fluvialis* can also cause gastroenteritis (Hlady and Klontz, 1996). This disease state is usually characterized by watery and/ or bloody diarrhea, abdominal cramps, headache, nausea, and vomiting (Honda and Iida, 1993; Honda et al., 1976).

Many outbreaks of gastroenteritis in North America have been linked to *V. parahaemolyticus*. For example, in 1997, there were 209 laboratory-confirmed cases of *V. parahaemolyticus* infection, many of which were linked to consumption of oysters harvested from British Colombia, Washington, Oregon, and California. This particular year, mean water temperatures in this region ranged from 54-66^{o}F from May through September; this constituted a 2-9°F increase in temperature compared to 1996 (CDC 1998). In 1998, 12

culture-confirmed cases of *V. parahaemolyticus* gastroenteritis were attributed to consumption of raw oysters and clams harvested from Long Island Sound, Connecticut, New Jersey, and New York, also areas much further north than previously observed (CDC 1999).

More recently, in 2004 62 passengers on 4 different Alaskan cruises experienced gastrointestinal illness after eating oysters; nine clinical samples from select patients tested positive for *V. parahaemolyticus* to the exclusion of all other pathogens. Four serotypes were identified, with all of the isolates positive for the *tdh* gene. When 96 environmental samples from the 17 oyster farms in Alaska collected between July and October were analyzed, 32% were found to be positive for *V. parahaemolyticus*. In July and August, water temperatures exceeded 15°C, especially at the location from which most of the implicated oysters were obtained. This follows the hypothesis of increased risk of *V. parahaemolyticus* related-illness when water temperatures exceed 15°C. In addition to elevated water temperature, animal migration changes and the dumping of ballast water from boats, both of which result in "seeding" of previously uncontaminated waters, was likely to have contributed to this outbreak (McLaughlin et al., 2005). Finally, 72 confirmed cases of *V. parahaemolyticus* infection were reported in the northern states of New York, Oregon, and Washington in 2006, a prevalence far exceeding normal annual CDC estimates collected for the years 2000-2004 (CDC 2006a).

1.4 Detection Methods

Officially, quantitative analysis of the concentrations of *V. vulnificus* and *V. parahaemolyticus* in molluscan shellfish and their overlay waters is done using the Most

Probable Number (MPN) method described in the FDA Bacteriological Analytical Manual (Figure 1.1). This method includes enrichment, isolation, and enumeration. Briefly, serial dilutions of oyster meat are prepared from an initial tissue sample homogenized 1:1 with phosphate buffered saline (PBS). From each dilution, three Alkaline Peptone Water (APW) tubes (pH 8.5±0.2) are inoculated and incubated at 35±2°C for 18-24 hrs. Tubes showing turbidity are streaked onto the selective agars modified Cellobiose-Polymysin B-Colistin (mCPC) for *V. vulnificus* and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) for *V. parahaemolyticus*. These are then incubated at between 35°C and 40°C for 18-24 hrs. Colonies of *V. vulnificus* on mCPC appear flat, round, opaque, yellow, and with a 1-2 mm diameter. Colonies of *V. parahaemolyticus* on TCBS appear green or bluish, opaque, round, and with 2-3 mm diameters. Plates showing typical colonies are reported as positive, and the associated MPN pattern used to determine the approximate count of *V. vulnificus* or *V. parahaemolyticus*. To ensure correct identification of colonies having typical characteristics of color and morphology, biochemical identification of select isolates may be done prior to estimating counts. In this case, three or more suspected colonies from mCPC and TCBS agars are tested by streaking on an arginine-glucose slant (AGS), inoculating T_1N_0 and T_1N_3 broths, and performing oxidase, urease, and ONPG tests. These biochemical tests can be replaced with the diagnostic strip API 20E from bioMerieux Inc., USA (Kaysner and DePaola, 2004).

Another FDA BAM-approved method for enumeration of *V. vulnificus* and *V. parahaemolyticus* is DNA colony lift hybridization (Figure 1.2). This method is applied after direct plating and allows for enumeration and confirmation at the same time. The method

uses species-specific alkaline phosphatase-labeled probes. Briefly, homogenized oyster samples (and their dilutions, as applicable) are directly plated on *Vibrio vulnificus* agar (VVA) and tryptone salt agar (T_1N_3) for *V. vulnificus* and *V. parahaemolyticus,* respectively, followed by overnight incubation at 37°C. Whatman #541 filters are then placed on surface of each plate to lift colonies. This is followed by the chemical lysis step using 0.5M NaOH/1.5M NaCl, with heat treatment by microwave. The filters are then incubated with agitation at 54°C and 55°C (for *V. vulnificus* and *V. parahaemolyticus*, respectively) with a hybridization buffer containing species-specific alkaline phosphatase-labeled probes. After several washing steps, the filters are exposed to NBT/BCIP (Nitro blue tetrazolium chloride / 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) solution at 35°C, covered from light, and the reaction allowed to develop over a period of 1-2 hours (Kaysner and DePaola, 2004). The NBT/BCIP reaction is based on the principle that the BCIP, which is the Alkaline Phosphatase (AP) substrate, is oxidized, after dephosphorylation, by NBT, yielding a dark-blue precipitating dye. The NBT is therefore deoxidized in the reaction producing a dark-blue precipitating dye. Having both compounds (NBT and BCIP) producing blue precipitate intensifies the dye and thus provide a more sensitive detection (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany).

Colony lift hybridization has been extensively studied (Jones, et al., 2009; Kaysner and DePaola, 2004) and is, overall believed to be more specific than MPN because it uses alkaline phosphatase-labeled probes targeting the thermolabile hemolysin (*tlh*) and cytolysin (*vvh*A) genes of *V. parahaemolyticus* and *V. vulnificus*, respectively. However, because the method does not include an enrichment step, the lower limit of detection of colony lift

hybridization is not as good as that for MPN. The probes are also quite expensive, and the method may be difficult to optimize and interpret.

Nucleic acid amplification methods, in the form of polymerase chain reaction (PCR), can be applied either immediately after the enrichment step of MPN, constituting a combined MPN-PCR method, or after selective agar incubation as a confirmatory step. Campbell and Wright (2003) developed the first *TaqMan* real-time PCR assay for the direct enumeration of *V. vulnificus* in oysters. This method does not include an enrichment step, but does include a DNA extraction and concentration method. The primers and probe sequences corresponding to the cytolysin/hemolysin (*vvh*A) gene are used for detection (Table 1.2), with detection limits of 6 CFU/reaction. When directly compared to colony lift hybridization, the two methods showed excellent correlation ($r^2 = 0.99$). In 2004, Panicker et al. described a rapid SYBR Green-based real-time PCR assay developed for the detection of *V. vulnificus* in shellfish and Gulf Coast waters. These investigators used oligonucleotide primers targeting the *V. vulnificus* hemolysin (*vvh*) gene (Table 1.2). After seeded oyster homogenate was subjected to a 5-hour enrichment, the method was able to detect 1 cfu/g. The same investigators developed a *Taqman*-based real-time PCR assay targeting the same (*vvh*A) gene (Panicker and Bej, 2005). The primers and probe for this assay are shown in Table 1.2. Detection limits for samples not preceded by enrichment, were 10^3 cfu/ml. Wright et al (2007) later compared *Taqman*- and SYBR Green I-based real-time PCR assays for the detection of *V. vulnificus* in validation of PHP methods. The primers used for both assays, as well as the *Taqman* probe, were reported previously (Campbell and Wright, 2003) (Table 1.2). Both assays demonstrate 100% sensitivity and specificity for *V. vulnificus.*

In 2003, Blackstone and colleagues developed a *Taqman* real-time PCR assay for the detection of pathogenic (*tdh*+) *V. parahaemolyticus* in oysters. The primers and probe used in this study are shown in Table 1.1. This real-time PCR assay was found to be highly specific for the *tdh*+ *V. parahaemolyticus* strains and also very sensitive (detection limits of 1 cfu/reaction). A similar study was done in 2004 by Kaufman et al., in this case targeting the thermolabile hemolysin (*tlh*) gene as a means by which to quantify total *V. parahaemolyticus*. The primers and Taqman probe used in this study are detailed in Table 1.1

Currently, three sets of primers are commonly used for *V. parahaemolyticus* detection and confirmation (those targeting the *tlh, trh,* and *tdh* genes) (Table 1.1), frequently in a multiplex assay design. The *tlh* gene is a *V. parahaemolyticus* species-specific marker (indicates total *V. parahaemolyticus*), encoding the nonpathogenic thermolabile hemolysin protein (TLH) (Nordstrom et al., 2007). The *tlh, tdh,* and *trh* genes together can be used to determine total *V. parahaemolyticus*, nonpathogenic, and pathogenic strains. If an isolate is only positive for the *tlh* gene then it is determined as nonpathogenic. If it is also positive for one or both of the *tdh* or *trh* genes, then it is designated as potentially pathogenic. Nordstrom et al. (2007) were the first to use this method in conjunction with MPN enrichment, and also included an internal amplification control (IAC) to detect false-negative results due to inhibition from oyster matrix. These investigators reported reaction efficiencies of 82, 94, and 95% for the *tlh, tdh,* and *trh* genes, respectively, with the ability to detect as few as 1 CFU/reaction for each target in a multiplex assay (Nordstrom et al., 2007).

More recently, Jones et al. (2009) evaluated a rapid multiplex real-time PCR method for detecting *V. parahaemolyticus* and *V. vulnificus* simultaneously in MPN samples and

compared it to the DNA colony hybridization methods. Using previously reported primers and probes targeting the *tlh* gene of *V. parahaemolyticus* and the *vvh*A gene of *V. vulnificus* (Campbell and Wright 2003; Nordsrtom et al. 2007), these investigators also included internal amplification control (IAC) probe to ensure PCR integrity and detect reaction inhibition (Table 1.3). The limit of detection for the real-time PCR assay in pure culture was as low as 6.4 and 12.4 CFU/reaction for *V. vulnificus* and *V. parahaemolyticus,* respectively. However, in the presence of the oyster matrix, the detection limit increased by about 2 $\log_{10}$ (Jones et al., 2009). Overall, this method was found to be in 96% concordance with colony lift hybridization, but took less time and labor.

1.5 Control and Post Harvest Process (PHP)

The first official effort to promote shellfish sanitation was the establishment of the National Shellfish Sanitation Program (NSSP) in 1925 by the U.S. Public Health Service (Now the U.S. Food and Drug Administration). The NSSP was initially established to provide protection to consumers from pathogens originating from human fecal contamination of shellfish growing/harvesting waters. At the time of the establishment of the NSSP, indigenous bacterial pathogens like *V. vulnificus* and *V. parahaemolyticus* had not yet been identified. Even after their identification (*V. vulnificus* in 1979 and *V. parahaemolyticus* 1951), these pathogens have historically been overlooked.

To further promote shellfish sanitation and protect the health and safety of the consuming public, the Interstate Shellfish Sanitation Conference (ISSC) was organized in 1982 (ISSC, 2011). In 1984, the Memorandum of Understanding (MOU) between the FDA

and the ISSC was established to recognize the ISSC as the official cooperative body between state and federal public health and control agencies, the shellfish industry, and academia (NSSP, 2009a). The ISSC adopts uniform procedures for the interstate NSSP, which are then implemented by all shellfish control agencies. The ISSC also provides comprehensive guidelines for state shellfish control programs relative to the harvesting, processing, and shipping conditions of shellfish; communicates with, and informs all relevant stakeholders about important issues and recent developments in shellfish sanitation; and manages three task forces to promote its mission in shellfish growing, processing and distribution, and administration.

In 2001, the ISSC and the FDA designed a seven-year *V. vulnificus* control plan for inclusion in, and to expand the focus of, the National Shellfish Sanitation Program (NSSP). This control plan requires any state that has experienced, since 1995, two or more laboratory-confirmed cases of *V. vulnificus* septicemia traced to state's harvest waters, to develop strategies to reduce any *V. vulnificus* oyster- related illnesses. The initial phase of the plan targeted a 40% *V. vulnificus* illness reduction rate for the years 2005 and 2006 combined. The final phase of the plan was to achieve a 60% illness reduction for the years 2007 and 2008. These reductions were to be based upon a baseline rate of 0.306 cases per million populations, which was the average for the years 1995-1999, based on data reported by California, Louisiana, Florida, and Texas (DiStefano et al., 2011; Shames 2011; NSSP 2009b). In 2003, the state of California took extra measures by requiring post-harvest processes (PHP) to be applied on all Gulf Coast oysters harvested from April thru October

and intended for raw consumption in that state. From 2003 thru 2008, California had only two reported cases of *V. vulnificus* illness.

As part of the illness reduction plan, the Gulf States and the ISSC have developed extensive educational programs targeting at-risk populations (immunocompromised), health professionals, and the general public. The ISSC has performed two surveys to assess the success of these educational programs. The first survey, done in 2002, established a baseline level of consumer knowledge about the consumption patterns and risks associated with raw oysters. The second survey was done as a follow up to determine whether educational programs had any impact on consumer behavior. The ISSC concluded that there was no significant difference in consumer knowledge, or change in raw oyster consumption, in association with the implemented educational programs (Shames 2011). By the end of 2008, the four reporting states (California, Louisiana, Florida, and Texas) only resulted in a 35% illness reduction rate (DiStefano et al., 2011; Shames 2011; NSSP 2009b).

In October 2009, the FDA announced its dissatisfaction with current NSSP *V. vulnificus* illness reduction plans because the plans failed to achieve the target of 60% illness reduction. They made clear their intentions for reformulating their procedures to largely eliminating *V. vulnificus.* This would be achieved by means of including post-harvest processing (PHP) treatments for Gulf Coast oyster harvested in the warm months of the year. Candidate PHP treatments included High Hydrostatic Pressure (HHP), mild heat treatment, Individual Quick Freezing (IQF) with extended frozen storage, and gamma irradiation. This move by the FDA contradicted the 1984 Memorandum of Understanding (MOU) with the ISSC that states the two groups would work concordantly. After formal written

communications between ISSC and FDA, the two groups agreed to work collaboratively and announced that the FDA would conduct an independent study to assess the feasibility and cost of implementing PHP in the Gulf Coast before making a final decision to mandate its use (DiStefano et al., 2011; Shames 2011).

On another note, the Food Safety Modernization Act (FSMA) passed by congress in 2011 requires the FDA to submit a report to the Committee on Energy and Commerce of the House of Representatives and the Committee on Health, Education, Labor, and Pensions of the Senate at least 90 days prior to any issuance of regulation, guidance, or suggested amendment to the NSSP's Model Ordinance that relates to PHP. Such report is to include assessments of the costs and feasibility of the implementation of PHP technologies or any equivalent controls, estimates of the projected public health outcomes, criteria to ensure that shellfish from all nations of origin are equally treated by PHP, evaluation of alternative ways to prevent or reduce to acceptable levels the incidence of foodborne illness, and the extend to which the FDA has consulted and coordinated with other regulatory agencies and States on PHP measures (FSMA 2011). The Food Safety Modernization Act (FSMA) also requires that the Comptroller General of the United States to prepare a review and evaluation of the FDA's report; comparison of the FDA's proposed guidance or regulation to those of other regulated foods on the basis of the risks found in the seafood and the other foods compared and their incidence rates; and to evaluate the PHP impact on the level of competitiveness of domestic oyster industry in both of the international and local markets (FSMA 2011).

Accordingly, the Research Triangle Institute (RTI International) (Research Triangle Park, North Carolina 27709) conducted an independent study for the FDA in 2011. The RTI report titled *Analysis of How Post-harvest Processing Technologies for Controlling Vibrio vulnificus Can Be Implemented* analyzed the feasibility of PHP for Gulf Coast oysters intended for the raw half-shell market. Their report found that (1) "cool pasteurization" (mild heat) was a feasible technology already in use at one Gulf Coast operation; (2) HHP was also feasible and in use at three Gulf Coast operations; (3) low-dose gamma irradiation was feasible for operations within reasonable distances from the Mulberry, FL irradiation facility; (4) IQF with extended storage was only feasible in cool weather months and already in use at eight Gulf Coast operations; and (5) there was insufficient evidence supporting the efficacy of high salinity treatment (relaying in salt-water) and that it was currently not feasible due to permitting issues.

In September of 2011, a U.S. Government Accountability Office (GAO) report to the House of Representatives reviewed the RTI report and criticized both the FDA and the ISSC on their efforts of reducing *V. vulnificus* illnesses (Shames 2011). Specifically, the report stressed that since 2004, the ISSC had not evaluated any of its consumer education programs and that the FDA has never conducted evaluations on their own consumer education programs. They also argue that neither the FDA nor the ISSC have ever directly evaluated the effectiveness of their time and temperature control plans for reducing *V. vulnificus* shellfish-borne illnesses. The GAO report also mentions the absence of data regarding compliance levels. Taken together, the GAO concluded that with the current state of affairs, compliance with time and temperature controls is highly unlikely (Shames 2011).

The current NSSP-proposed guidelines are that, to be recognized as a valid PHP, the process must reduce the concentrations of *V. vulnificus* and/or *V. parahaemolyticus* to non-detectable levels, defined as < 30 MPN/g and a minimum 3.52-$\log_{10}$ reduction (NSSP 2009a; NSSP 2009c). As described above, many PHP technologies are currently in use, and could become FDA-approved if they are appropriately validated. The data supporting their efficacy (or lack thereof) is discussed in greater detail below.

1.5.1 *High Hydrostatic Pressure Processing (HHP)*

This non-thermal processing technology was first applied to food products in 1899. The technique uses hydrostatic compression pressure in the range of 100-1000 MPa, which provides a very effective means by which to destroy vegetative bacterial cells, both Gram positive and Gram negative. It is particularly promising as it results in limited impact on sensory properties of food products, including color and flavor. The method also has little negative effect on nutritional value.

Both *V. vulnificus* and *V. parahaemolyticus* are very sensitive to high-pressure treatment (FDA 2011b; Ma and Su 2011; Prapaiwong et al 2009). For example, Ma and Su (2011) studied the efficacy of HHP in reduction of *V. parahaemolyticus* in artificially contaminated Pacific oysters (*Crassostrea gigas*). The product was treated at 293 MPa for 90, 120, 150, 180, and 210 sec at about 8±1°C. Log_{10} reductions exceeding 3.52 were observed after 120 seconds or more of HHP treatment. In another study, Kural et al (2008) investigated if HHP could achieve a 5-$\log_{10}$ reduction of *V. parahaemolyticus* in Gulf Coast oysters (*Crassostrea virginica*). Artificially contaminated oyster meat was temperature

abused (held at 21°C for 24 hours), blended, and then subjected to HHP. Five $\log_{10}$ reductions were observed after treatment for 2 min at ≥350 MPa and temperatures ranging from 1-35°C, or at ≥300 MPa at 40°C. In similar studies applied to the most pressure resistant strain of *V. vulnificus*, Kural and Chen (2008) observed that both lower temperatures (<20°C) and elevated temperatures (>30°C) increased the efficacy of HHP treatment. A treatment of ≥250 MPa for ≤4 minutes at -2 or 1°C produced a >5 $\log_{10}$ reduction in levels of *V. vulnificus* in artificially contaminated Gulf Coast oysters.

1.5.2 *Mild Heat Processing*

This treatment involves submerging shell-stock oysters in a water bath at specific time-temperature combinations, followed by rapid chilling. For pathogens with notable heat sensitivity, including *V. vulnificus and V. parahaemolyticus,* this treatment has been found to be quite effective (FDA 2011b). The process is mainly used by North and South Carolinas oyster processors as a shucking aid, and is also known as the commercial heat-shock process. If performed appropriately, this process maintains the visual and organoleptic properties of raw oyster meat (Hesselman et al., 1999).

