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Title: Commercial Application of High Pressure Processing for Inactivating *Vibrio parahaemolyticus* in Pacific Oysters (*Crassostrea gigas*)

Abstract approved:

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Vibrio parahaemolyticus is a Gram-negative, halophilic pathogen that occurs naturally in coastal and estuarine environments. This human pathogen is frequently isolated from a variety of seafood, particular oysters, and is the leading cause of gastroenteritis associated with seafood consumption. Several outbreaks of *V. parahaemolyticus* infections linked to consumption of raw oysters have been documented. Contamination of oysters with *V. parahaemolyticus* is a concern for public health. This study investigated the efficacy of high pressure processing (HPP) in inactivating *V. parahaemolyticus* in raw Pacific oysters (*Crassostrea gigas*) and identified a process condition capable of achieving greater than 3.52-log reductions of *V. parahaemolyticus* in raw oysters for commercial application.

Raw Pacific oysters were inoculated with a clinical strain of *V. parahaemolyticus* 10293 (O1:K56) to levels of 10^{4-5} cells per gram and processed at 293 MPa (43K PSI) for 90, 120, 150, 180 and 210 s. Populations of *V. parahaemolyticus* in oysters after processes were analyzed with the 5-tube most probable number (MPN) method. A minimum HPP of 293 MPa for 120 s at groundwater temperature (8±1 °C) was identified capable of achieving greater than 3.52-log reductions of *V. parahaemolyticus* in Pacific oysters.

The HPP (293 MPa for 120 s at 8±1 °C) was validated at a commercial scale according to the FDA's National Shellfish Sanitation Program Post Harvest Processing (PHP) Validation/Verification Interim Guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Negative results obtained by the MPN method were confirmed with a multiplex PCR detecting genes encoding thermolabile hemolysin (*tl*), thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*).

Oysters processed at 293 MPa for 120 sec had a shelf life of 6-8 days when stored at 5 °C or 16-18 days when stored in ice. This validated HPP was accepted by the FDA as a post harvest process to eliminate *V. parahaemolyticus* in raw oysters.

Commercial Application of High Pressure Processing for Inactivating *Vibrio parahaemolyticus* in Pacific Oysters (*Crassostrea gigas*)

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Chapter 1

Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that is naturally distributed in the marine environments (McCarter 1999). This pathogen is frequently isolated from a variety of seafoods and recognized as a significant causative agent of human gastroenteritis worldwide associated with consumption of raw seafood, particularly oysters (Yeung and Boor 2004; Su and Liu 2007). Oysters are filter-feeding bivalve mollusk and can filter up to 34 liters of water every hour (Prieur and others 1990), thus can accumulate a large number of *V. parahaemolyticus* in tissues from the growing environment with concentrations up to 100-fold of the density of the bacterium in surrounding water (Fuhrman 1999; Morris 2003).

The United States produces more than one billion pounds of shellfish each year with more than 30 million pounds of oysters. It is estimated that 20 million Americans consume raw shellfish, making raw shellfish the biggest seafood hazard in the U.S. (Rippey 1994). Outbreaks of *V. parahaemolyticus* infections linked to the consumption of contaminated raw oysters occurred in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the United States between 1997 and

1998, in New Jersey and New York in 2002, in Alaska in 2004, and in New York, Oregon and Washington in 2006 (Agasan 2002; CDC 1998, 1999, 2006; DePaola and others 2000; McLaughlin and others 2005; Mulnick 2002). The United States Centers for Disease Control and Prevention (CDC) reported an 85% increase in the incidence of *Vibrio*-associated infections in 2009 from the 1996-1998 baselines and estimated that 4,500 cases of *V. parahaemolyticus* infection occur each year in the U.S. (CDC 2008, 2010). The increased incidence of *V. parahaemolyticus* infection is a public health concern, particularly for people who eat raw or undercooked oysters.

The U. S. Food and Drug Administration (FDA) guideline for *V. parahaemolyticus* contaminants in seafood has action levels equal to or greater than 10^4 CFU/g (FDA 2011). To minimize *V. parahaemolyticus* infection associated with shellfish consumption, the National Shellfish Sanitation Program (NSSP) has established a guideline for post harvest processing (PHP) of shellfish, which requires a process to reduce *V. parahaemolyticus* in shellfish to an end point of less than 30 cells per gram with a minimum 3.52-log reduction (FDA 2007). Several PHP methods, include low-temperature pasteurization, quick-freezing followed by frozen storage, high pressure treatment, and low-dose irradiation have been studied for inactivating *V. parahaemolyticus* in oysters. In Japan, oysters intended to be eaten raw should not contain more than 100 MPN/g of *V. parahaemolyticus* (JETRO 2009). However, the European Union Commission Regulation (EC) No. 2073/2005, which sets microbiological criteria for foodstuffs, sets no specific criteria for pathogenic *Vibrio*

vulnificus and *V. parahaemolyticus* in seafood traded within the European Community (EC 2005).