In 1999, Hesselman and colleagues evaluated a commercial heat shock process for its ability to inactivate pathogens in Gulf Coast oysters (*Crassostrea virginica*). Using naturally-contaminated oysters having *V. vulnificus* concentrations of 10^2 to 10^4 MPN/g and applying a heat treatment that achieved an internal oyster meat temperature of ≥50°C for 1-4 min, between 1 and 4 $\log_{10}$ inactivation of *V. vulnificus* was observed. These results showed initial promise, leading the investigators to conclude that, with optimization, a method might

be able to provide the recommended 3.52 $\log_{10}$ reduction required by the NSSP for a legitimate PHP.

In another study, investigators sought to "pasteurize" shell-stock Gulf Coast oysters using a mild heat treatment during which the internal temperature of the oyster meat was held at 50^{o}C for 0, 5, 10 and 15 min (Andrews et al., 2000). Both naturally and artificially contaminated oysters were treated by this method and then stored on ice for 14 days. The heat treatment effectively reduced the levels of *V. vulnificus* and *V. parahaemolyticus* from concentrations exceeding 1.0 x 10^5 to <3 MPN/g after 10 min or less. When followed by ice storage, the low concentrations of *V. vulnificus* and *V. parahaemolyticus* were maintained. Interestingly, after 4 days storage at refrigeration temperatures without a prior heat treatment, *V. vulnificus* reductions of over 2 $\log_{10}$ MPN/g were observed.

1.5.3 *Depuration*

In depuration, or controlled purification, freshly harvested shellfish are washed and placed in a clean aquatic environment having controlled environmental conditions (e.g., temperature, water flow, salinity) that promotes optimal physiological functions, allowing the shellfish to naturally open and purge particulates and microbes (Chae et al, 2009; Lewis et al, 2010). Chae et al (2009) studied the efficacy of depuration as a means of reducing *V. parahaemolyticus* and *V. vulnificus* in Gulf Coast oysters (*Crassostrea virginica*). Specifically, depuration was evaluated at different temperatures and time combinations. When the process was conducted at 22°C for 48 hours, reductions of only 1.2 and 2.0 $\log_{10}$ MPN/g were observed for *V. parahaemolyticus* and *V. vulnificus*, respectively. When lower

temperatures were used (15°C), reduction of *V. parahaemolyticus* and *V. vulnificus* increased to 2.1 and 2.9 $\log_{10}$ MPN/g, respectively. Increasing the duration of depuration from 48 to 96 hours also improved reduction for both pathogens to 2.6 and 3.3 $\log_{10}$ MPN/g, respectively, but further reduction in depuration temperature (from 10 and 5°C) had little effect on depuration efficacy.

Lewis et al (2010) tested a flow-through depuration system for potential elimination of *V. vulnificus* from Eastern oysters (*Crassostrea virginica*). In their experiments, both naturally contaminated and artificially inoculated oysters were used. Microbiological analysis was done immediately after depuration and also 1, 2, 3, and 6 days after depuration was completed. These investigators observed that depuration efficacy was better for artificially contaminated oysters compared to naturally contaminated specimens; the former demonstrated >3.52 $\log_{10}$ reduction in the concentration of *V. vulnificus,* while removal of *V. vulnificus* was more recalcitrant for naturally contaminated oysters. Increasing flow rate from 11 L/m to 68 L/m resulted in a reduction of *V. vulnificus* counts from 1.1 x 10^5 MPN/g to 3 MPN/g for naturally contaminated oysters depurated for a total of 6 days, although there was variability among replicates. It was also noted that salinity >30 ppt may facilitate efficient depuration of *V. vulnificus*. While depuration may appear effective in some of these studies, there are substantial challenges in consistency and optimization of the method as applied to elimination of pathogenic *Vibrio* spp. in oysters. Hence, this method is not applied commercially in the Gulf region.

1.5.4 *Ionizing radiation*

Ionizing radiation or "irradiation" is used in a variety of foods for the elimination or reduction of parasites, insects, and bacterial pathogens. The technique is very effective for elimination of pathogenic *Vibrio* spp. in molluscan shellfish (FDA 2011b). For example, researchers have found that doses ≤ 0.1 kGy are effective against *V. cholerae* and *V. vulnificus*. Further, doses ≤ 2.0 kGy can be applied to oyster meat without any significant changes to organoleptic properties (Drake et al., 2007). Mahmoud (2009) studied the efficacy of X-ray radiation for inactivation of *V. vulnificus* in pure culture and in artificially contaminated half shell and whole shell American oysters (*Crassostrea virginica*). These investigators observed that doses of ≥ 0.75, 1.0, and 3.0 kGy were necessary to achieve 6-$\log_{10}$ reductions in pure cultures of *V. vulnificus*, half shell, and whole shell oysters, respectively.

1.5.5 *Other PHP Technologies*

Other PHP technologies are currently under development in academia and/or industry but have substantial technical challenges. An interesting PHP approach is the use of Generally Recognized as Safe (GRAS) compounds. GRAS compounds are typically added at different concentrations during depuration to facilitate inactivation and removal of *V. vulnificus*. Supplementing depuration with GRAS compounds constitutes a new process, bringing with it the potential for consumer concerns that often accompany alternative processing technologies.

Results using such GRAS compounds have been mixed. For example, In 2003 Birkenhauer and Oliver evaluated diacetyl for reduction of *V. vulnificus* in the Gulf Coast oysters. They found that this compound is only effective at reducing the pathogen in shucked oysters. In another study, Pelon et al (2005) used oyster extract that has a natural antimicrobial activity. They observed that, after incubating *V. vulnificus* cultures at 4°C with this oyster extract for various periods of time, $\log_{10}$ reductions were as follows: 3 $\log_{10}$ after 18 h, and 5 $\log_{10}$ after 42 h. They suggested that their findings could be used to optimize bio-depuration of shell-stock oysters. Wang et al (2010) examined the use of chlorine dioxide (ClO_2) during depuration, finding that concentrations of 20 mg/L completely eliminated *V. parahaemolyticus* after only a 6 hour depuration period. The process also increased the shelf-life of the product to12 days if stored at 4°C. However, oysters treated with ClO_2 developed a chocolate-like color on their gills that was undesirable in consumer testing.

1.5.6 *The Promise of PHP*

Some post-harvest processing technologies do present high potential in improving the safety of molluscan shellfish, especially for product intended for the raw market. Some [e.g., High Hydrostatic Pressure (HHP), Mild Heat Processing, and Individual Quick Freezing (IQF) with extended frozen storage] have already demonstrated the ability to meet the requirements of a 3.52-$\log_{10}$ reduction and a final concentration of <30 MPN/g. There are other potential PHP methods being used in, for example, North and South Carolina, but for purposes other than food safety. These methods may be adjusted and validated for pathogenic *Vibrio* inactivation efficacy with the potential to become approved PHP

technologies. The heat shock treatment used to facilitate oyster shucking is one such example.

For the most part, the candidate PHP methods described above have also demonstrated, in sensory panel studies, no significant impact on product appearance, aroma, flavor, or taste. For example, Andrews and Coggins (2004) conducted a sensory study, finding no significant difference between appearance, aroma, flavor, or taste of the oysters processed by IQF, "cool pasteurization" and HHP, although these oysters were not compared to an untreated controls. In another panel study, Coggins (2004) evaluated consumer acceptability of oysters processed by IQF, Cool Pasteurization, and HHP with respect to product appearance, aroma, and flavor, in this case, comparing to untreated control oysters; no significant differences were observed between any of the products.

Oysters originating from the Gulf Coast region dominate the production for the entire United States, constituting 60% of the entire harvest. Louisiana alone harvests 14.7 millions of pounds of oysters each year, with an estimated value of $50 million. The Gulf States combined produce 23 millions of pounds of oysters annually, with the value of $72 million (Shames 2011). Clearly, this is a vibrant industry producing a high value product.

However, this industry has universally resisted the regulatory requirement for PHP. Regardless of the PHP method used, the requirement of treatment would force Gulf Coast producers to divert their summer harvest. Even though some of these promising PHP methods are already being used by various processors in the Gulf Coast region, the ability to process the entire Gulf Coast summer market is beyond the current capacity of these facilities combined. Because of this, a regulatory mandate would require significant capital

investment in new equipment and technologies. The expansion of PHP capacity will be very expensive, ranging from costs associated with facility expansion and up-fit, employee training, and maintaining facilities and equipment. As detailed in the RTI report (DiStefano et al., 2011), this will be a challenge to oyster harvesters and processors alike.

There could also be issues associated with consumer acceptance, despite promising sensory results. In a mail survey to assess consumer preference for different PHP methods, Hanson et al (2003) found that 61% preferred the depuration process to irradiation, ozonation, and pressurization. Since depuration is not effective, and given some strong push-back by consumer advocates regarding the public acceptability of alternative processes such as ionizing radiation and high pressure, it is possible that there may be problems with consumer acceptance of product. On the other hand, Andrews and Coggins (2004) reported that 77% of the participants in their sensory study said they would consume more PHP oysters if they were free of pathogens. Therefore, educating consumers of raw oysters about the importance of PHP could potentially increase consumption that might at least in part, cover the cost of investing in PHP.

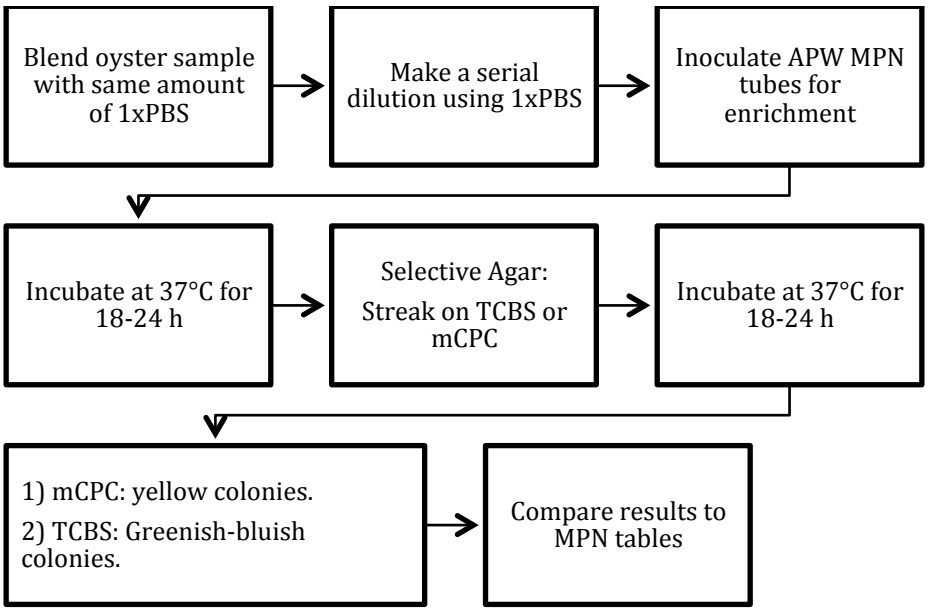

Figure 1.1- MPN with Selective Agar for *V. vulnificus* and *V. parahaemolyticus*

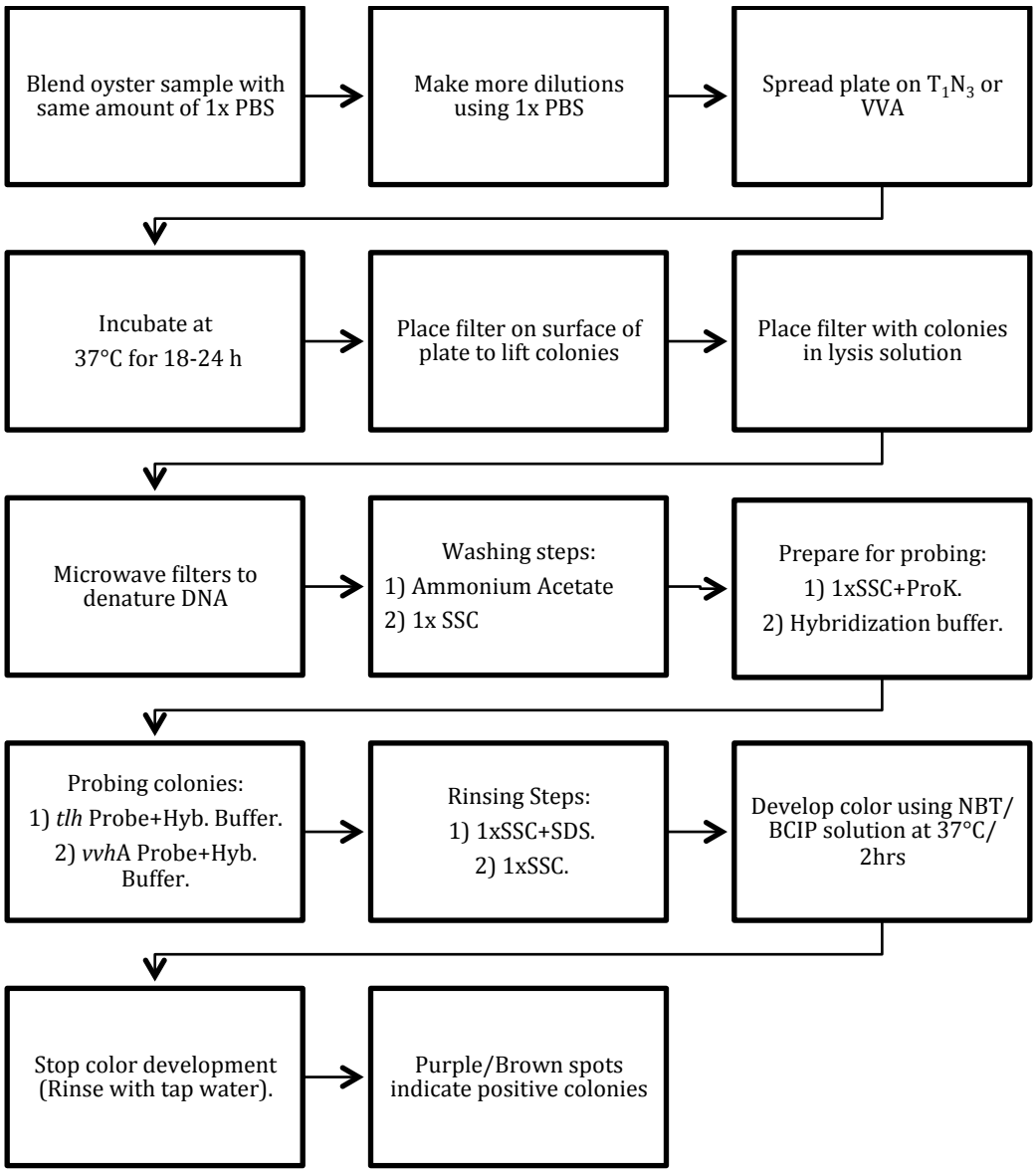

Figure 1.2- DNA colony lift hybridization for *V. vulnificus* and *V. parahaemolyticus*

Table 1.1: Real-Time PCR methods and sequences used to identify *V. parahaemolyticus.*

Gene	Sequence	Application	Reference
tdh	Forward 5'-TCCCTTTTCCTGCCCCC-3' Reverse 5'-CGCTGCCATTGTATAGTCTTTATC-3' Probe 5'-FAM-TGACATCCTACATGACTGTG-MGBNFQ-3'	Taqman Multiplex Real-time PCR	Nordstrom et al (2007)
trh	Forward 5'-TTGCTTTCAGTTTGCTATTGGCT-3' Reverse 5'-TGTTTACCGTCATATAGGCGCTT-3' Probe 5'-TET-AGAAATACAACAATCAAAACTGA-MGBNFQ-3'		
tlh	Forward 5'-ACTCAACACAAGAAGAGATCGACAA-3' Reverse 5'-GATGAGCGGTTGATGTCCAA-3' Probe 5'-TxRED-CGCTCGCGTTCACGAAACCGT-BHQ2-3'		
IAC[1]	Forward 5'-GACATCGATATGGGTGCCG-3' Reverse 5'-CGAGACGATGCAGCCATTC-3' Probe 5'-Cy5-TCTCATGCGTCTCC CTGGTGAATG TG-BHQ2-3'		
tlh	Forward 5'-CGAGAACGCAGACATTACGTTC-3' Reverse 5'-TGCTCCAGATCGTGTGGTTG-3' Probe 5'-FAM-TCGCCGCTGACAATCGCTTCTCAT-BHQ1-3'	Real-time PCR	Kaufman et al (2004)
tdh	Forward 5'-AAACATCTGCTTTGAGCTTCCA-3' Reverse 5'-CTCGAACAACAAACAATATCTCAT CAG-3' Probe 5'-FAM-TGTCCCTTTCCTGCCCCCGG-TAMRA-3'	Real-time PCR	Blackstone et al (2003)

[1]Internal Amplification Control: used to prevent false negative results during PCR.

Table 1.2: Real-Time PCR Primers and Probe Sequences for *Vibrio vulnificus*

Gene	Sequence	Application	Reference
*vvh*A	Forward 5'-TTCCAACTTCAAACCGAACTATGAC-3' Reverse 5'-ATTCCAGTCGATGCGAATACGTTG-3' Probe 5'-ROX-AACTATCGTGCACGCTTTGGTACC GT-BHQ2-3'	Tagman Real-time PCR	Panicker and Bej (2005)
*vvh*A	Forward 5'-CTCACTGGGGCAGTGGCT-3' Reverse 5'-CCAGCCGTTAACCGAACCA-3' Probe 5'-ROX-AACTATCGTGCACGCTTTGGTACC GT-BHQ2-3'	Tagman Real-time PCR	Panicker and Bej (2005)
*vvh*A	Forward 5'-TTCCAACTTCAAACCGAACTATGAC-3' Reverse 5'-ATTCCAGTCGATGCGAATACGTTG-3'	SYBR green Real-time PCR	Panicker et al (2004)
*vvh*A	Forward 5'-TGTTTATGGTGAGAACGGTGACA-3' Reverse 5'-TTCTTTATCTAGGCCCCAAACTTG-3' Probe 5'-FAM-CCGTTAACCGAACCACCCGCAA-BHQ-3'	Taqman Real-time PCR	Campbell and Wright (2003)

Table 1.3: Multiplex Real-time PCR Primers and Probe sequences for *V. vulnificus* and *V. parahaemolyticus*

Gene	Sequence	Application	Reference
tlh	Forward 5'-ACTCAACACAAGAAGAGATCGACAA-3' Reverse 5'-GATGAGCGGTTGATGTCCAA-'3 Probe 5'-TxRED-CGCTCGCGTTCACGAAACCGT-BHQ2-3'	Taqman Multiplex Real-time PCR	Jones et al (2009)
vvhA	Forward 5'TGTTTATGGTGAGAACGGTGACA-3' Reverse 5'-TTCTTTATCTAGGCCCCAAACTTG-3' Probe 5'-6-FAM-CCGTTAACCGAACCACCCGCAA-TAMARA-3'		
IAC[1]	Forward 5'-GACATCGATATGGGTGCCG-3' Reverse 5'-CGAGACGATGCAGCCATTC-3' Probe 5'-TET-TCTCATGCGTCTCCCTGGTGAA TGTG-BHQ-1-3'		

[1]Internal Amplification Control: used to prevent false negative results during PCR.

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CHAPTER 2

Evaluation of the Efficacy of a Commercial Heat Shock Method for Reduction of *Vibrio vulnificus* and *Vibrio parahaemolyticus* in Gulf Coast Oysters

2.1 Abstract

Pathogenic *Vibrio* species (*V. vulnificus* and *V. parahaemolyticus*) are the most common cause of foodborne disease associated with the consumption of raw or undercooked seafood, especially molluscan shellfish. The failure to adequately control these pathogens prompted the U.S. Food and Drug Administration in 2009 to propose that Gulf Coast oysters harvested in warm months and intended for the raw (half-shell) be subjected to a post-harvest process (PHP). The purpose of this study was to evaluate, and modify if necessary, an existing heat-shock treatment routinely used by North Carolina shellfish dealers to facilitate oyster shucking, for its efficacy in reducing *Vibrio vulnificus* and *Vibrio parahaemolyticus* levels. In addition, the study was to determine if this method met FDA and ISSC requirements for recognition under the NSSP guidelines as an approved Post-Harvest Processing (PHP) method. Naturally contaminated Gulf Coast oysters having levels of *V. vulnificus* and *V. parahaemolyticus* exceeding 1 x 10^4 MPN/g were subjected to a mild heat process under pilot-scale laboratory conditions (consisting of a 200 liters stainless steel tank with recirculating system and direct steam injection) and commercial-scale conditions (heat shock tanks containing approximately 400 liters of water heated by gas-fired burners). Treatments consisted of 60 ± 0.5°C for 2, 4, 6, 8, and 10 min followed by rapid chilling. Control and treated oysters were quantitatively analyzed for surviving *V. vulnificus* and *V.*

parahaemolyticus populations using the Most Probable Number (MPN) method. A treatment of 8-10 min at 60°C was necessary to reduce *V. vulnificus* and *V. parahaemolyticus* by about 4 $\log_{10}$, in keeping with the NSSP definition of a PHP method, i.e., one that reduces the concentrations of these pathogens to non-detectable levels, defined as < 30 MPN/g and a minimum 3.52-$\log_{10}$ reduction. These results indicate that the commercial heat-shock treatment has the potential to be used by industry as a PHP method for control of pathogenic *Vibrio* spp. in raw oysters once given approval by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration.

2.2 Introduction

Members of the *Vibrio* genus are the most significant cause of foodborne disease associated with the consumption of seafood, and molluscan shellfish are the leading product category causing these illnesses. Disease caused by pathogenic *Vibrio* spp. can be severe or mild and generally fall into three categories: wound infections and primary septicemia (predominantly caused by *V. vulnificus*) and gastroenteritis (predominantly caused by *V. parahaemolyticus*). Each year in the U.S. there are about 100 cases of *V. vulnificus*, over 34,000 cases of *V. parahaemolyticus* infection, and greater than 17,000 illnesses caused by other *Vibrio* species. *Vibrio vulnificus* infection is particularly severe, with a mortality rate exceeding 50% (number of deaths per year in U.S. average 12) The consumption of raw, molluscan shellfish is responsible for the vast majority of these pathogenic *Vibrio* infections (Scallan et al., 2011).

The pathogenic *Vibrio* species are ubiquitous in marine environments, where their levels can be quite high during the warm summer months, particularly in the Gulf of Mexico. Further, as filter feeders, molluscan shellfish concentrate these pathogens in their digestive tracts such that levels can be as high as 10^4-10^5 CFU/g in summertime oysters. Because contamination cannot be avoided, there has been a steady increase in concern over the public health risk posed by pathogenic *Vibrio* species in molluscan shellfish.

Accordingly, in 2001, the Interstate Shellfish Sanitation Conference (ISSC) and the U.S. Food and Drug Administration (FDA) designed a seven-year *V. vulnificus* control plan for inclusion in the National Shellfish Sanitation Program (NSSP). This control plan required any state that had experienced, since 1995, two or more laboratory-confirmed cases of *V. vulnificus* septicemia traced to the state's harvest waters, to develop strategies to reduce any *V. vulnificus* oyster-related illnesses. The initial phase of the plan targeted a 40% *V. vulnificus* illness reduction rate for the years 2005 and 2006 combined. The final phase of the plan was to achieve a 60% illness reduction for the years 2007 and 2008. Despite efforts on the part of the Gulf of Mexico states and the ISSC (including targeted educational programs and time/temperature controls from harvest to consumption), only a 35% illness reduction rate had been realized by the end of 2008 (DiStefano et al., 2011; NSSP 2009b; Shames et al., 2011).

The relative failure of the control plan to achieve a 60% illness reduction over nearly a decade prompted FDA to propose in 2009 the requirement that a Post-Harvest Processing (PHP) treatment be applied to Gulf Coast oysters harvested in the warm months of the year (April through October). The focus of these treatments is the elimination of *V. vulnificus*

from summertime Gulf Coast oysters destined for the half-shell (raw) market. The NSSP has proposed that, to be recognized as a valid PHP, the process must reduce the concentrations of *V. vulnificus* and/or *V. parahaemolyticus* to non-detectable levels, defined as <30 MPN/g and a minimum 3.52-$\log_{10}$ reduction (NSSP 2009a; NSSP 2009c). Recognized PHP methods include mild heat treatment ("cool pasteurization"), High Hydrostatic Pressure (HHP), Individual Quick-Freezing (IQF) with extended frozen storage, mild thermal processing, ionizing radiation, controlled purification (depuration, relaying), and treatment with Generally Recognized as Safe (GRAS) compounds (Drake et al., 2007; Hanson et al., 2003). In a comprehensive study on the commercial feasibility of implementing PHP, the only three processes that were identified as economically feasible (based on efficacy and availability/location of processing facilities) are cool pasteurization (mild heat), HHP, and low dose gamma irradiation.

Heat shocking is a form of mild heat processing that involves submerging shell-stock oysters in water for specific time-temperature combinations, followed by rapid chilling. For pathogens with notable heat sensitivity, including *V. vulnificus and V. parahaemolyticus,* these treatments have been found to be quite effective (FDA 2011). The commercial heat shock process is mainly used by North and South Carolinas oyster processors as a shucking aid, and if performed appropriately, this process maintains the visual and organoleptic properties of raw oyster meat (Hesselman et al., 1999). However, this method has not been validated with respect to its efficacy in eliminating the pathogenic *Vibrio* species with a focus on the PHP standards set in the NSSP. The purpose of this study was to evaluate, and modify if necessary, an existing heat-shock treatment routinely used by North Carolina shellfish

dealers to facilitate oyster shucking, for its efficacy in reducing *Vibrio vulnificus* and *Vibrio parahaemolyticus* levels. In addition, the study was to determine if this method met FDA and ISSC requirements for recognition under the NSSP guidelines as an approved Post-Harvest Processing (PHP) method.

2.3 Materials & Methods

2.3.1 Oyster Samples

Commercially harvested summertime oysters originating from the Gulf of Mexico, obtained from local North Carolina dealers, were used in all experiments. For the pilot scale, oysters were harvested from Texas and Florida. For the commercial scale, four trials were run on oysters harvested on September 13, 2011, from harvest area number 23 of Louisiana. Four other trials were run on oysters harvested on October 5, 2011, from harvest area number 406 of Texas. Pilot scale processing of oysters was performed by personnel at the North Carolina State University (NCSU) Seafood Laboratory in Morehead City, NC, while commercial scale processing was performed by Seafood Lab personnel in cooperation with Ardys Oysters in Supply, NC. Microbiological testing was conducted at the Jaykus laboratory on the NCSU campus.

2.3.2 Temperature Monitoring

In preparation for continuous temperature monitoring, a cordless drill with a 3 mm bit was used to pierce the top shells of each of three oyster specimens. Teflon-coated, 24-gauge type T thermocouples from Omega Engineering (Stamford, Connecticut) were inserted into

the oyster shell and secured with rubber bands. Hot glue was used to seal the gap between the drilled hole and thermocouple, as well as to secure the thermocouple to the oyster shell. Heat penetration data were collected using an OM-CP-OctTemp2000 eight-channel data logger from Omega Engineering (Stamford, Connecticut). Temperatures were taken at 0.083 min time intervals.

2.3.3 Pilot Study

For each experiment, three oysters with thermocouples were placed in mesh polypropylene bags along with approximately twelve additional oysters from the same batch. The bags were lowered into a 200 L round, stainless steel tank which was pre-heated by live steam injection to a temperature of 60°C. The water was recirculated and the temperature maintained manually. After a residence time of 2, 4, 6, 8, and/or 10 min, one bag each was removed and placed into a 19 L bucket containing an ice-water slurry. The oysters remained in the slurry until the internal temperature was reduced to ≤13°C at which time the probed oysters were removed (discarded) and the remaining oysters were packed on ice and shipped on ice to NCSU for microbiological analysis, which was initiated within 24 h of oyster processing. A total of six trials were undertaken, with the criteria for inclusion in this study being initial (untreated control oysters) *V. vulnificus* and *V. parahaemolyticus* levels exceeding 10^4 MPN/g. Photographs for the pilot scale set-up are shown in Figure 2.9.

2.3.4 Commercial Study

The commercial heat shock method was conducted at 60°C for 6, 8, and 10 minutes. Oysters for the commercial study were obtained and prepared in the same manner as described above, except that in place of mesh polypropylene bags, oysters were placed in wire baskets that were lowered into heat shock tanks containing approximately 400 L of water heated by gas-fired burners that were thermostatically controlled at 60°C; manual recirculation of the water was done throughout the treatments. Wire baskets were removed from the tanks after 6, 8, and 10 min and the oysters cooled in ice-water slurry, packed, and shipped as described above. A total of eight commercial trials were undertaken and included in this study. Photographs for the commercial scale set-up are shown in Figure 2.10.

2.3.5 Microbiological Analysis

Control and treated oysters were quantitatively analyzed for surviving *V. vulnificus* and *V. parahaemolyticus* populations using the Most Probable Number (MPN) methods as described in the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) (Kaysner and DePaola 2004). Briefly, about 10-12 oysters were shucked and homogenized in phosphate buffered saline (PBS) (1:1), followed by the preparation of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) in PBS. From each dilution, three alkaline peptone water (APW) tubes were inoculated with 1 ml and incubated at 37°C for 18-24 hrs. Tubes showing turbidity were streaked onto selective agars [Modified Cellobiose-Polymyxin B-Colistin (mCPC) agar for *V. vulnificus* / Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar for *V. parahaemolyticus*] and incubated at 37°C for 18-24 hrs. Colonies that were 1-2 mm in

diameter, yellow, opaque, round, and flat on mCPC plates were considered positive for *V. vulnificus,* while colonies that were 2-3 mm in diameter, opaque, round, and green or bluish on TCBS plates were considered positive for *V. parahaemolyticus*.

2.3.6 Data Analysis

MPN scores for each individual pilot and commercial scale run were recorded and compared to 0.1, 0.01, and 0.001, 3-tube-MPN tables for enumeration (Kaysner and DePaola 2004). F-values were calculated for each commercial run. F-value is defined as the total area under the process lethality curve, and used to compare different processes and determine if a given process is adequate to control the target organism (defined here as *V. vulnificus* and *V. parahaemolyticus*). The F-value calculation was made using 70°C as the reference temperature and 8.5°C as the z_D-value for both targeted organisms, *V. vulnificus* and *V. parahaemolyticus* (Ama et al., 1994; Asselt and Zwietering 2006; Bang and Drake 2002; ICMF 1996).

The formula was as follows:

$F = 10^{(Ti-Tref)/z} \Delta t$

Where:

T_i is the midpoint temperature for a given time

T_{ref} is the reference temperature (70°C)

z_D is the *z*-value

Δt is the time interval between temperature readings.

The mean temperature of the three probed oysters in each trial was used in the F-value (process lethality) calculations. The mean temperature of the three probed oysters in each trial and the water temperature of each trial were used to generate Figures 2.3, 2.4, and 2.5. The data corresponding to the most conservative (lowest) F-value of the eight individual runs for each treatment time (6, 8, and 10 min) was used to produce Figure 2.6, 2.7, and 2.8. For purposes of comparison, total $\log_{10}$ reductions for these conservative estimates were estimated using the formula F-value/D_{ref}. Microsoft Excel software was used for all the calculations and to produce the graphs.

$$F = D_{ref} \ \text{Log}_{10} (N_0/N)$$

$$F/D_{ref} = \text{Log}_{10} (N_0/N)$$

2.4 Results

2.4.1 Vibrio vulnificus

For the pilot study, a total of six trials were undertaken, with the criteria for inclusion in this study being initial (untreated control oysters) *V. vulnificus* levels exceeding 1 x 10^4 MPN/g. Two oyster lots fulfilled this criterion, with maximum inactivation after 8 min of treatment ranging from 3.76 to slightly over 4.0 $\log_{10}$ MPN/g, based on assay limit of detection and initial levels of contamination (Figure 2.1). In general, the required 3.52 $\log_{10}$ reduction was achieved after a 6 min treatment, with additional treatment time producing relatively little added value.

For commercial scale studies, a total of eight trials were undertaken, all of which met the inclusion criteria of *V. vulnificus* initial (control) levels exceeding 1 x 10^4 MPN/g. Table

2.1 summarizes the *V. vulnificus* data within the context of the NSSP PHP requirements. Yellow highlighted data correspond to treatments directly meeting the regulatory requirements of <30 MPN/g and a minimum 3.52-$\log_{10}$ reduction of *V. vulnificus*. For all treatment times (6, 8, and 10 min), a total of eight trials were undertaken. In general, 8 and 10 min treatment times resulted in near complete elimination of the pathogen, which ranged from 3.8-4.6-$\log_{10}$ reductions. The 8 min treatment effectively fulfilled the NSSP PHP requirements in all cases.

2.4.2 Vibrio parahaemolyticus

For the pilot study, a total of six trials were undertaken, with the criteria for inclusion in the study being initial (untreated control oysters) *V. parahaemolyticus* levels exceeding 1 x 10^4 MPN/g. Figure 2.2 displays the results for *V. parahaemolyticus*; in this case, four oyster lots fulfilled the inclusion criteria. Overall, *V. parahaemolyticus* was more heat resistant than *V. vulnificus*, with maximum $\log_{10}$ reductions (4.0-4.5 $\log_{10}$ MPN/g, based on assay limit of detection and initial levels of contamination) reached in only one trial. On average, about a 3 $\log_{10}$ inactivation was observed after an 8 min heat treatment. Based on these initial results, treatments of 8 or 10 min at 60^{o}C were considered promising in meeting the NSSP PHP requirements for control of *V. parahaemolyticus* given the initial contamination levels of the oysters used in these experiments.

For commercial scale studies, a total of eight trials were undertaken, all of which met the inclusion criteria of *V. parahaemolyticus* initial (control) levels exceeding 1 x 10^4 MPN/g. Table 2.2 summarizes the *V. parahaemolyticus* data within the context of the NSSP

PHP requirements. As was the case for *V. vulnificus*, the 10 min treatment resulted in near complete elimination of the pathogen, which ranged from 3.7-4.6 $\log_{10}$ reductions. Two out of eight (25%) of the 8 min trials failed to attain the NSSP standard of <30 MPN/g.

2.4.3 Temperature monitoring

Continuous temperature monitoring was conducted during all of the commercial runs (temperature monitoring unit accidently shut off for trials 5 and 6 of the 8 min treatment, hence data for those trials is not available). Figure 2.3 displays heating and cooling curves for the 6 min treatments. For these trials, the water temperature during heating met our process criteria of approximately 60^{o}C in four out of eight trials. Maximum internal oyster temperature (IOT) was approximately at 45^{o}C for Trials 1 and 2, and 50^{o}C for Trials 5 and 6. An OIT of 13^{o}C was recorded after 15 min of cooling in the ice slurry. For the 8 min treatment trials (Figure 2.4), the water temperature during heating met our process criteria of approximately 60^{o}C in two out of eight trials. Maximum internal oyster temperature (IOT) was approximately at 54^{o}C for Trials 3 and 4. An OIT of 13^{o}C was recorded after 18 min of cooling in the ice slurry. For the 10 min treatment trials (Figure 2.5), the water temperature during heating met our process criteria of approximately 60^{o}C in six out of eight trials. Maximum internal oyster temperature (IOT) was approximately at 58.5^{o}C for Trials 1, 2, 5, and 6, and 54^{o}C for Trials 7 and 8. An IOT of 13^{o}C was recorded after approximately 21-24 min of cooling in the ice slurry.

2.4.4 F-value

An F-value calculation was made for each of the three commercial time-temperature combinations using 70°C as the reference temperature and 8.5°C as the *z*-value (Ama et al., 1994; Asselt and Zwietering 2006; Bang and Drake 2002; ICMF 1996). For the 6 min treatment, the minimum F-value achieved was $F^{8.5\circ C}_{70°C} = 0.002$ min and the maximum was 0.2 min. The data for the 8 min treatment times was similarly variable, ranging from a low of $F^{8.5\circ C}_{70°C} = 0.037$ min to a high of 0.239 min. For the 10 min treatment time, the $F^{8.5\circ C}_{70°C}$ range was 0.044 min to a high of 0.315 min. For the projected Log_{10} inactivation of *V. vulnificus* and *V. parahaemolyticus*, the ratio of the most conservative (lowest) F-value of the eight individual runs for each treatment time (6, 8, and 10 min) and the D_{ref} of 0.006 min was 0.37 for the 6 min treatment, 6.5 for the 8 min treatment, and 7.7 for the 10 min treatment.

2.5 Discussion

The purpose of this study was to evaluate, and modify if necessary, an existing heat-shock treatment routinely used by North Carolina shellfish dealers to facilitate oyster shucking, for its efficacy in reducing *Vibrio vulnificus* and *Vibrio parahaemolyticus* levels. In addition, the study was to determine if this method met FDA and ISSC requirements for recognition under the NSSP guidelines as an approved Post-Harvest Processing (PHP) method. For a process to be recognized as a valid PHP, it must reduce the concentrations of *V. vulnificus* and/or *V. parahaemolyticus* to non-detectable levels, defined as <30 MPN/g and a minimum 3.52-log_{10} reduction (NSSP 2009a; NSSP 2009c).

Few previous studies have been conducted to evaluate the efficacy of the so-called commercial heat shock method. For example, Hesselman et al. (1999) conducted studies using naturally-contaminated Gulf Coast oysters (*Crassostrea virginica*) subjected to commercial heat shock, the same method used in our study, finding that when applying a heat treatment that achieved an internal oyster meat temperature of $\geq$50°C for 1-4 min, a 1 to 4 $\log_{10}$ inactivation of *V. vulnificus* was observed.

Andrews et al. (2000) later sought to "pasteurize" both naturally and artificially-contaminated Gulf Coast oysters using a mild heat treatment during which the internal temperature of the oyster flesh was held at 48-50^{o}C for 0, 5, 10 and 15 min, followed by subsequent storage on ice for 14 days. The heat treatment effectively reduced the levels of *V. vulnificus* and *V. parahaemolyticus* from concentrations exceeding 1.0 x 10^5 to <3 MPN/g after 10 min or less. When followed by ice storage, these low concentrations of *V. vulnificus* and *V. parahaemolyticus* were maintained.