High pressure processing (HPP), also described as high hydrostatic pressure (HHP), or ultra high pressure (UHP) processing, can be applied to food processing for inactivating microorganisms at ambient or low temperature with negligible impairment of taste, texture, appearance, and nutritional values (Styles and others 1991; Berlin and others 1999; Chen and others 2006; Grove and others 2006; Considine and others 2008; Corbo and others 2009). Numerous studies have reported the effectiveness of HPP on inactivating *V. parahaemolyticus* including the serotype O3:K6 strain in raw oysters without adverse impacts to sensory properties but an added benefit of shucking oysters during the process (Cruz-Romero and others 2008a, 2008b; Prapaiwong and others 2009). Styles et al. (1991) reported that a process of 170 MPa for 10 and 30 min eliminated *V. parahaemolyticus* (10^6 CFU/mL) in clam juice and phosphate buffer, respectively. Berlin et al. (1999) showed that a treatment of 200 MPa for 10 min at 25 °C achieved greater than 6-log reductions of *V. parahaemolyticus* in homogenized raw oysters. Calik et al. (2002) found that treatments of 345 MPa for 30 and 90 s at 22 °C were capable of reducing *V. parahaemolyticus* in pure culture (7.6×10^6 - 5.5×10^8 CFU/ml) and in oysters (8.4×10^5 - 3.4×10^7 CFU/g), respectively, to non-detectable levels (<10 CFU/mL or CFU/g). Cook (2003) reported that a treatment of 300 MPa for 180 s at 28 °C could achieve a 5-log reduction of *V. parahaemolyticus* O3:K6 strains in oysters. Kural et al. (2008) studied effects of temperature and pressure levels on *V. parahaemolyticus* inactivation and

reported treatments of at least 350 MPa for 120 s at temperatures between 1 and 35 °C and of 300 MPa for 120 s at 40 °C could achieve a 5-log reduction of *V. parahaemolyticus* in oysters. A treatment of 345 MPa for 7.7 min was also reported capable of reducing *V. parahaemolyticus* in oysters by 5.4-log (Koo and others 2006).

While a number of studies on inactivation of *V. parahaemolyticus* in oysters by HPP under various conditions have been reported, no study has been conducted to identify a condition for achieving greater than 3.52-log reductions of *V. parahaemolyticus* in raw oysters for commercial application and validate the process at a commercial scale according to the FDA's National Shellfish Sanitation Program (NSSP) Post Harvest Processing (PHP) Validation/Verification Interim Guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus* (FDA 2007). The shellfish industry in the Pacific Northwest has been using HPP of 293 MPa (43 K PSI) at groundwater temperature for shucking oysters. It would be beneficial to the industry to identify a processing condition at such a pressure level to eliminate *V. parahaemolyticus* contamination in oysters. This study aimed at identifying and validating a HPP for achieving greater than 3.52-log reductions of *V. parahaemolyticus* in raw Pacific oysters (*Crassostrea gigas*) for commercial application and determination the shelf life of the processed oysters stored at 5 °C or in ice.

Chapter 2

Literature Review

2.1 Overview of *Vibrio parahaemolyticus*

2.1.1 Classification and virulence factors of *V. parahaemolyticus*

The genus *Vibrio* comprises 30 species, of which 13 are known pathogenic to humans. The bacteria are Gram-negative, halophilic, mesophilic and asporogenous rod that is straight or has a single rigid curve (Drake and others 2007). Among them, *V. cholera*, *V. parahaemolyticus*, and *V. vulnificus* are recognized as the most common causes of seafood-borne illnesses.

Classification of *V. parahaemolyticus* historically has been made based on serotyping using antibodies specific to O (somatic) and K (capsular) antigens (FDA 2004a). Currently, 12 O antigen types and 76 K antigen types have been recognized with at least 14 pathogenic serotypes, including O1:K32, O1:K56, O2:K28, O3:K6, O3:K29, O4:K4, O4:K8, O4:K10, O4:K11, O4:K12, O4:K55, O4:K68, O5:K15, and O5:K17, being identified. In addition, the species *V. parahaemolyticus* can be distinguished from other *Vibrio* species based on the presence of the species-specific thermolabile hemolysin gene (*tl*) (Taniguchi and others 1985).

While *V. parahaemolyticus* has often been involved in seafood-associated foodborne illnesses, only a small percentage (generally less than 5%) of the total *V. parahaemolyticus* present in the environment or seafood is pathogenic to humans (Nishibuchi and Kaper 1995). Pathogenic strains of *V parahaemolyticus* are identified based on the ability to produce thermostable direct hemolysin (TDH) and/or thermostable related hemolysin (TRH). The *tdh* and *trh* genes encoding thermostable direct hemolysin (TDH) and thermostable related hemolysin (TRH), respectively, are

recognized as major virulence factors of pathogenic strains of *V. parahaemolyticus* with the *tdh* gene being detected in the vast majority of clinical isolated strains.

The TDH is a cardiotrophic, cytotoxic protein comprising 165 amino acid residues with a molecular weight of 42,000 Daltons (Jay and others 2005). The TDH acts on cellular membranes as a pore-forming toxin that changes ion flux in intestinal cells, resulting in a secretory response and diarrhea (Raimondi and others 2000; Zhang and Austin 2005). The TDH is thermal stable and cannot be inactivated by heating at 100 °C for 15 min (Fukui and others 2005). It was first identified for its ability to produce a beta-type haemolysis on Wagatsuma blood agar known as Kanagawa phenomenon (KP) (Yeung and Boor 2004). However, KP-negative strains have also been isolated from patients with *V. parahaemolyticus* gastroenteritis. An isolate of *V. parahaemolyticus* from an outbreak in the Republic of Maldives in 1985 did not carry the gene encoding TDH, but produced a TRH (Honda and others 1988). Shirai and others (1990) surveyed 285 strains of *V. parahaemolyticus* and revealed an association of the TRH-positive strains with gastroenteritis. The TRH is immunologically similar but physiochemically different to TDH and has approximately 70% identity of nucleotide sequence with the TDH (Kishishita and others 1992). However, the TRH is labile to heat treatment at 60 °C for 10 min. The mechanism of the TRH in causing infection appears to be similar to that of TDH. It induces Ca^{2+}-activated Cl^- channels that leads to altered ion flux (Honda and Iida 1993).

Both the *tdh* and *trh* genes have been cloned and sequenced. A polymerase chain reaction (PCR) using DNA probes for genes encoding TDH and TRH was

developed for detecting pathogenic *V. parahaemolyticus* (Tada and others 1992). Bej et al. (1999) developed a multiplex PCR procedure for amplification of *tl*, *tdh* and *trh* for detecting total and pathogenic *V. parahaemolyticus* in shellfish. Presence of *tl*, *tdh* or *trh* gene in bacterial cells detected by the PCR assay resulted in appearance of bands of 450 bp, 269 bp or 500 bp, respectively, on electrophoresis gel.

In addition to *tdh* and *trh* genes, Abbott et al. (1989) first reported that urea hydrolysis may be used as a marker to predict virulent strains of *V. parahaemolyticus* and found that the urease-positive phenotype was associated with the O4:K12 serotype. Several studies also reported that clinical strains carrying the *tdh* or *trh* gene were urease-positive (Kaysner and others 1994; Osawa and others 1996). A later study revealed that the urease and TRH were genetically linked between the urease gene (*ureC*) and *trh* on the chromosome of virulent *V. parahaemolyticus* strains (Park and others 2000). DePaola et al. (2003) demonstrated that presence of *trh* or *ure* gene in addition to the *tdh* gene could increase the virulence of the *tdh*+ strains of *V. parahaemolyticus*.

In addition to the *tdh*, *trh*, and *ure* genes, other virulence factors, such as adherence factors and various enzymes, have been proposed to play a role in the pathogenicity of *V. parahaemolyticus*. Furthermore, a heat labile protein (serine protease) purified and characterized from a clinical strain without carrying *tdh* or *trh* gene was also regarded as a potential virulent factor (Lee and others 2002). The purified protease had significant effects on the growth of Chinese hamster ovary,

HeLa, Vero, and Caco-2 cells. It lysed erythrocytes and caused tissue hemorrhage and death in mice when injected either intraperitoneally or intravenously.

2.1.2 Ecology of *V. parahaemolyticus*

Vibrio parahaemolyticus can grow at temperatures ranging from 5 to 44 °C and over a broad pH values ranging from 4.8 to 11.0 with the optimal growth condition at a temperature between 30 and 35 °C and a pH between 7.6 and 8.6 (Sakazaki 1983). This foodborne pathogen occurs naturally in the ocean and estuarine environments and is frequently isolated from a variety of seafoods such as crayfish, codfish, sardine, mackerel, flounder, clam, octopus, mussel, shrimp, crab, lobster, crawfish, scallop, and oyster (Liston 1990; Drake and others 2007). *V. parahaemolyticus* is sensitive to low temperature and its growth is retarded at hydrostatic pressures higher than 20 MPa as encountered in the deep sea (Schwartz and Colwell 1974). Therefore, *V. parahaemolyticus* is more frequently isolated from shallow water and estuarine environments.