In 1995, AmeriPure patented a mild heat treatment termed "cool pasteurization". There is currently one facility in Franklin, Louisiana, which processes Gulf Coast oysters using this method (DiStefano et al., 2011). Cool pasteurization differs from commercial heat shock in some important ways. For example, oysters processed by cool pasteurization are first washed and individually banded to prevent shells from opening during the process. The oysters are loaded onto trays, placed in carts, and then submerged in a water tank held at 126°F (52.2°C) for 24 min, followed by transfer to another tank held at 40°F (4.4°C). The major difference between commercial heat-shock and cool pasteurization lies in the time and temperature criteria, as the commercial heat-shock process uses a higher temperature (60°C)

and a hold time about one-half (10 min) of that used in cool pasteurization (52.2°C and 24 min). In addition, the AmeriPure facility in Louisiana is designed for a processing capacity of up to 10,500 oysters per cycle (run) (DiStefano et al., 2011), whereas the commercial heat-shock process is generally used to facilitate shucking, and is applied in batch operations using much smaller product volumes (8-10 baskets of oysters per tank). Clearly, the two processes use very different equipment set-ups, which ultimately will affect delivery of an adequate heat process. To date, this commercial heat shock method has not been validated to achieve the NSSP reductions necessary to classify it as a valid PHP.

Overall, the 10 min trials consistently produced $\log_{10}$ reductions greater than 3.52 and counts <30 MPN/g for both *V. vulnificus* and *V. parahaemolyticus*, and hence meet the NSSP process requirements for a valid PHP, at least within the confines of this particular study. The NSSP PHP requirements of <30 MPN/g and 3.52 $\log_{10}$ were also met in all 8 min trials for *V. vulnificus* and in six 8 min trials for *V. parahaemolyticus*. For the 6 min treatment trials, the NSSP PHP requirements of <30 MPN/g and 3.52 $\log_{10}$ failed to be met in four *V. vulnificus* and six *V. parahaemolyticus* trials. Unfortunately, in some instances the initial concentration of *V. vulnificus* and *V. parahaemolyticus* in untreated (control) oysters exceeded the MPN assay limit of detection (>1.1 x 10^5 MPN/g) or, after treatment, fell below the assay detection limits (<3 MPN/g). Although this limited the ability in accurately determine a specific $\log_{10}$ inactivation value, the sheer fact that the lower limit of detection was reached after starting with such a high initial load in naturally contaminated oysters supports the efficacy of the 10 min treatment time for elimination of pathogenic *Vibrio* species. In future experiments, one

could manipulate sample dilutions to increase the upper limit of detection, and reduce the lower limits of detection.

In commercial trials, the process criteria of approximately 60°C water tank temperature was met at four 6 min treatment trials (Trials 1, 2, 5, and 6), two 8 min treatment trials (Trials 3 and 4), and six 10 min treatment trials (Trials 1, 2, 5, 6, 7, and 8). The failure to meet the 60°C water temperature in some trials may be due to inadequate circulation of water during processing and/or the lack of a properly functioning thermostat that connects between the water tank and the heating source. Such thermostat would maintain the water tank temperature at 60°C as it would turn the heating source off if the temperature exceeds the designated 60°C and it would turn it back on if it went below 60°C.

Variability in internal oyster temperature (IOT) in 6 min trials (two trials at 45°C and two at 50°C) or in 10 min trials (four trials at 58.5°C and two trials at 54°C) can be a function of natural variation in oyster size and shape, as larger oysters would theoretically heat-up and cool down at a slower rate than would smaller oysters. Also, another consideration is the openings of oysters during processing. For example, hot water can leak inside the oyster through the openings between the shells and the meat, resulting in uneven distribution of heat. Banding oysters using rubber bands to keep shells closed during the process can possibly prevent this, but oysters were not banded in this study. In addition, in this study, the oysters were placed on top of each other inside wire baskets, and the location of each individual oyster can affect the amount of heat it receives. Oysters at the center of the wired basket can possibly receive less heat than oysters positioned on the sides of the basket.

As would be expected, the time to reach a target cool-down temperature of about 13^{o}C increased with higher IOT (and increased hold time in the cooling tanks). *Vibrio* species are mesophilic and hence cooling oysters rapidly after the heat shock treatment is necessary to prevent amplification of surviving cells. Also, this process is designed to heat oysters to such a time and temperature combination to eliminate the pathogenic Vibrios yet retain a "raw" quality to the oysters. Failing to rapidly cool the oysters could result in changing the sensory qualities of the raw oyster meat.

Three oysters were probed and placed in the wire baskets such that one was located in the center, one along the side and one at the top of the oysters inside the basket to take into account the temperature variations. The temperature profiles for the heat-shock process offered in Figures 2.3, 2.4, and 2.5 represent the mean of the three probed oysters for each trial of the eight commercial trials for each treatment time (6, 8, and 10), as well as the associated water temperatures. Despite a target temperature of 60^{o}C, it was very difficult to control the gas-heated water baths to achieve this temperature. This may be due to inadequate circulation of water during processing and/or the lack of a properly functioning thermostat that connects between the water tank and the heating source. Such thermostat would maintain the water tank temperature at 60°C as it would turn the heating source off if the temperature exceeds the designated 60°C and it would turn it back on if it went below 60°C.. These Figures 2.3, 2.4, and 2.5 also clearly show that the final IOTs increased as a function of time, but oysters were never held for a pre-determined amount of time at a given temperature.

The F-value calculation is used to estimate process efficacy. The F-value is defined as the time at a given temperature required to destroy a pre-determined percentage of the target

microbial population whose thermal resistance is characterized by the z_D. The predetermined amount of target microbe inactivation is usually expressed as $\log_{10}$ inactivation, and reflects what would be necessary to produce a product having a likelihood of contamination considered to result in negligible risk to human health. For example, a 12 $\log_{10}$ inactivation is considered appropriate for the elimination of *C. botulinum* in high acid, commercially sterile foods. However, many processes are not designed to hold the product at a certain target temperature for a specified period of time, but rather reflect the sum total of the heat treatment to which the product was exposed. In this case, F-values are defined as the total area under the process lethality curve.

The $\log_{10}$ reduction estimated using the calculated F-values are technically relevant only if the probed oysters were also processed for microbial enumeration. In other words, if the same individual oyster has been tested, $\log_{10}$ reductions by microbiological testing would correlate directly to $\log_{10}$ reductions calculated by the formula F-value/D_{ref}. Because this was not the case in our experimental design, as well as the fact that initial and final microbial loads frequently were outside MPN assay limits of detection, it is not possible to accurately compare $\log_{10}$ reductions predicted by F-value/D_{ref} to those obtained by microbiological testing.

However, within these confines, the F-value/D_{ref} ratio can be used to approximate process efficacy. Using the most conservative (lowest) F-value of the eight individual runs for each treatment time (6, 8, and 10 min), the process efficacy ($\log_{10}$ inactivation) estimate for the 6 min treatment was 0.37, while $\log_{10}$ reduction for the same trial by microbiological testing was >4.56 and >2.46 for *V. vulnificus* and *V. parahaemolyticus,* respectively. For the

8 min treatment, $\log_{10}$ reductions calculated by F-value/D_{ref} were 6.54, while $\log_{10}$ reduction for the same trial by microbiological testing exceeded 4.49 for both *V. vulnificus* and *V. parahaemolyticus*. Log_{10} reductions for the 10-minute treatment were estimated to be 7.72, and microbiological testing gave >4.11 and >3.87 $\log_{10}$ reduction for *V. vulnificus* and *V. parahaemolyticus,* respectively. Because initial levels of oyster contamination and assay detection limits precluded determination of $\log_{10}$ inactivation exceeding 4-5, we were unable to evaluate if the 8 and 10 min treatment times were actually able to achieve the predicted inactivation of 6-8 $\log_{10}$. However, these proximate F-value calculations suggest that these two treatment times should be more than sufficient to provide the targeted 3.52 $\log_{10}$ reduction.

There are several issues that need to be considered before valid PHP processes are commercially implemented. One unknown factor is the impact of heat shock method on the sensory qualities of molluscan shellfish. In a sensory study conducted by Andrews and Coggins (2004), there was no significant difference between appearance, aroma, flavor, or taste of the oysters processed by Individual Quick Freezing (IQF), Cool Pasteurization (mild heat), and High Hydrostatic Pressure (HHP). However, in this case, none of the treated oysters were compared to untreated controls. In a parallel study, Coggins (2004) evaluated the same product parameters (appearance, aroma, and flavor) of oysters processed by the same three methods, but in this case, compared treated oysters to untreated controls. Again, no significant differences were observed between any of the products. Further controlled sensory studies would be necessary to definitively determine if there are significant sensory differences between raw product and product processed by commercial heat shock.

There are other issues, aside from sensory qualities, that could also affect consumer acceptance of oysters processed by PHP. For example, Hanson et al (2003) found that 61% of consumers preferred oysters processed for safety via depuration, when compared to those processed by ionizing irradiation, ozonation, and pressurization. This can perhaps be interpreted as consumer preference to more "natural" foods, as the depuration process relies on allowing the shellfish to naturally open and purge particulates and microbes under controlled environmental conditions (e.g., temperature, water flow, salinity) (Bang and Drake 2002; Hesselman et al., 1999). On the other hand, Andrews and Coggins (2004) reported that 77% of the participants in their sensory study said they would consume more PHP oysters if they were free of pathogens.

Another major issue is the cost of implementing PHP technologies. In a comprehensive commercial feasibility and cost study, Research Triangle Institute (RTI) International estimated that implementing ionizing radiation for Gulf Coast oysters processing would cost $0.06 per oyster. The costs for of implementing cool pasteurization and high hydrostatic pressure were estimated to be $0.04 - $0.05 and $0.04 - $0.07 per oyster, respectively (DiStefano et al., 2011). A large proportion of these expenses would be associated with capital equipment costs such as conveyors, compressors, hoists, and ultraviolet water purification systems. Given the fact that the heat-shock facilities in North Carolina are generally of lower capacity, perhaps due to lower demand and a smaller market, these capital equipment costs might be lower. However, scale-up of facilities to process larger volumes of product might require capital expenses not necessarily documented in the RTI report.

This study shows the feasibility of commercial heat shock as a potential PHP for reduction of pathogenic *Vibrio* species in Gulf Coast shell oysters in keeping with NSSP standards. The process validation (time/temperature to deliver an adequate reduction in targeted organisms) described in this work is the first step necessary for industry to gain ISSC approval to use commercial heat shock as an approved PHP. The next step required to move this work forward would be the design and operation of commercial-scale equipment for delivery of the process conditions established in this study. Taking into consideration adjustments and modifications of heat shock equipment employed in this study for better process temperature control as follows: installment of water pump for the recirculation of water in tank; installment of a thermostat connected to heating source for automatic control; covering the water tank to contain temperature; possibly submerging oysters in water tank using wired beds rather than wired baskets for better temperature distribution. Also, commercial trials were performed during the study but were not conducted at full capacity of heating tanks and to become an approved process, these would need to be validated during full-scale commercial operations. In addition, current heat shock operations do not include a rapid cooling step, which is necessary to reduce the impact on sensory qualities (appearance) in PHP oysters. These studies need to be undertaken by commercial processors who want to optimize the heat-shock method for use as a PHP method with minimal effects on the appearance and sensory quality of the oyster meats.

Finally, commercial use of heat shock operations as a PHP method for shell-stock oysters must be verified by the state Shellfish Authority through laboratory analysis in an FDA certified laboratory. Currently, the NC Shellfish and Recreational Water Quality Office

is not set up to perform the necessary microbiological (*Vibrio* species) methods necessary to allow North Carolina oyster processors to seek ISSC approval of the commercial heat shock process as a valid PHP method. The need for the state shellfish program to expand the laboratory for inclusion of pathogenic Vibrios is self-evident and must be addressed if the North Carolina shellfish industry hopes to expand its capacity to process not only Gulf Coast oysters but potentially harvest and process oysters originating from conditionally closed North Carolina harvest waters as well. This action could provide economic benefits to coastal communities (oyster harvesters) and commercial processors of oysters across the state.

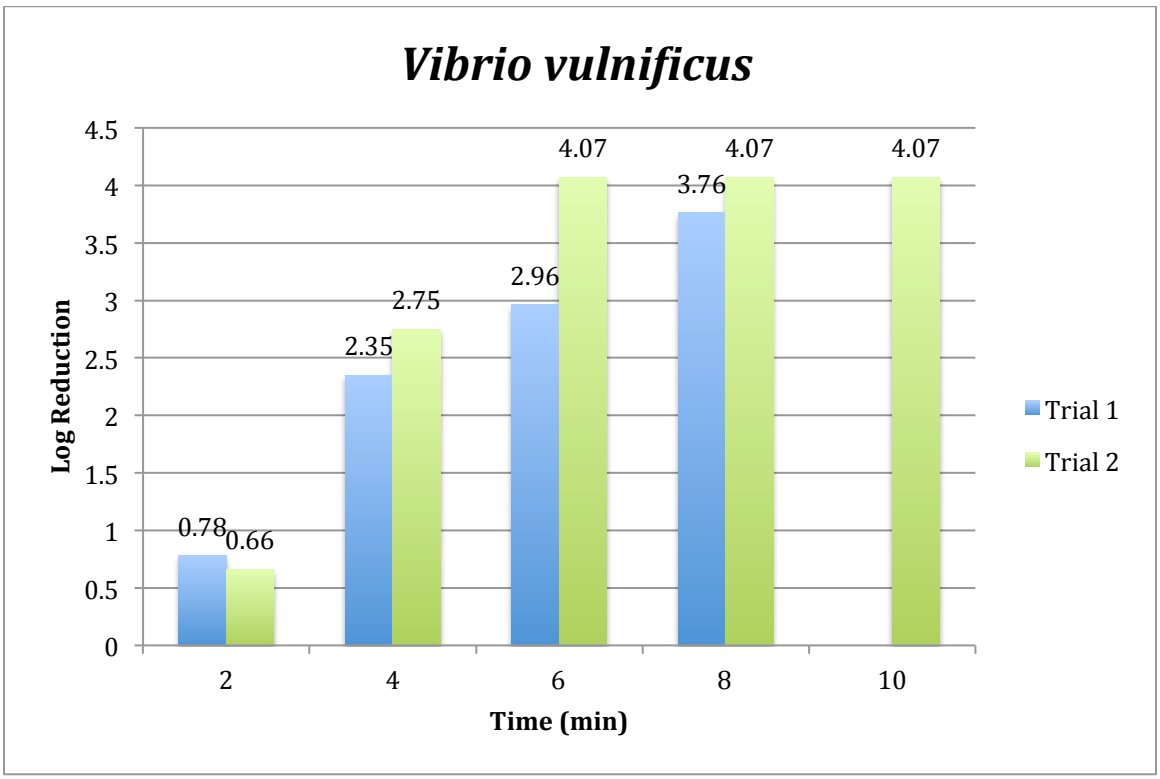


Figure 2.1 Log_{10} inactivation of *Vibrio vulnificus* in naturally contaminated summertime Gulf Coast oysters for two individual trials of a pilot scale heat shock process at approximately 60°C.

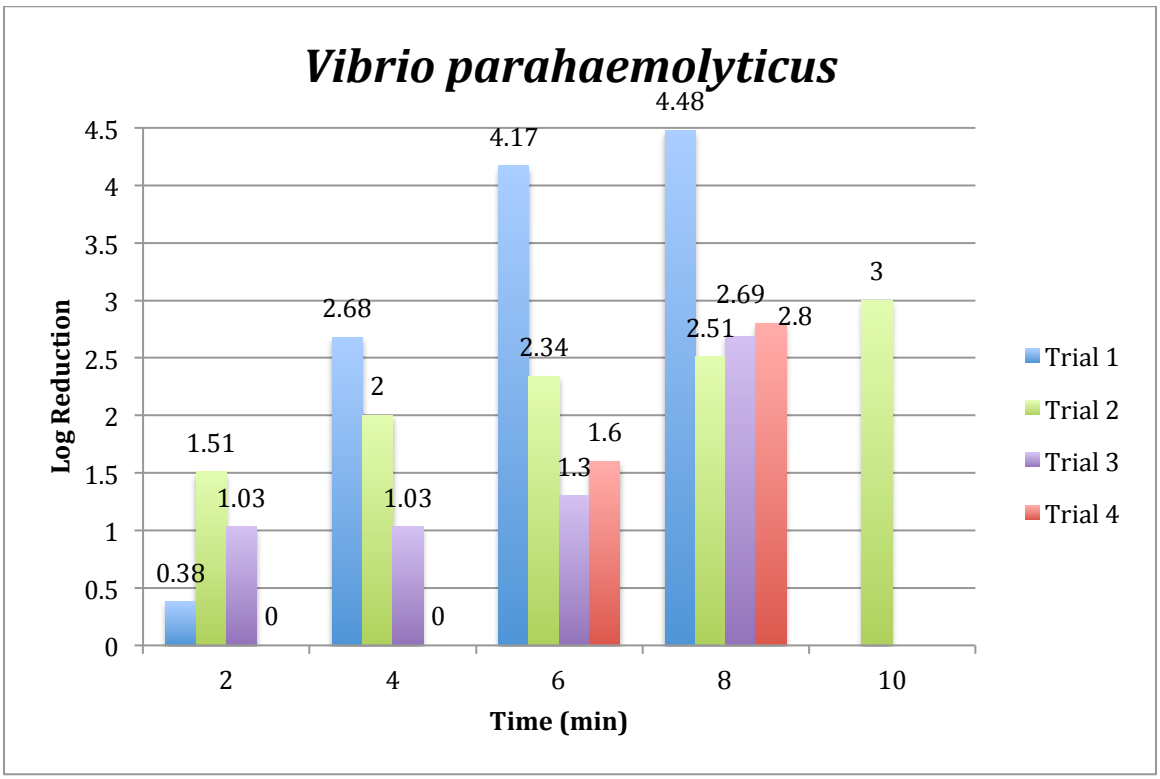


Figure 2.2 Log_{10} inactivation of *Vibrio parahaemolyticus* naturally contaminated summertime Gulf Coast oysters for four individual trials of a pilot scale heat shock process at approximately 60°C.

Table 2.1: *Vibrio vulnificus* concentrations (MPN/g) and associated log_{10} reductions after application of a commercial heat-shock treatment for 6, 8, or 10 min at approximately 60°C .