The density of *V. parahaemolyticus* in seawater is known to relate to water temperature (Su and Liu 2007). As water temperatures rise, the number of *V. parahaemolyticus* detected in water, sediment, and various marine species increases (Johnson and others 2010). Sutton's study (1974) conducted at the coast of Sydney found that the viable counts of *V. parahaemolyticus* in oyster rose with increasing water temperature, from non-detectable level (< 3.0 MPN/100g) at 10 ºC to 1.5 log MPN/g at 22 ºC. Since *V. parahaemolyticus* tends to be more abundant in warmer

seasons than in colder seasons, shellfish harvested in summer usually have much higher concentrations than those harvested in winter. DePaola et al. (2000) reported that populations of *V. parahaemolyticus* in oysters harvested from Galveston Bay were relatively high (100 to 1,000 per gram) when water temperature was between 27.8 to 31.7 °C during the summer. Similarly, a survey of 370 lots of oysters from restaurants and seafood markets throughout the U.S. between June 1998 and July 1999 indicated a seasonality of *V. parahaemolyticus* densities in market oysters, with the highest densities (some exceeded 1,000 MPN/g) in the summer (Cook and others 2002). Duan and Su (2005) reported that levels of total and pathogenic *V. parahaemolyticus* (20-43 MPN/g) were higher in oysters harvested in July and August at the Tillamook and Yaquina Bays in Oregon when compared with levels in oysters (<3 MPN/g) harvested in winter. Another seasonal-cycle study of *V. parahaemolyticus* identified water temperature, turbidity, and dissolved oxygen as factors governing density of *V. parahaemolyticus* in oysters in Maryland from November 2004 to October 2005 (Parveen and others 2008).

Vibrio parahaemolyticus is ubiquitous in salt water containing NaCl concentrations ranging from 1 to 8% with an optimal growth condition of 2 to 4% (Sakazaki 1979). Martinez-Urtaza et al. (2008) investigated the occurrence and distribution of *V. parahaemolyticus* in the Rias of Galicia, Spain from January 2002 to December 2004 in association with environmental and oceanographic variables. They concluded the salinity was a determining factor for the temporal and spatial distribution of *V. parahaemolyticus.* A study conducted by Zimmerman et al. (2007)

during the summer of 2004 in the northern Gulf of Mexico reported that both salinity and turbidity affected densities of *V. parahaemolyticus* in water and in oysters.

Vibrio parahaemolyticus is widely distributed in the seawater around the world and has been isolated in many countries including China (Chen and others 1991; Yam and others 2000), Japan (Hara-Kudo and others 2003), Korea (Lee and others 2008), Thailand (Pan-Urai and others 1973), Indonesia (Lesmana and others 2001), Vietnam (Neumann and others 1972), India (Deepanjali and others 2005), Russia (Libinzon and others 1977), Australia (Sutton 1974), United Kingdom (Little and others 1997), France (Robert-Pillot and others 2004), Spain (Martinez-Urtaza and others 2004; Lozano-Leon and others 2003), Germany (Lhafi and Kuhne 2007), Italy (Ottaviani and others 2010), Mexico (Vitela and others 1993), Chile (Fuenzalida and others 2007), Peru (Gil and others 2007), Canada (Flick and Granata 2010), and the United States (CDC 1998, 1999, 2006, 2010). Due to increase in ocean temperature, *V. parahaemolyticus* has been detected in coastal waters as far north as the southern coast of Alaska (Daniels and others 2000; McLaughlin 2005).

In some extreme conditions, for instance, starvation, sudden temperature shifts, low or high salt concentrations, suboptimal pH, depletion of nutrients, and certain wavelengths of light, *V. parahaemolyticus* tends to enter a viable but non-culturable (VBNC) state (Jiang and Chai 1996; Colwell 2000; Wong and Wang 2004). The VBNC cells do not form colonies on nutrient media and, therefore, cannot be detected by cultural methods. However, the cells are considered alive because metabolic activity can still be detected.

2.1.3 Incidence of *V. parahaemolyticus* food poisoning

Vibrio parahaemolyticus is one of the most commonly reported causes of seafood-associated infections. It was first identified as a foodborne pathogen after a large outbreak in Japan in 1951 (Fujino and others 1953). Since then, outbreaks of *V. parahaemolyticus* infections have been reported in both the Eastern and Western hemispheres, such as Asia (Wong and others 2000; Yam and others 2000; Deepanjali and others 2005), North America (CDC 1998, 1999, 2006; McLaughlin and others 2005), and Europe (Lozano-Leon and others 2003).

In the U.S., *V. parahaemolyticus* is recognized as the leading causative agent of seafood associated bacterial gastroenteritis (52% of all *Vibrio* isolates in 2009) (CDC 2010). The first confirmed outbreak of *V. parahaemolyticus* illness in the U.S. occurred in Maryland in 1971, involving 425 cases of gastroenteritis associated with consumption of improperly cooked crabmeat (Molenda and others 1972). The largest outbreak of *V. parahaemolyticus* gastroenteritis recorded in the U.S. occurred in the Port Allen of Los Angeles in the summer of 1978, affecting 1,133 of 1,700 persons attending a dinner (Montville and Matthews 2005). Later, an outbreak related to the consumption of raw oysters occurred in the Pacific Northwest region in 1981 (Nolan and others 1984). Since 1997, several large outbreaks have been reported in the U.S.. Almost all the cases of foodborne *Vibrio* infections were associated with seafood consumption, primarily raw oyster consumption. Among them, the largest outbreak of *V. parahaemolyticus* infection linked to raw oyster consumption occurred in 1998 with

416 illnesses caused by a strain of O3:K6 serotype (DePaola and others 2000). These outbreaks are summarized in Table 1.1.

Table 1.1 Outbreaks of illnesses from *Vibrio parahaemolyticus* associated with consumption of raw oysters in the United States

Year	Location	Number of Cases	Reference
1997	Pacific Northwest [a]	209	CDC 1998
1998	Washington	43	DePaola and others 2000
1998	Texas	416 [b]	DePaola and others 2000
1998	Northeast Atlantic	10	CDC 1999
2002	New York	7	Agasan 2002
2002	New Jersey	11	Mulnick 2002
2004	Alaska	14	McLaughlin and others 2005
2006	New York	83	CDC 2006
2006	Washington, Oregon	94	CDC 2006

[a] The Pacific Northwest includes California, Oregon, Washington State, and British Columbia.
[b] 296 cases in Texas and 120 cases in other states that were traced back to oysters harvested from Texas.

The predominant syndrome of *V. parahaemolyticus* infection is gastroenteritis, accounting for up to 80% of cases. Symptoms of illness usually occur within 24-72 hr, and may include diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills. In addition, wound infection through exposure of an open wound to contaminated sea water and primary septicaemia may also occur in people having

underlying medical conditions such as liver disease or immune disorders (Butt and others 2004; Drake and others 2007).

Vibrio parahaemolyticus infections may occur throughout the year and usually with a peak in spring and summer, corresponding to the warmer water temperatures. In addition, *V. parahaemolyticus* infections may be associated with certain geographical locations because of differences in regional climate and the amounts of oysters harvested. Approximately one-half of the oysters produced in the U.S. are harvested from the Gulf of Mexico, while about one-forth are harvested from the Pacific Northwest region with less than one-tenth being harvested from the Mid-Atlantic region.

2.1.4 Control and prevention of *V. parahaemolyticus* infection

2.1.4.1 Guidelines

The recent 85% increase in the incidence of *Vibrio*-associated infections in 2009 from the 1996-1998 baselines reported by the CDC (CDC 2008, 2010) pointed out that *V. parahaemolyticus* infection continues to be a health threat to the consumers. To minimize the risk of seafood-associated *V. parahaemolyticus* infection, a number of control strategies, include monitoring of harvest waters and microbiological analysis of oysters, identification and implementation of process controls, and consumer education, can be implemented. Federal agencies, state governments, and private industry all bear responsibility for reducing seafood-associated infections.

In response to the severe outbreaks that occurred in 1997 and 1998, the "Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio parahaemolyticus* In Raw Oysters" was initiated by the U.S. Food and Drug Administration (FDA) in 1999, and was modified based on public comments and new data to better understand the infection factors and evaluate the impact of various control methods later on. The risk assessment comprises four components: (1) hazard identification, (2) hazard characterization, (3) exposure assessment, and (4) risk characterization with four seasons and six oyster harvest areas (Gulf Coast-Louisiana, Gulf Coast-non Louisiana, Mid-Atlantic, Northeast Atlantic, Pacific Northwest-Dredged, and Pacific Northwest-Intertidal) (FDA 2005).

The FDA recommended a limit of 10,000 cells per gram of *V. parahaemolyticus* in oysters for human consumption (FDA 1997). However, epidemiological investigation of the four outbreaks occurred between 1997 and 1998 indicated that the overall levels of *V. parahaemolyticus* detected in some oysters from implicated harvest sites were lower than 1,000 cells per gram with a few as low as 100 cells per gram (Kaysner and DePaola 2000). Since not all *V. parahaemolyticus* strains are pathogenic to humans, the FDA's risk assessment report suggested testing pathogenic *V. parahaemolyticus* in oysters upon harvest as a step to protect consumers from *V. parahaemolyticus* infection associated with raw oyster consumption.