Vv		T_0	T_6	T_8	T_{10}
Trial #1	MPN/g	> 1.1 x 10^5	4.3 x 10^2	< 3	< 3
	Log rd	N/A	>2.41	>4.56	>4.56
Trial #2	MPN/g	> 1.1 x 10^5	< 3	< 3	< 3
	Log rd	N/A	>4.56	>4.56	>4.56
Trial #3	MPN/g	> 1.1 x 10^5	3.6	3.6	< 3
	Log rd	N/A	>4.49	>4.49	>4.56
Trial #4	MPN/g	> 1.1 x 10^5	9.3 x 10^3	< 3	< 3
	Log rd	N/A	>1.07	>4.56	>4.56
Trial #5	MPN/g	1.1 x 10^5	6.2	< 3	< 3
	Log rd	N/A	4.25	4.56	4.56
Trial #6	MPN/g	1.1 x 10^5	2.3 x 10^1	< 3	1.1 x 10^1
	Log rd	N/A	3.68	4.56	4
Trial #7	MPN/g	4.6 x 10^4	6.4 x 10^1	7.4	3.6
	Log rd	N/A	2.86	3.79	4.11
Trial #8	MPN/g	4.6 x 10^4	2.1 x 10^3	< 3	< 3
	Log rd	0	1.34	4.19	4.19

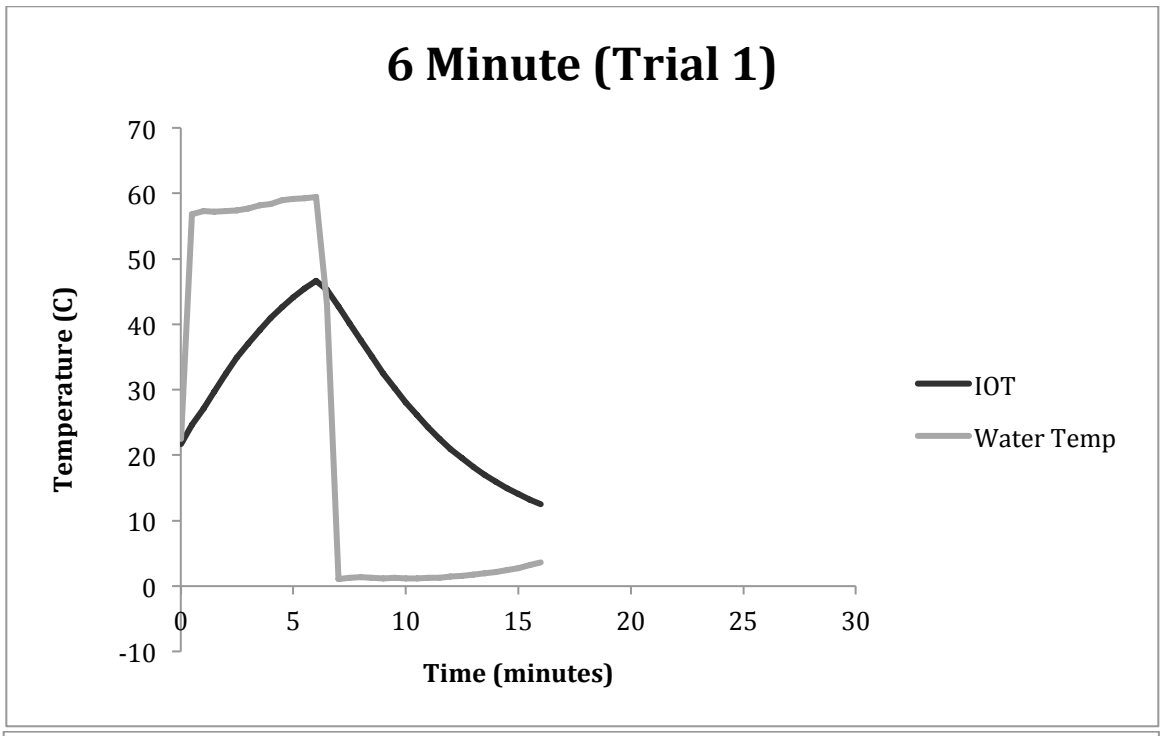
Yellow highlighted meets NSSP PHP requirements: < 30 MPN/g and a minimum of 3.52 log_{10} reduction

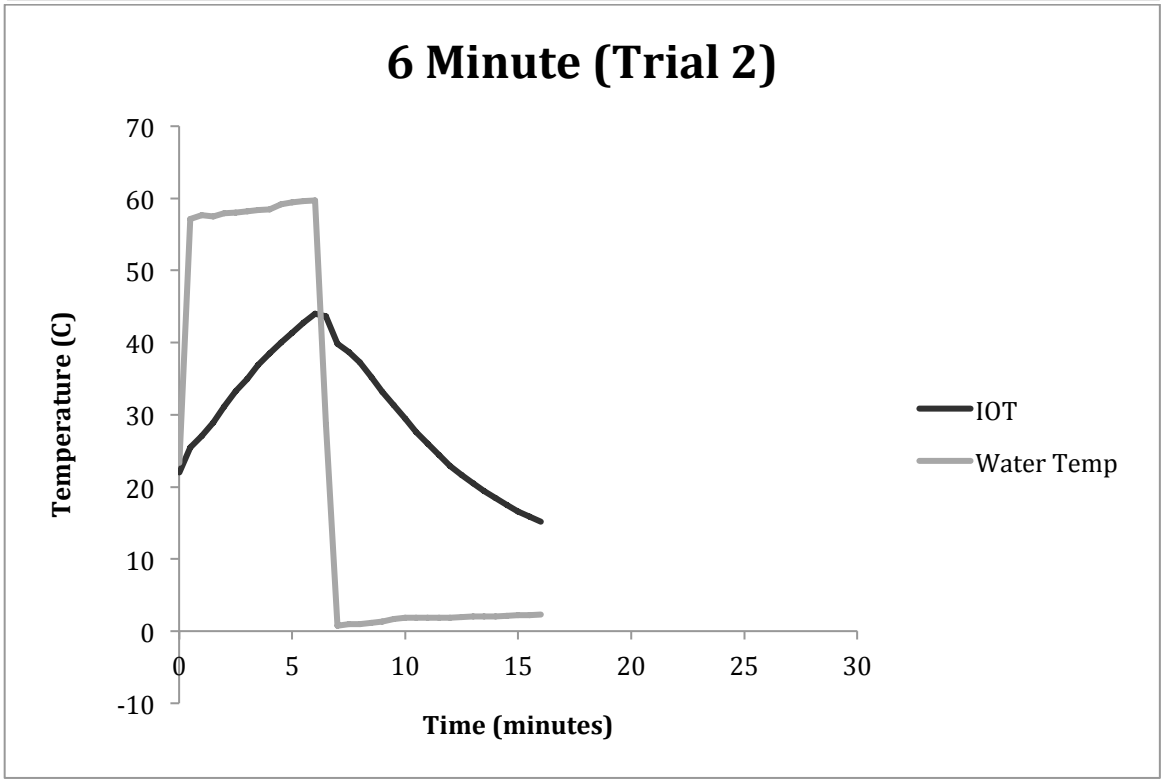
Table 2.2: *Vibrio parahaemolyticus* concentrations (MPN/g) and associated log_{10} reductions after application of a commercial heat-shock treatment for 6, 8, or 10 min at approximately 60°C.

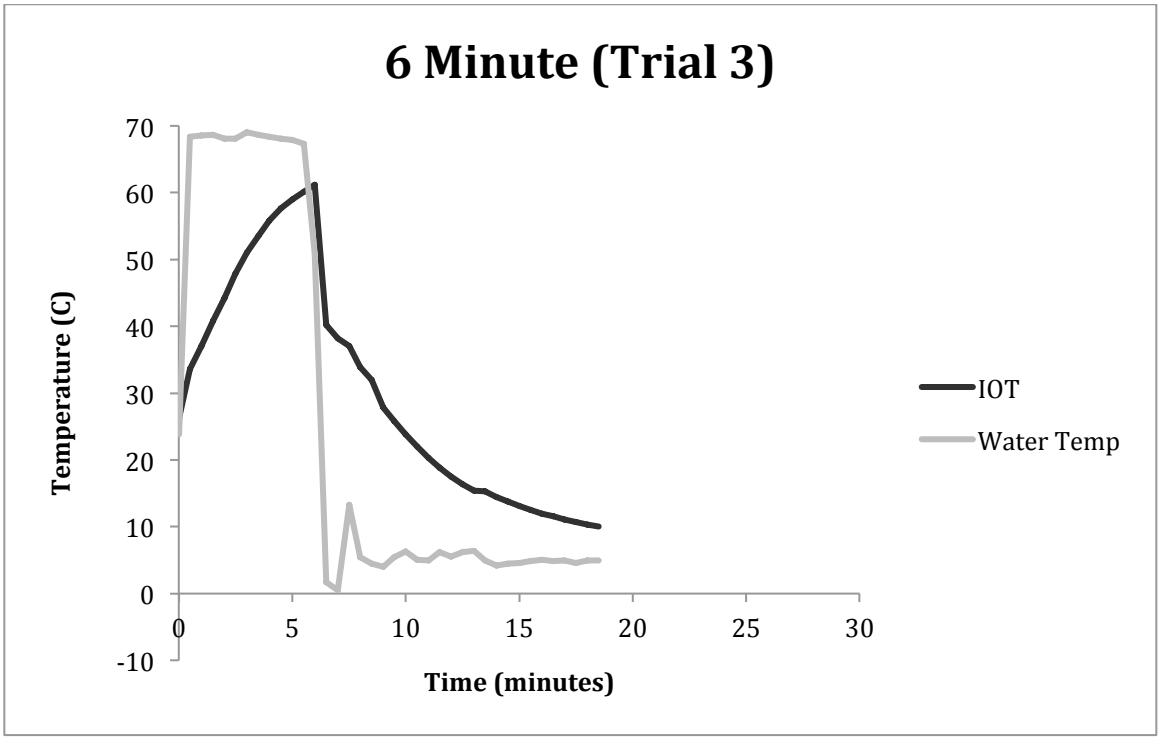
Vp		T_0	T_6	T_8	T_{10}
Trial #1	MPN/g	> 1.1 x 10^5	< 3	< 3	< 3
	Log rd	N/A	>4.56	>4.56	>4.56
Trial #2	MPN/g	> 1.1 x 10^5	3.8 x 10^2	< 3	< 3
	Log rd	N/A	>2.46	>4.56	>4.56
Trial #3	MPN/g	> 1.1 x 10^5	< 3	3.6	< 3
	Log rd	N/A	>4.56	>4.49	>4.56
Trial #4	MPN/g	> 1.1 x 10^5	4.3 x 10^3	3.6	< 3
	Log rd	N/A	>1.41	>4.49	>4.56
Trial #5	MPN/g	1.5 x 10^4	2.3 x 10^2	< 3	< 3
	Log rd	N/A	1.81	3.7	3.7
Trial #6	MPN/g	1.5 x 10^4	9.3 x 10^2	< 3	< 3
	Log rd	N/A	1.21	3.7	3.7
Trial #7	MPN/g	> 1.1 x 10^5	1.5 x 10^2	9.3 x 10^1	1.5 x 10^1
	Log rd	N/A	>2.87	>3.07	>3.87
Trial #8	MPN/g	> 1.1 x 10^5	1.5 x 10^4	9.3 x 10^1	1.5 x 10^1
	Log rd	N/A	>0.87	>3.07	>3.87

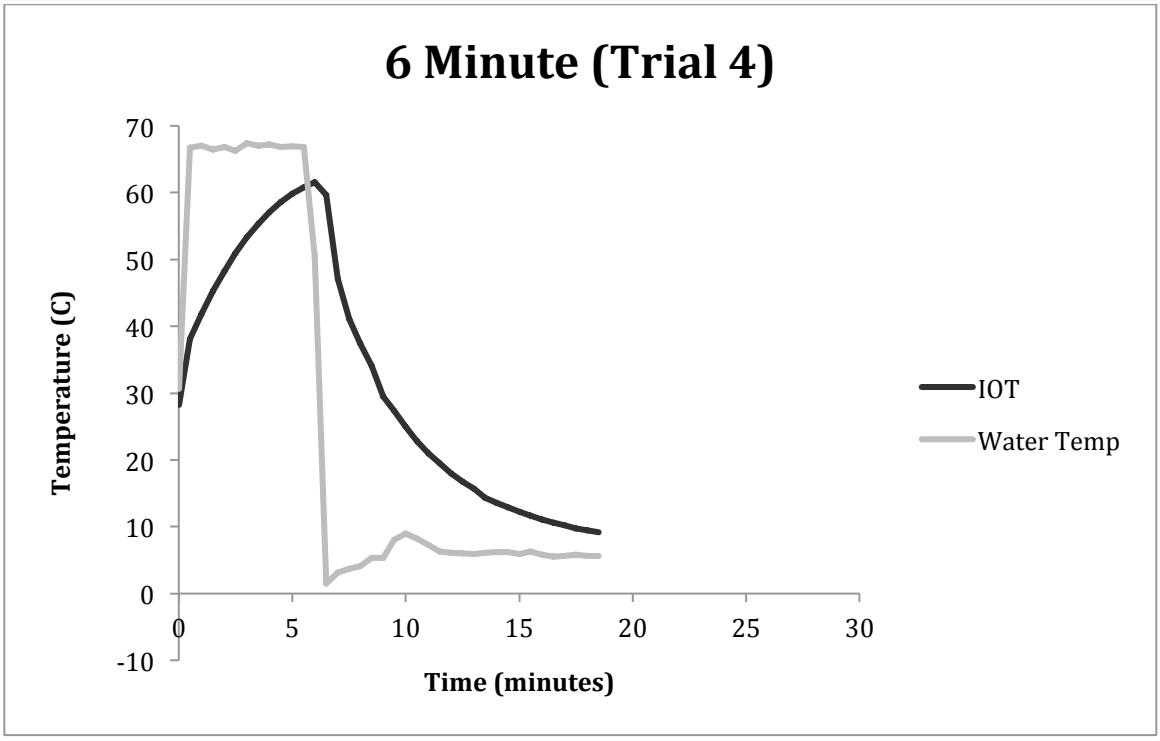
Yellow highlighted meets NSSP PHP requirements: < 30 MPN/g and a minimum of 3.52 log_{10} reduction

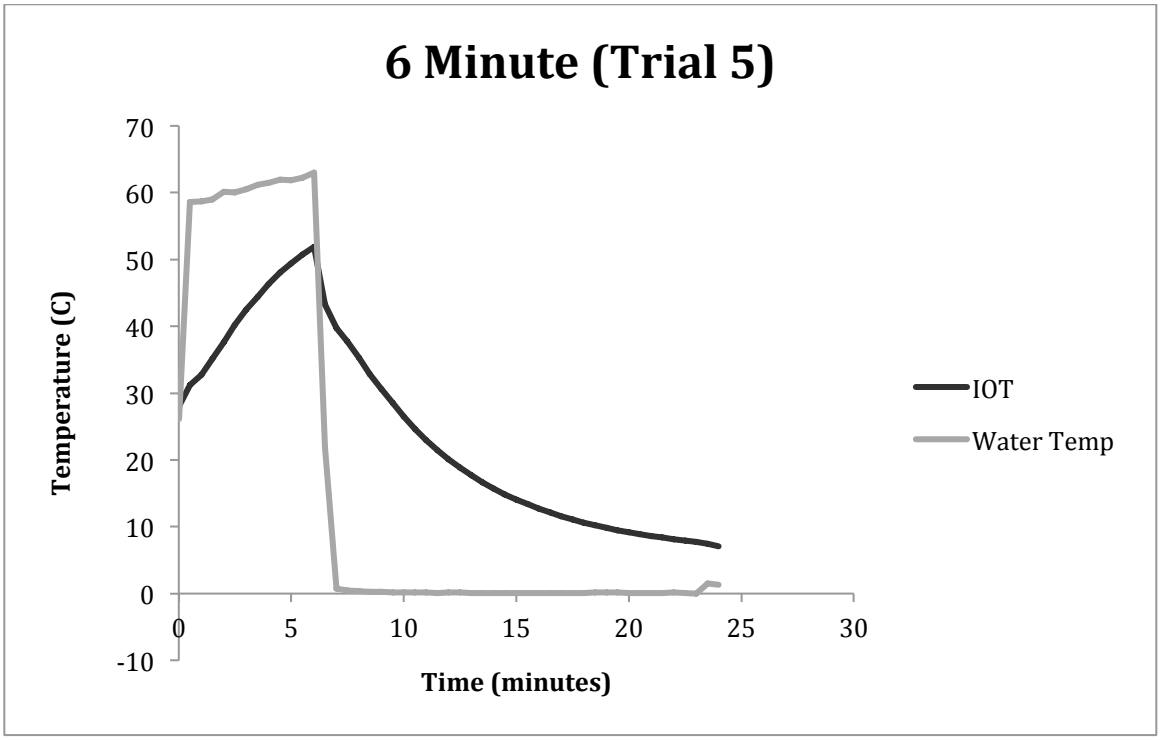
Figure 2.3 Heating-Cooling curves showing the means of three probed oysters for each commercial trial of the 6-minute treatment.