In order to regulate the harvesting, processing, and shipping of shellfish for interstate commerce, the National Shellfish Sanitation Program (NSSP) guidelines were established through the Interstate Shellfish Sanitation Conference (ISSC), which

is a cooperative organization formed in 1982 between the FDA and other federal agencies, state health departments and shellfish authorities, and private industry. The NSSP suggested several measures, such as post harvest processing (PHP), closing the area to oyster harvest, and restricting oyster harvest to product labeled for shucking by a certified dealer, to effectively control *V. parahaemolyticus* in seafood. For the validation of PHP, ten samples each consisted of 10-12 oysters with an adjusted geometric mean (AGM) of 10^4 MPN/g or greater should be used and the levels of *V. parahaemolyticus* in oysters need to be reduced to less than 30 MPN/g with a minimum 3.52 log reduction observed after treatments. The initial *V. parahaemolyticus* levels in oyster samples before and after the treatment should be determined by the three-tube and five-tube most probable number (MPN) method, respectively (FDA 2007). The validation process should be conducted three times on three different processing days.

Vibrio parahaemolyticus can multiply rapidly in oysters if the stock temperature of shellfish is not properly controlled. A time-to-temperature regulation was established by the NSSP to limit the maximum time of exposure of oysters to ambient temperatures. Shellfish intended for raw consumption are required to be cooled down to 10 °C (50 °F) or lower within 10 h of harvest if the average monthly maximum air temperature is ≥27 °C (81 °F), within 12 h if the average monthly maximum air temperature is between 19 and 27 °C (66-80 °F), and within 36 h if the average monthly maximum air temperature is <18 °C (66 °F) (FDA 2007).

In addition to temperature controls, harvest practices can also influence levels of *V. parahaemolyticus* in oysters. Before being transported to the processing plant, oysters may be exposed to a favorable growth temperature, which allows *V. parahaemolyticus* to multiply rapidly in oysters especially on a warm and sunny day. Nordstrom et al. (2004) found that the mean densities of *V. parahaemolyticus* in oysters after exposure to the ambient temperature generally increased to four to eight times of the initial level at the beginning of the low-tide exposure and suggested that avoiding harvest of oysters after intertidal exposure to ambient conditions could potentially reduce the incidence of *V. parahaemolyticus* infection associated with raw oyster consumption. In Mississippi, harvest of oysters for raw consumption is limited from mid-September through April. Oyster harvest areas are closed during the warmer summer months and at other times when heavy rains cause an influx of potentially contaminated water. These harvesting practices minimized the incidence of *V. parahaemolyticus* illnesses from consumption of Mississippi oysters (Andrews 2004).

While avoiding harvest of oysters after being exposed to ambient conditions, properly controlling storage temperature of oysters upon harvest, and application of PHP can reduce risks of *V. parahaemolyticus* infections associated with raw oyster consumption, consumers should be aware of the potential health risks associated with eating seafood that are raw or inadequately heated , particularly for people with medical conditions. It was estimated that people who consume raw oysters are 2.8 times more likely to experience *V. parahaemolyticus* illness than those who do not eat

raw oysters (FDA 2005). In several states, a warning sign is required to be posted where raw oysters are served or sold.

2.1.4.2 Depuration and relaying

Depuration is a controlled process that purges shellfish harvested from moderately contaminated areas through holding shellfish in tanks of either a flowthrough or recirculating system for 2 to 3 days. The process usually results in a reduction of total bacterial contaminants in shellfish and, therefore, prolongs the shell life of products. However, the process is not consistently effective in removing *Vibrio* spp, heavy metals, organic chemicals, or marine biotoxins such as those causing paralytic shellfish poisoning (PSP), diarrheal shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP). It is also less effective in removing viral contaminants, such as norovirus and hepatitis A.

There are many factors affecting the efficacy of depuration: oyster species, temperature, salinity, pH, dissolved oxygen and turbidity of seawater, suspended solids, harvest time and location, types of targeted microbe, initial levels of bacteria1 contamination, and designs of the depuration system (Richards 1988; Govorin 2000). Depurations combined with chlorination (Wells 1929), ultraviolet light (Kelly 1961), iodophors (Fleet 1978), and ozone (Croci and others 2002) have been applied separately or synergistically to enhance the reduction of bacterial contamination. However, none of them could effectively eliminate *V. parahaemolyticus* from shellfish. Ren and Su (2006) reported that an electrolyzed oxidizing (EO) water treatment

(chlorine: 30 ppm, pH: 2.82; oxidation-reduction potential: 1131 mV) of raw oysters for 4-6 h resulted in reductions of *V. parahaemolyticus* and *V. vulnficus* by 1.13 log and 1.05 log MPN/g, respectively. The chlorine contents and low pH value were believed to be the main factors contributing to the bactericidal effects of the EO water. However, long exposure (>12 h) of oysters to EO water (>30 ppm chlorine) led to the death of oysters.

Relaying is another purification process that involves moving shellfish from a contaminated harvesting area to an unpolluted open area for natural cleansing (Yeung and Boor 2004). Cook and Ellender (1986) found that the temperature and the microbiological quality of the relaying water had an impact on the length of time needed to reduce fecal coliform levels in oysters. Relaying can be used to reduce bacterial contaminants in shellfish. However, it is labor-intensive and requires a clean marine environment, which has been a big challenge for the shellfish industry to locate an unpolluted marine environment for the practice.

2.1.4.3 Thermal processes

Thermal processing (e.g. refrigeration, frozen storage, heat treatment, etc.) is the prevailing approach to achieve microbial stability and safety. Refrigeration is the most commonly used process to control the multiplication of *V. parahaemolyticus* in oysters. Thompson and Vanderzant (1976) reported that the density of *V. parahaemolyticus* in shucked oysters stored at 3 °C for 7 days decreased from more than 11,000 to 0.36 MPN/g. Cook and Ruple (1989) found that members of the

Vibrios increased in shellstock oysters harvested from the Gulf Coast and stored at 22 and 30 ºC, while growth was prevented when the oysters were stored at 10 ºC. Levels of *V. vulnificus* in shellstock oysters immersed in ice for 3 h and then stored in a refrigerator gradually reduced from 2.9 CFU/g to 1.6 and 1.2 log CFU/g after 7 and 14 days of storage, respectively (Quevedo and others 2005). Shen et al. (2009) reported that populations of *V. parahaemolyticus* in oysters decreased by about 2.0 log MPN/g after storage at 0 ºC or 5 ºC for 96 h. A subsequent frozen storage of the oysters at -30 ºC for 75 days led to around 3.80 and 5.08 log MPN/g reductions of *V. parahaemolyticus* in shell and shucked oysters, respectively.

Several studies have reported that frozen storage is capable of reducing certain levels of *V. parahaemolyticus* in oyster meat and half-shell oysters. Cook and Ruple (1992) reported that freezing reduced the levels of *Vibrio* spp. in shellfish, although it did not eliminate the organism even after storage of 12 weeks. Muntada-Garriga et al. (1995) reported that storing oyster homogenates at -18 and -24 °C for 15 to 28 weeks could completely inactivate viable cells of *V. parahaemolyticus* (10^{5-7} CFU/g) in the homogenates. Frozen storage of half-shell oysters for up to 4 months at -20 °C was capable of reducing low populations of *V. parahaemolyticus* (<1,000 CFU/g) to non-detectable levels (Andrews 2004). However, the process could not reduce high levels of *V. parahaemolyticus* (>1,000 CFU/g) in oysters to non-detectable levels within 6 months. More recently, a study demonstrated that a process of flash freezing (-95.5 ºC for 12 min) followed by 5 months storage at -21±2 ºC could reach more than 3.52 log MPN/g reductions of *V. parahaemolyticus* in half-shell Pacific oysters (Liu and others

2009). The Interstate Shellfish Sanitation Conference (ISSC) has adopted the flash freezing combined with frozen storage as an acceptable means of PHP to control *V. parahaemolyticus* in raw Pacific oysters. A number of oyster producers are now using this process to control *V. parahaemolyticus* in oysters, which must be validated and in compliance with HACCP (21 CFR 123). Nowadays, the Individually Quick Frozen (IQF) system has become popular around the world. The IQF is a post harvest processing system that freezes the half shelled oysters in trays going through a nitrogen (or carbon dioxide) freezing tunnel, and stored in freezers. One big advantage of the IQF is keeping the flavor and appeal of non-processed oysters.

Vibrio species are sensitive to heat. Cultures of *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* were reduced by more than 7.0 logs CFU/mL after being treated at 70 ºC for 2 min (Johnston and Brown 2002). Andrews et al. (2000) demonstrated that *V. parahaemolyticus* (10^5 MPN/g) artificially inoculated in oysters could be reduced to non-detectable level by exposing oysters to a low-temperature pasteurization (50 ºC for 10 min) process. However, the treatment for eliminating *V. parahaemolyticus* O3:K6 required a longer time (22 min) at temperature between 50 and 52 ºC (Andrews and others 2003). These results indicate that certain parameters, such as strain variation and bacterial levels in food, need to be considered in order to make the low-temperature pasteurization effectively against *V. parahaemolyticus*. In addition, exposure of *V. parahaemolyticus* to a mild heat treatment may increase its tolerance to subsequent heat processing. It has been reported that cells of *V.*

parahaemolyticus became more resistant to heat inactivation at 47 ºC after a heat-shock process at 42 ºC for 30 min (Wong and others 2002).