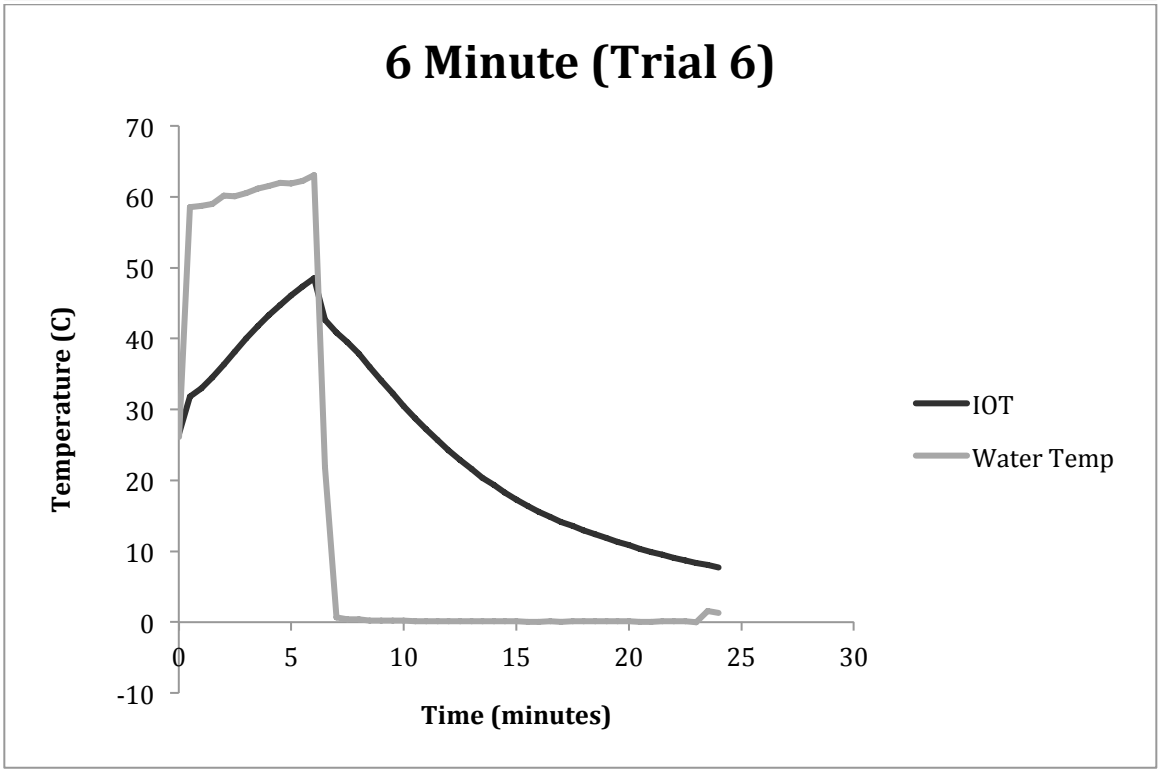

6 Minute (Trial 1)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

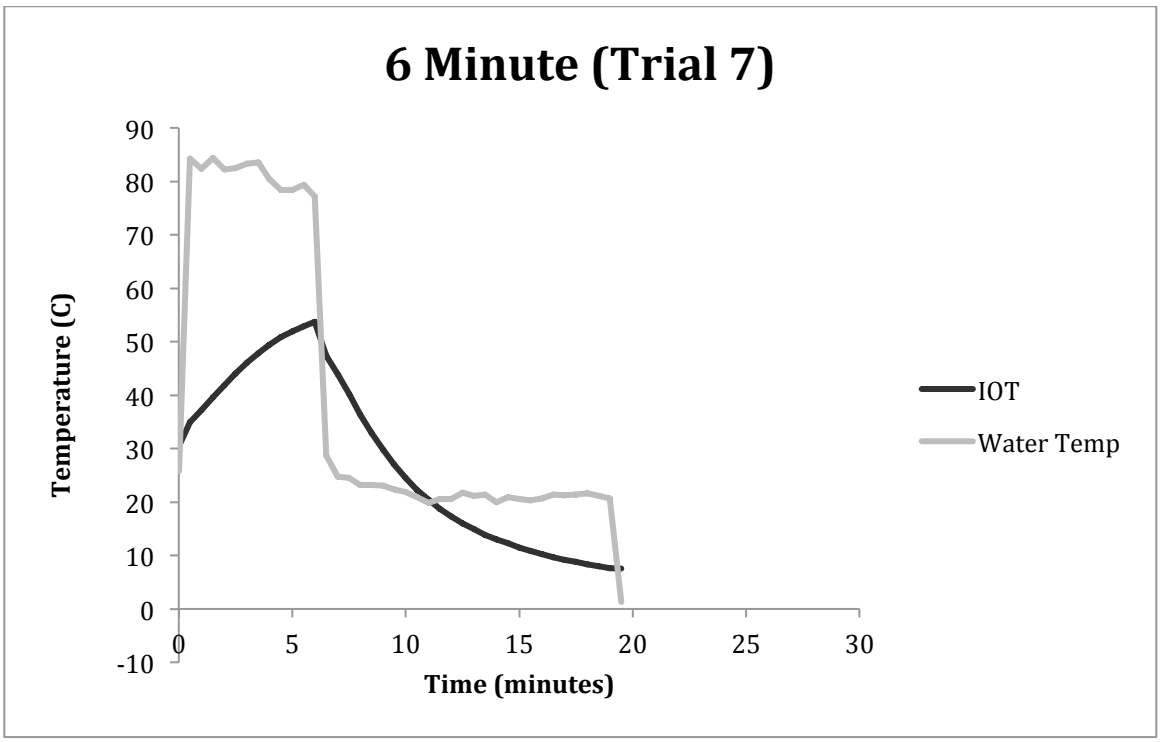

6 Minute (Trial 2)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


6 Minute (Trial 3)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


6 Minute (Trial 4)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


6 Minute (Trial 5)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


6 Minute (Trial 6)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


6 Minute (Trial 7)
Temperature (C)
90
80
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

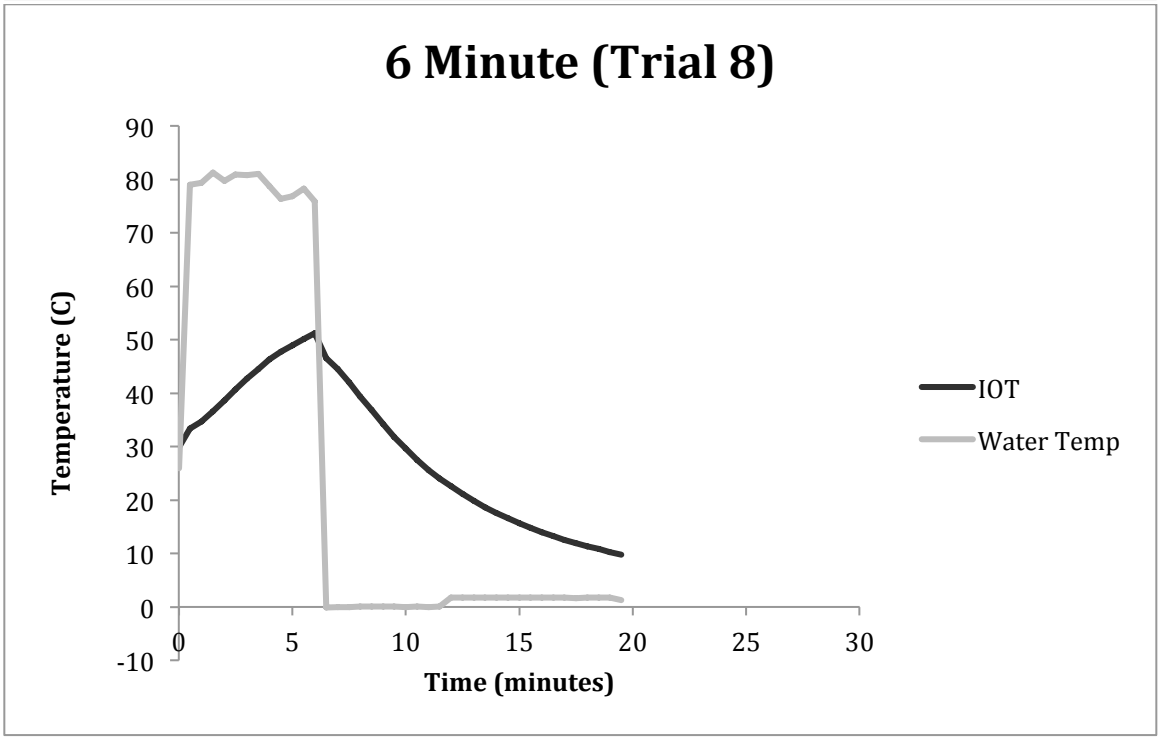
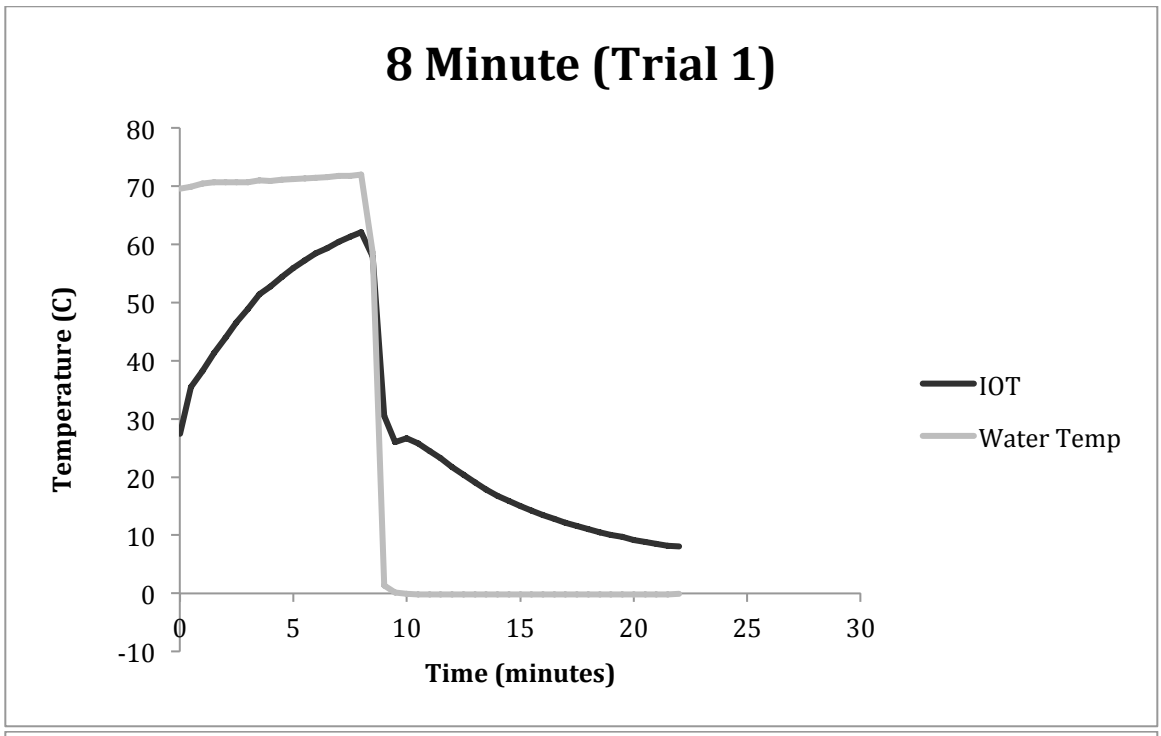
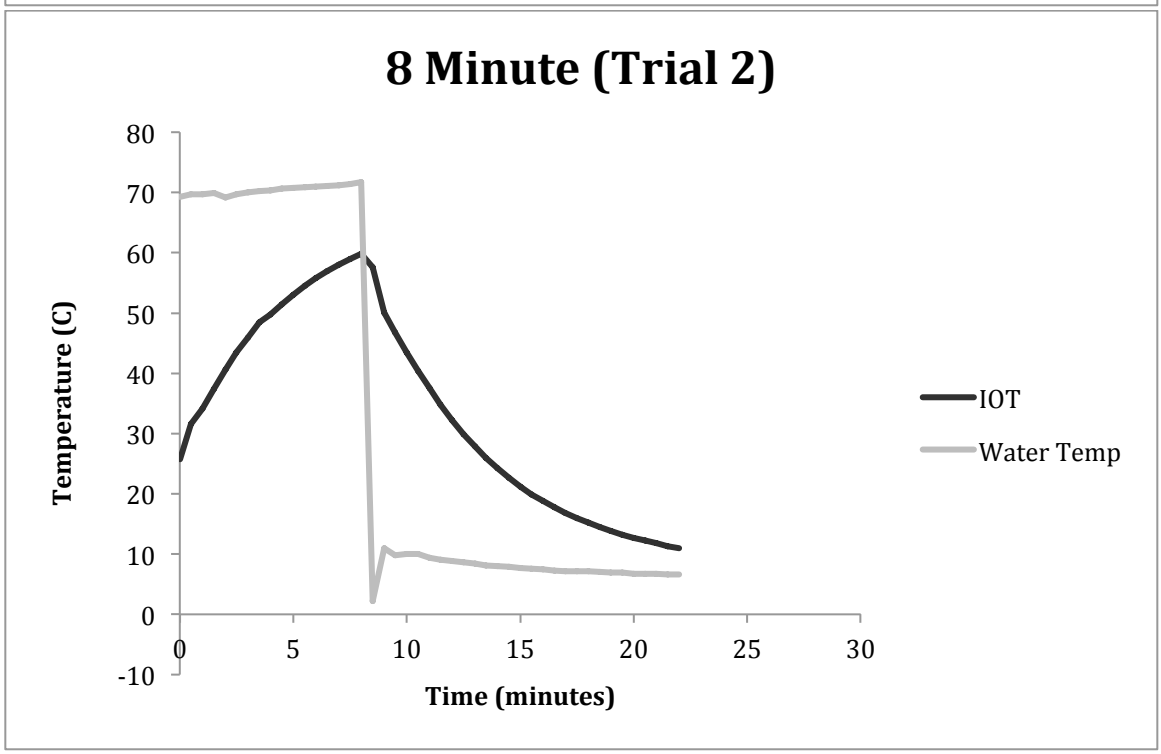
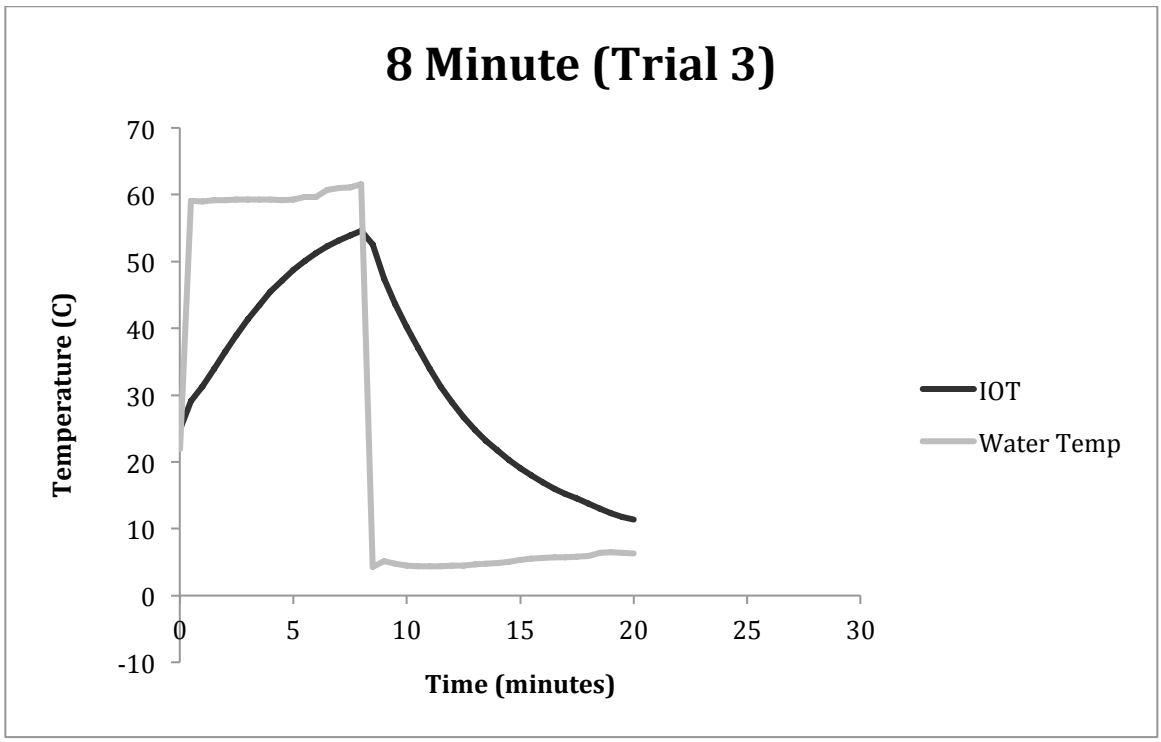
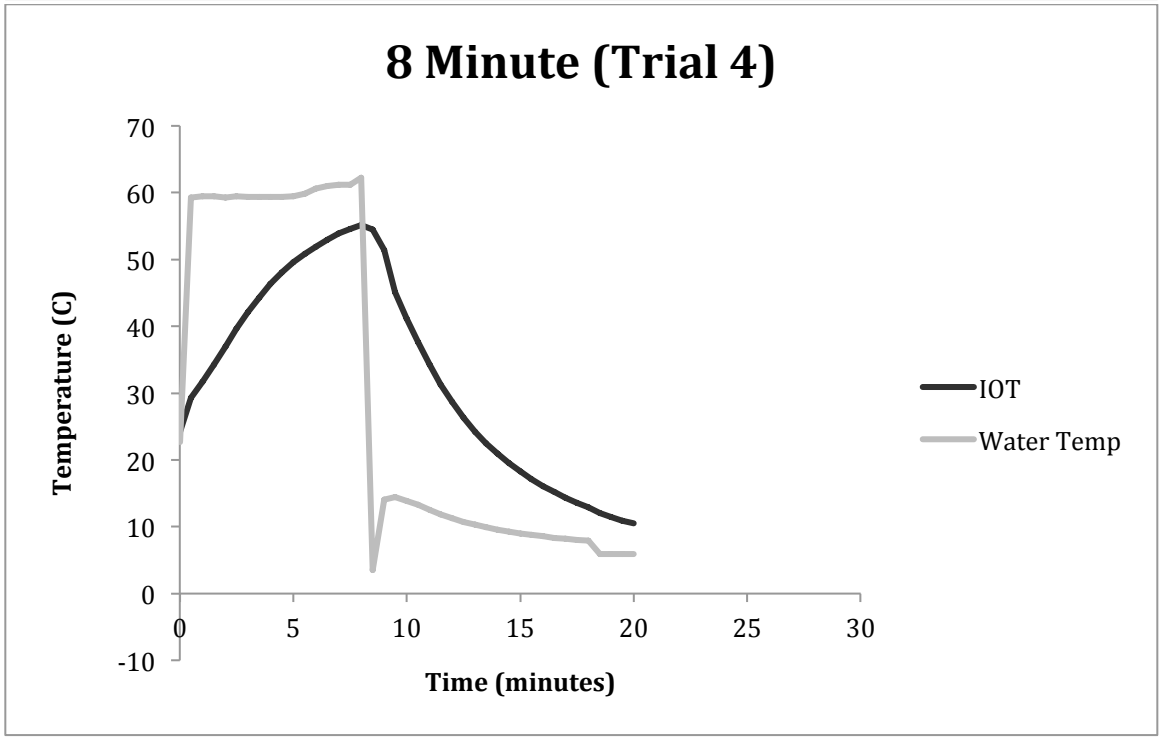

6 Minute (Trial 8)
Temperature (C)
90
80
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

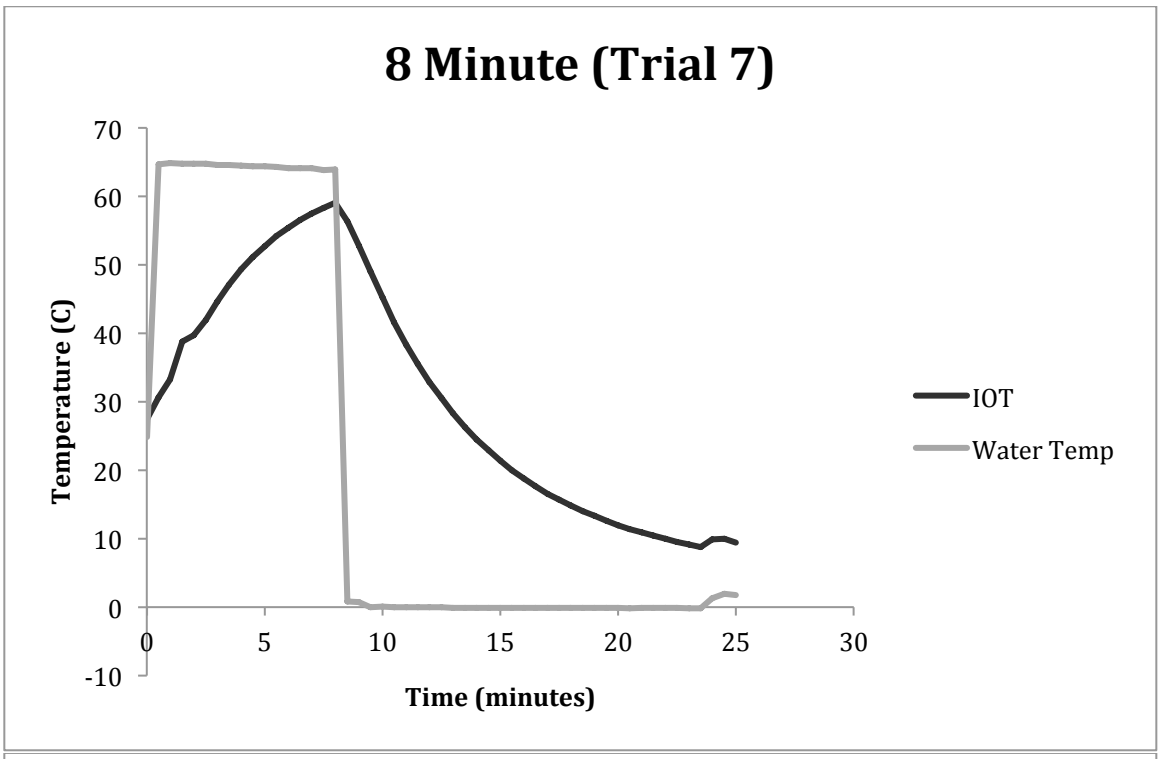
Figure 2.4 Heating-Cooling curves showing the means of three probed oysters for each commercial trial of the 8-minute treatment.


8 Minute (Trial 1)
Temperature (C)
80
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


8 Minute (Trial 2)
Temperature (C)
80
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


8 Minute (Trial 3)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


8 Minute (Trial 4)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


8 Minute (Trial 7)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

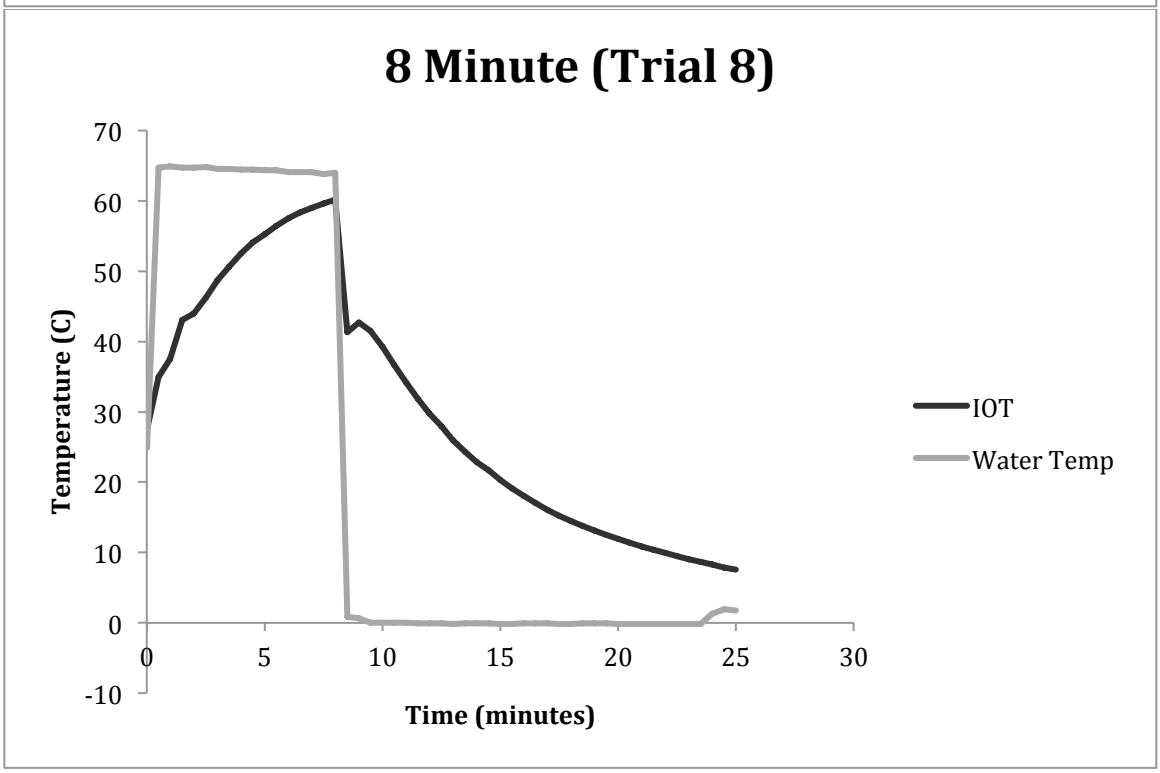
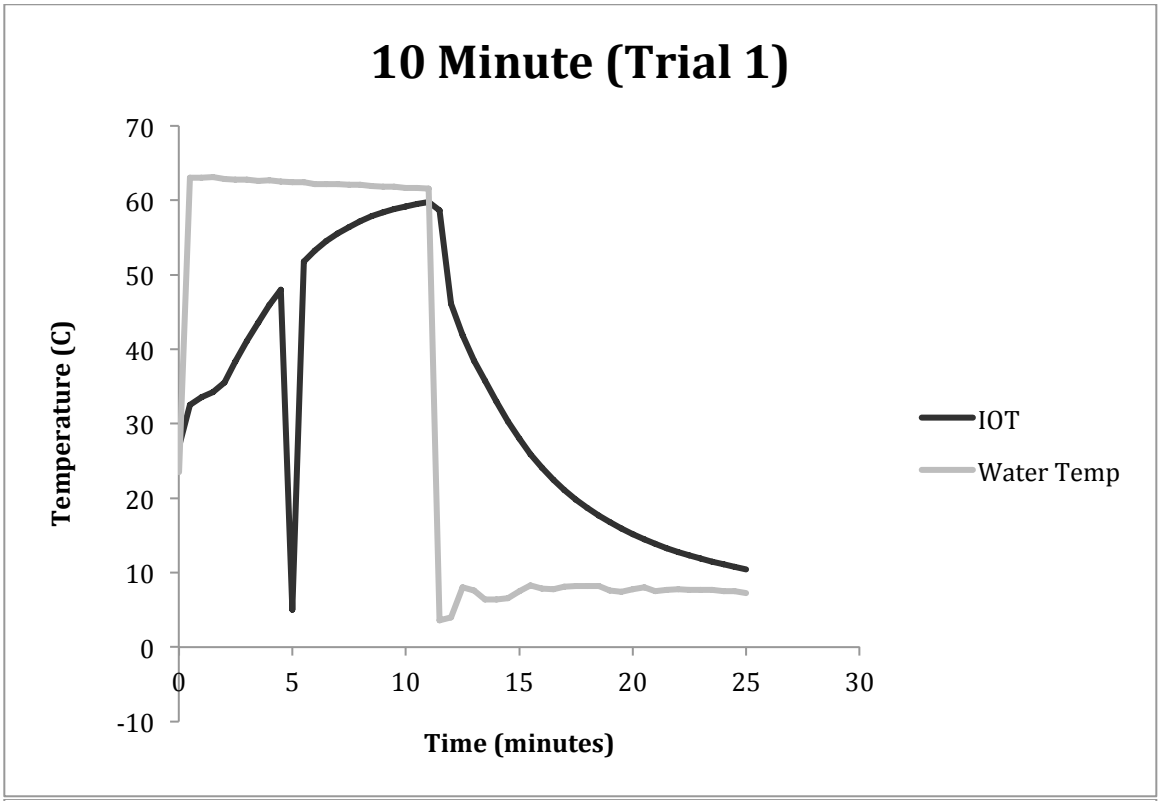
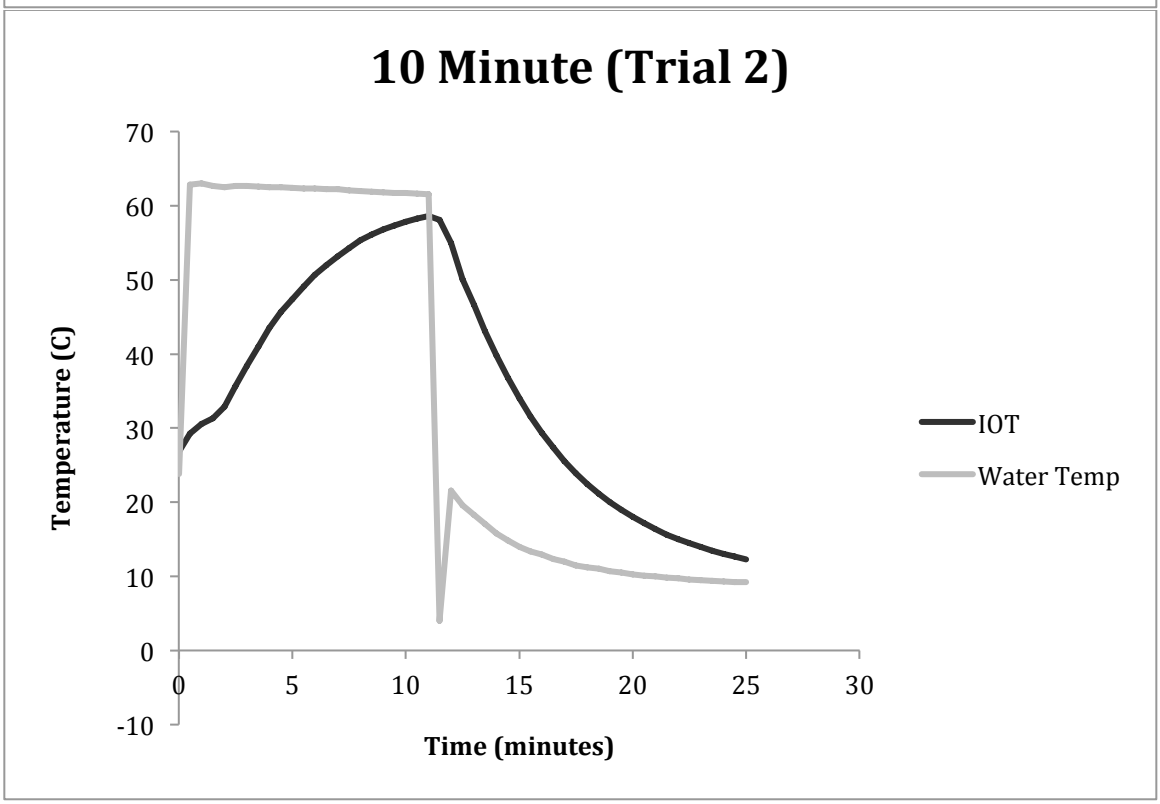
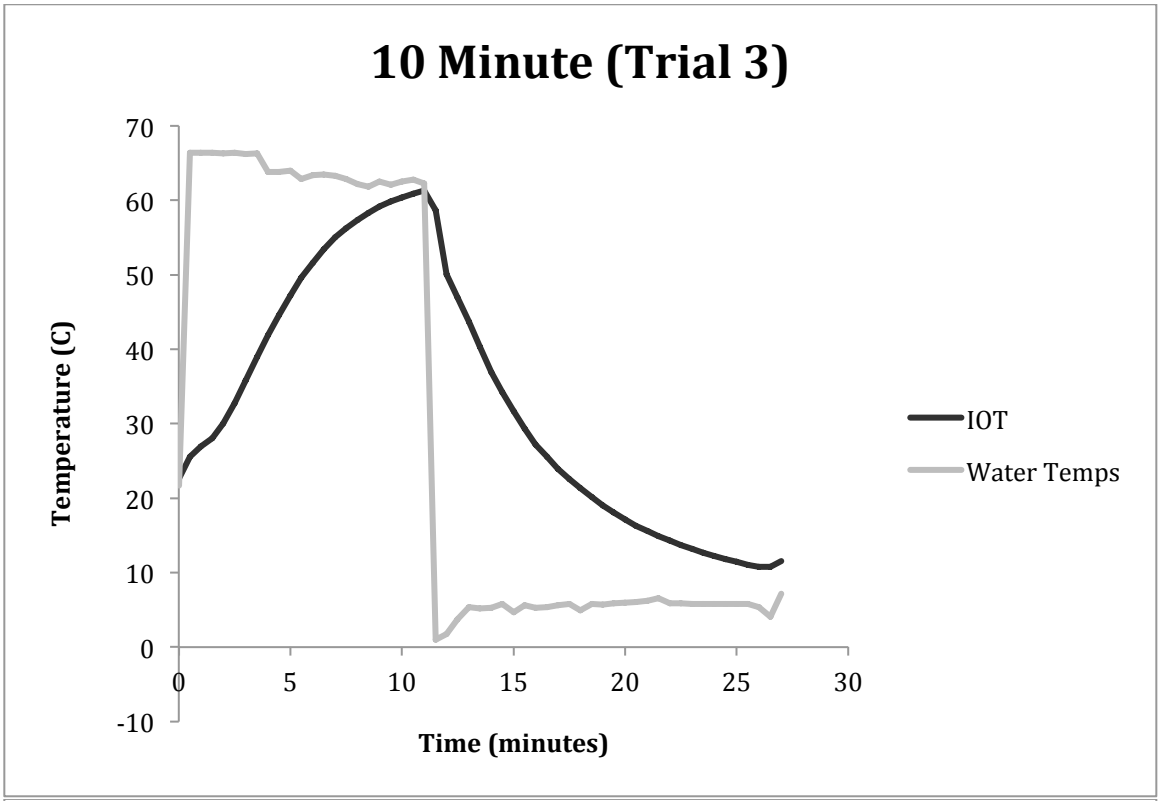
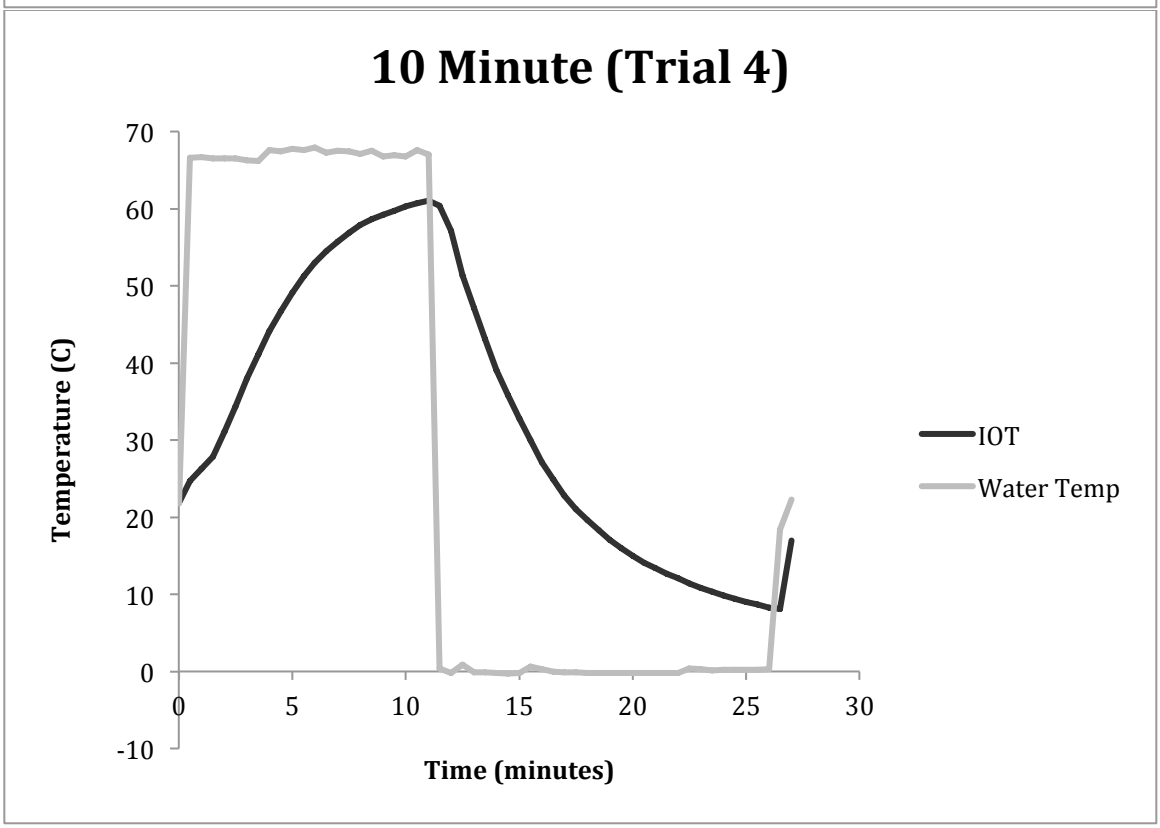

8 Minute (Trial 8)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

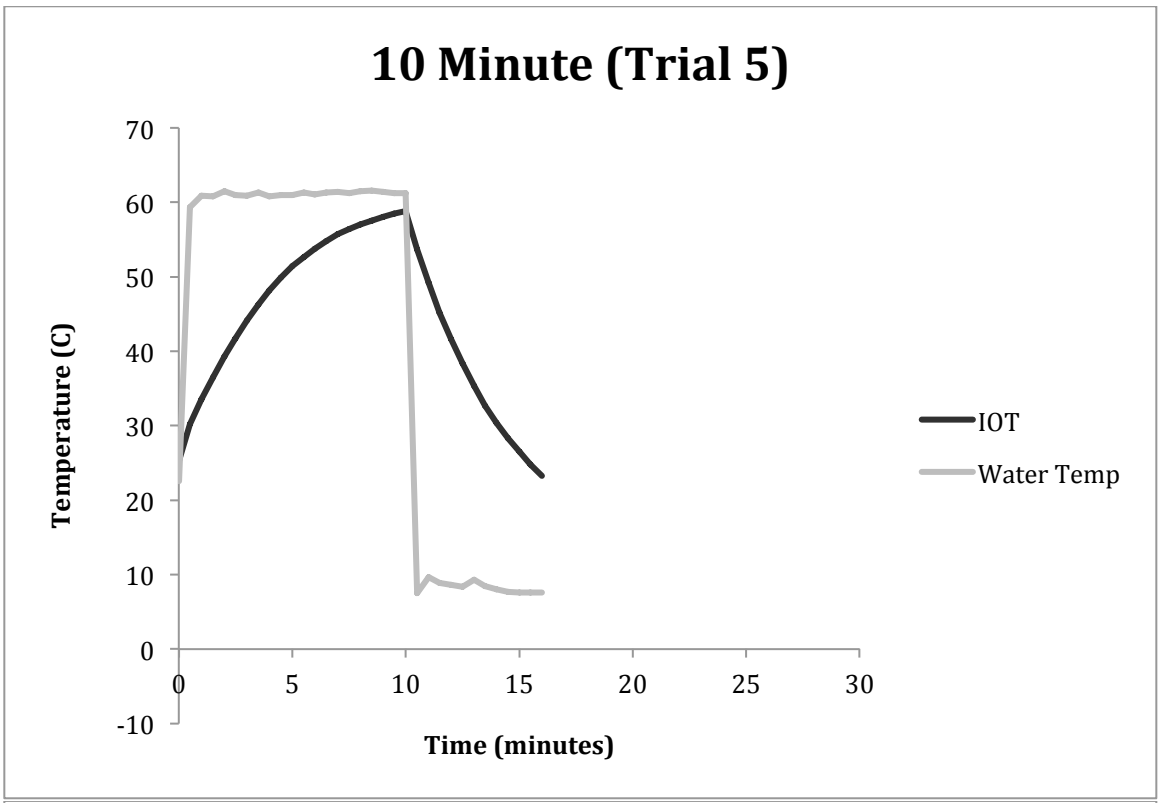
Figure 2.5 Heating-Cooling curves showing the means of three probed oysters for each commercial trial of the 10-minute treatment.

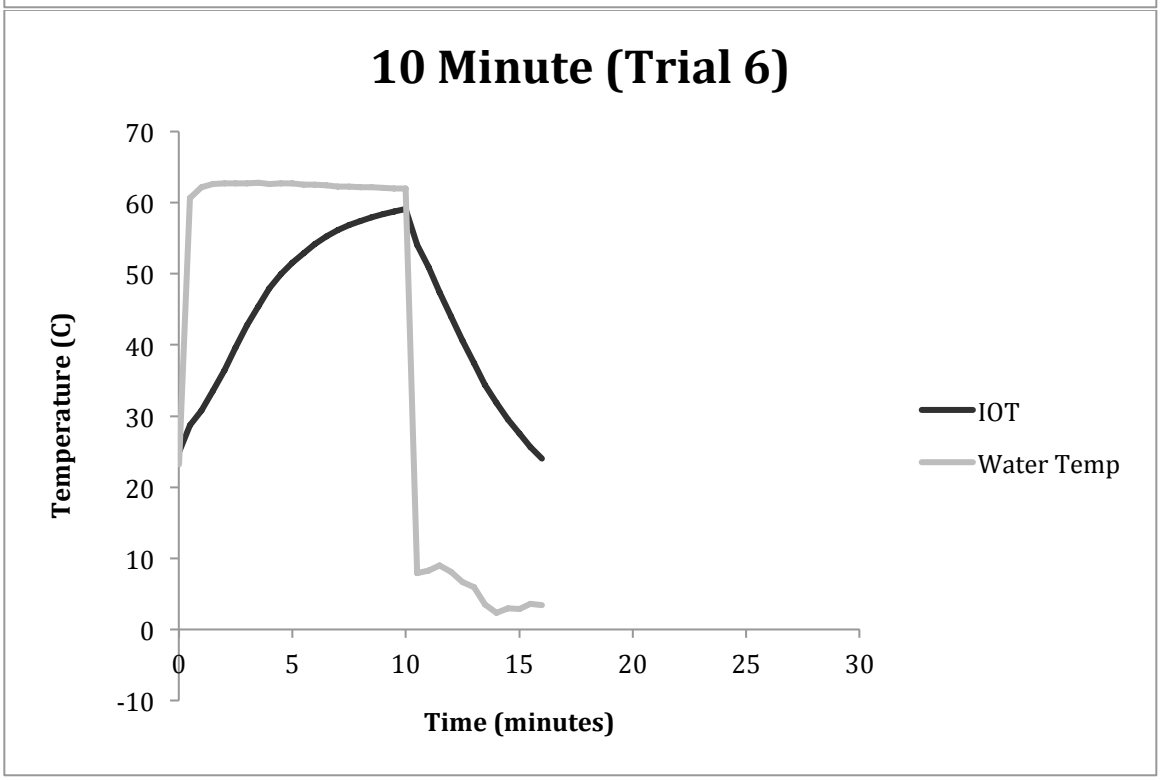

10 Minute (Trial 1)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

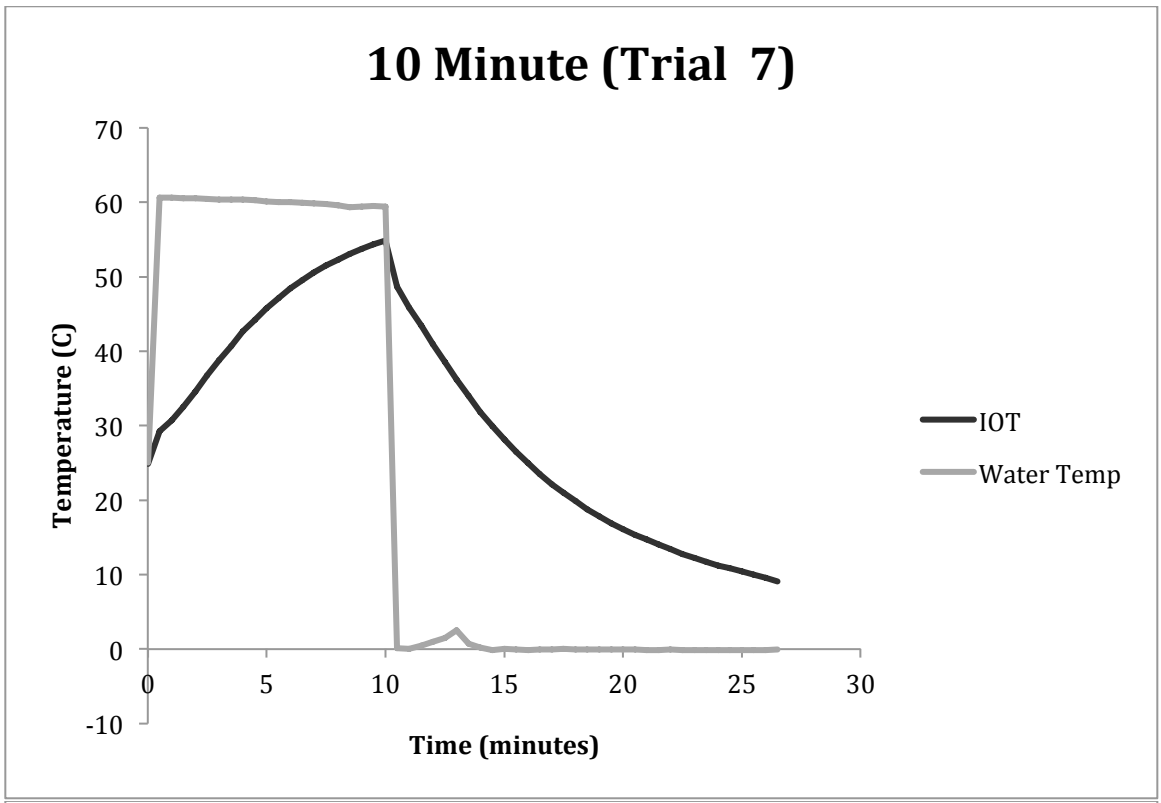

10 Minute (Trial 2)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

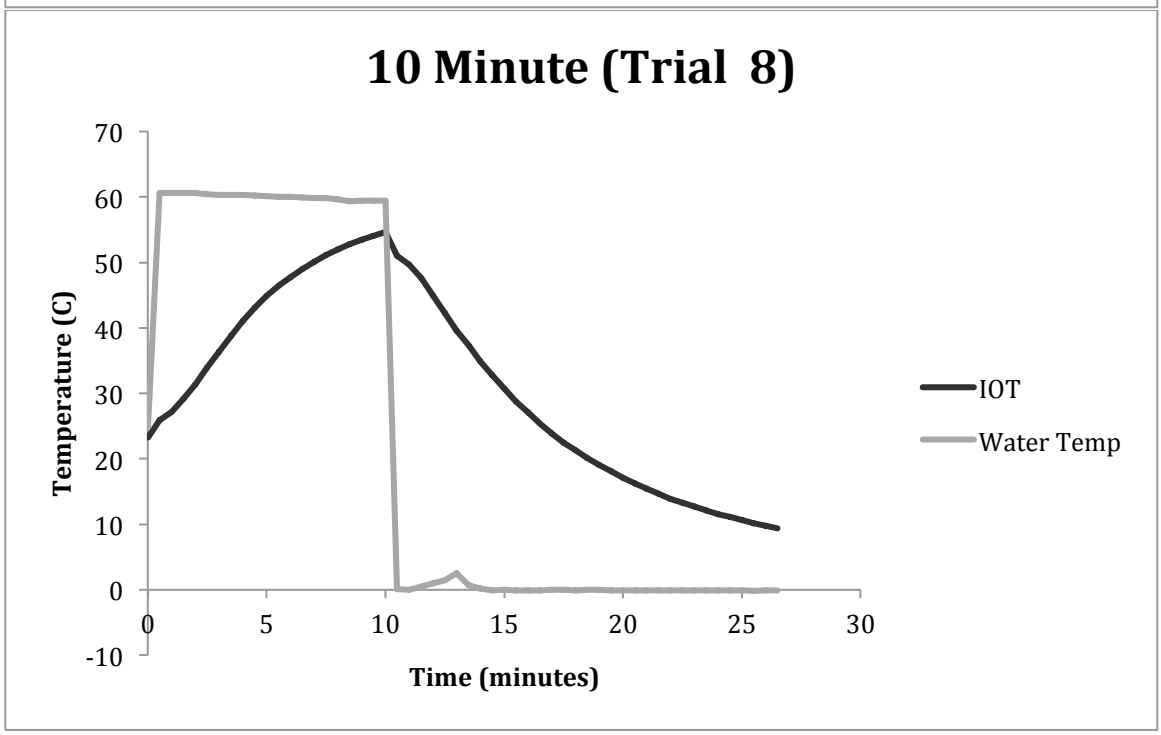

10 Minute (Trial 3)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temps


10 Minute (Trial 4)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


10 Minute (Trial 5)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


10 Minute (Trial 6)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


10 Minute (Trial 7)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp


10 Minute (Trial 8)
Temperature (C)
70
60
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
Time (minutes)
IOT
Water Temp

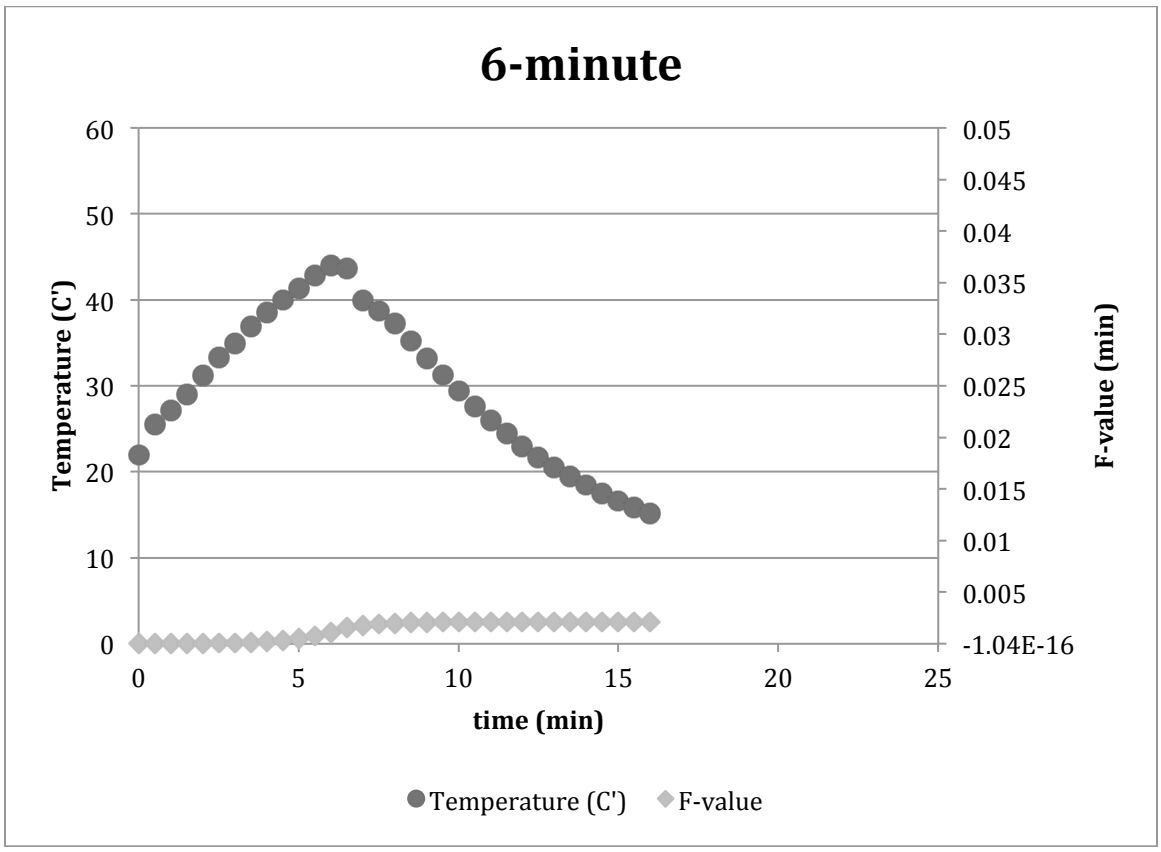


Figure 2.6 Lowest F-value of a 6-minute commercial trial and its mean IOT.

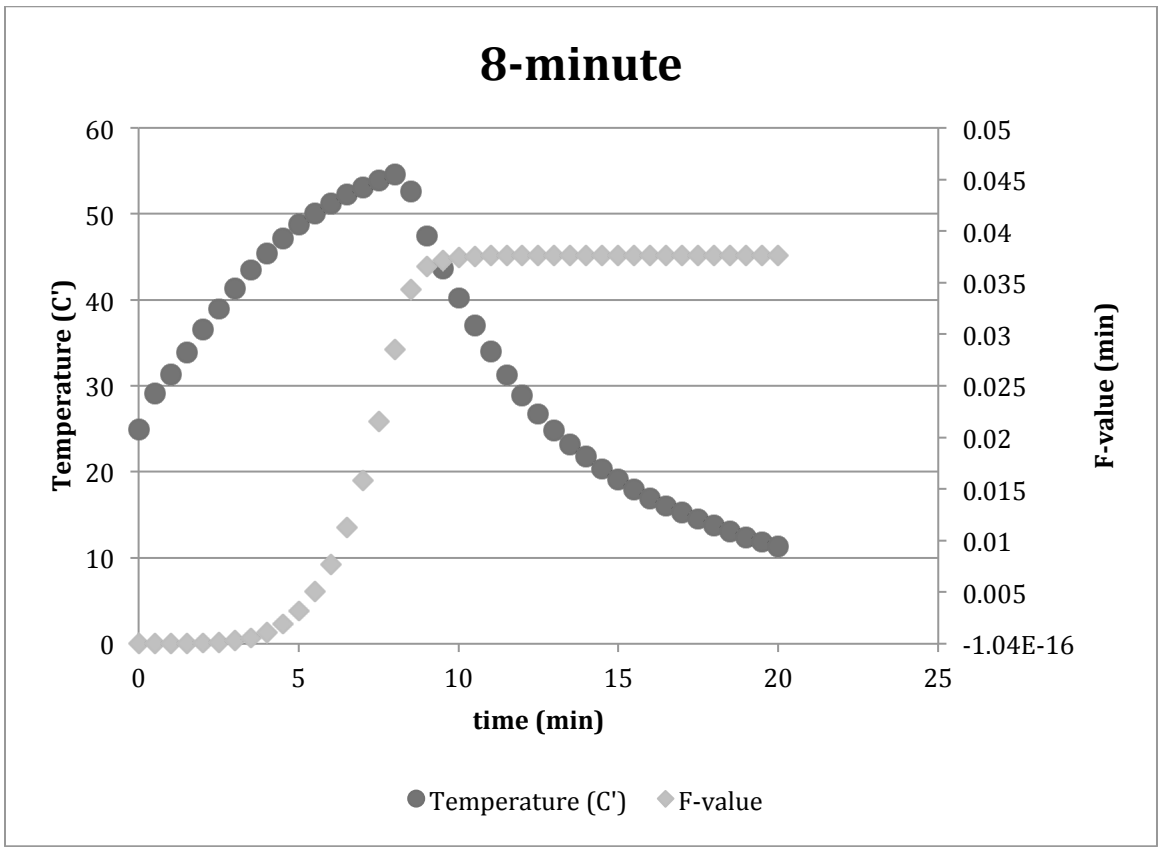


Figure 2.7 Lowest F-value of an 8-minute commercial trial and its mean IOT.

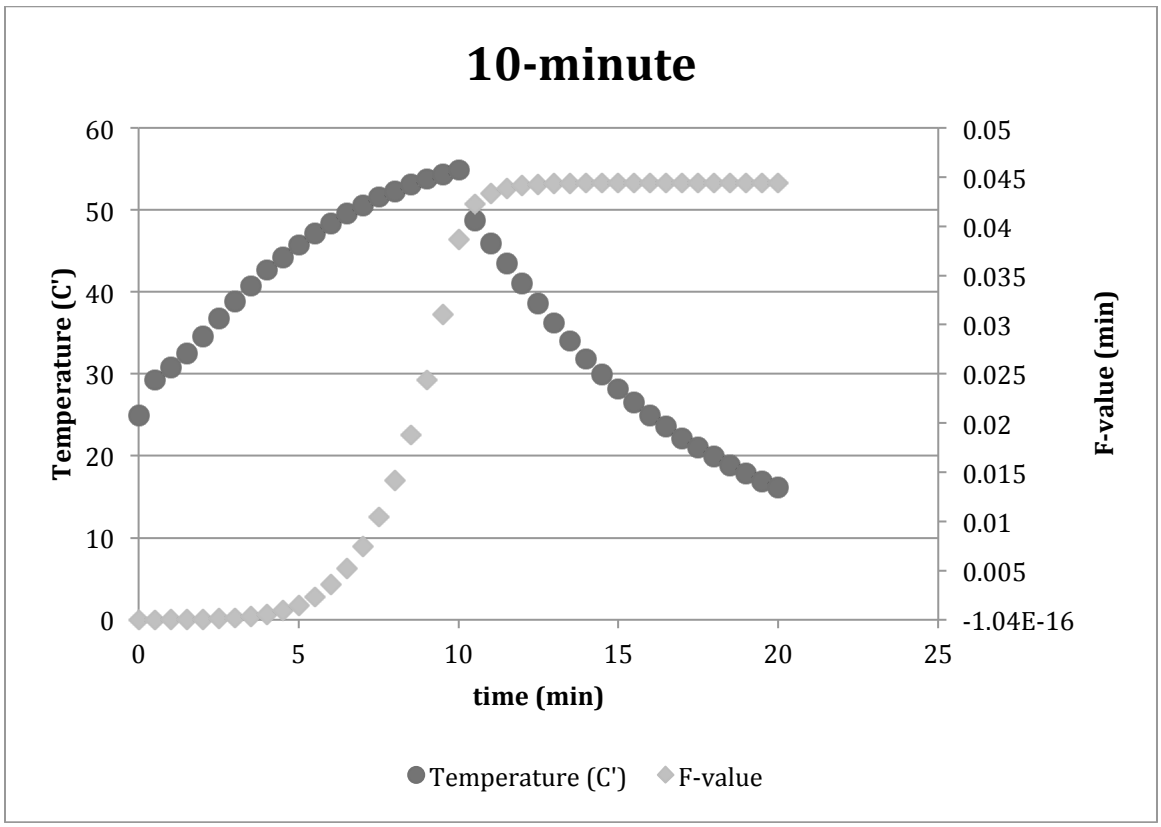


Figure 2.8 Lowest F-value of a 10-minute commercial trial and its mean IOT.

Figure 2.9 Photographs for the pilot scale set-up

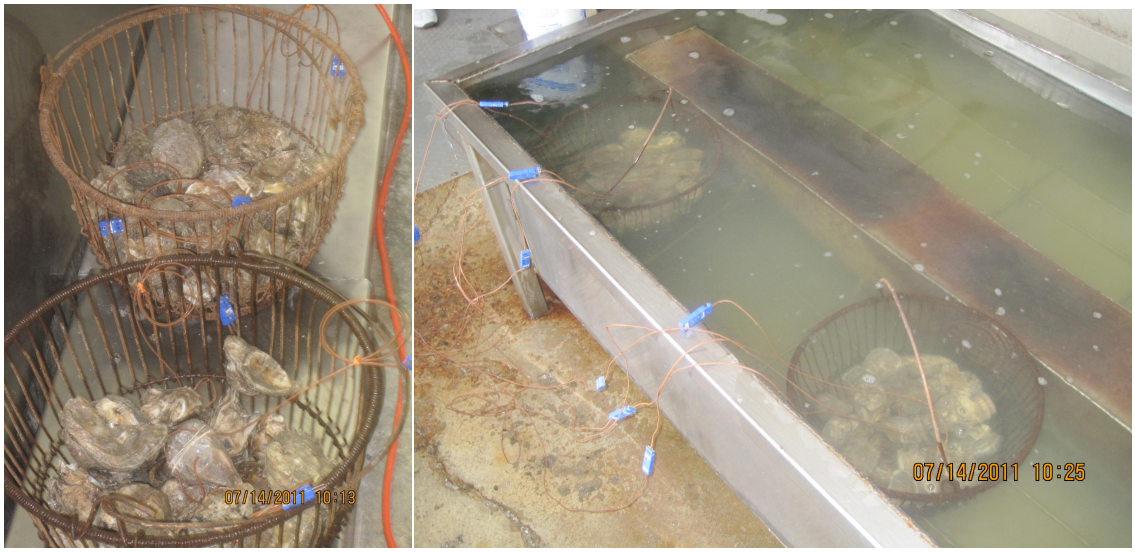


Figure 2.10 Photographs for the commercial scale set-up

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