2.1.4.4 Non-thermal processes

Thermal pasteurization and sterilization are the most important processes to achieve food safety. Unfortunately, thermally processed foods generally loose the characteristics of fresh products. Other technologies, including high pressure processing (HPP), ionizing radiation, ultraviolet radiation (UV), ultrasound, and pulsed electric field (PEF) have been extensively investigated in the past thirty years for preserving food and reducing pathogen contamination with little change in quality or sensory characteristics. These processes are referred as non-thermal techniques, as food is usually processed at ambient or refrigeration temperature.

HPP can inactivate spoilage and pathogenic microorganisms in liquid and solid foods with or without packaging at pressures between 100 and 800 MPa and between -20 ºC to 60 ºC with limited impairment of food quality. A number of research papers on effectiveness and applications of HPP in food have been published. This non-thermal processing is discussed in greater detail in section 2.2.

Irradiation is a batch process that can be easily adapted to continuous applications. The process is commonly referred as ionizing radiation and is often conducted with Gamma radiations or electron beams at doses of 2-10 kGy (Farkas 1998). Irradiation inactivates microorganisms by causing DNA damage and protein denaturation (Lucht and others 1998; DeRuiter and Dwyer 2002). The Gamma

irradiation is effective against vegetative and spore-forming bacteria (Zhang and others 2006; Baskaran and others 2007). Irradiation is approved by the U.S. FDA as a food additive for seafood, including oysters (FDA 2004b). Novak et al. (1966) found that a 2 kGy dose of gamma radiation could be applied for pasteurizing oyster meat to reduce total bacterial counts by 99% without significant changes in organoleptic quality.

Many studies have investigated the efficacy of irradiation in inactivating *Vibrio* species. Matches and Liston (1971) reported that, in most cases, *V. parahaemolyticus* was reduced by 4 to 6 log units using a dose of 0.3 to 0.4 kGy. Jakabi et al. (2003) showed that irradiation with Cobalt-60 gamma radiation at dose of 1.0 kGy was sufficient to reach a 6-log reduction in *V. parahaemolyticus*. Cells of *V. parahaemolyticus* 03:K6 (10^4 CFU/g) inoculated in oysters were eliminated when exposed to a gamma radiation at a dose of 1.5 kGy. Most oysters survived from irradiation processes at low dosages, and sensory analysis showed that consumers could not tell a difference between irradiated and non-irradiated oysters (Jakabi and others 2003). Although irradiation is effective in eliminating *V. parahaemolyticus* from oysters, the reluctance among consumers to accept irradiated food and the need to safely handle radioactive materials limit its usage.

Ultraviolet (UV) energy is a non-ionizing radiation with germicidal properties at wavelengths in the range of 200-280 nm, which may be utilized for pasteurization of liquids or disinfection of surface (Bintsis and others 2000).

Ultrasound, defined as waves with a frequency greater than 20 kHz, can also be used to preserve the quality of foods and has been applied to reduce bacteria in liquid food (Villamiel and others 1999). The lethal effect of ultrasound to bacterial cells is primarily due to the cavitation phenomenon, causing breakdown of cell walls, disruption of cell membranes, and DNA damage (Manvell 1997). High intensity ultrasound can also denature proteins and produce free radicals, which can adversely affect the flavor of fruit-based or high-fat foods (Williams 1994; Sala and others 1995). A study showed a 6-log reduction of *E. coli* O157:H7 cells in apple cider and a 5-log reduction of *Listeria monocytogenes* populations in milk following an ultrasound treatment (sonifier probe at 20 kHz, 100% power level, 150 W acoustic power, 118 W/cm2 acoustic intensity, 57 ºC) (D'Amico and others 2006).

PEF is based on the delivery of pulses at high voltage (typically 20 - 80 kV/cm) through foods placed between two electrodes (FDA 2000). The effect of PEF on inactivating microorganisms has been attributed to an electric potential across the membranes of biological cells, resulting in reversible and irreversible formation of pores depending on the intensity of the electrical field (Zhang and others 1995). PEF has been proposed for dairy products (skim milk, whole milk and yogurt) (Alvarez and Ji 2003; Sepulveda and others 2005), apple (Schilling and others 2007), and tomato juices (Nguyen and Mittal 2007).

2.2 Overview of high pressure processing

High pressure processing (HPP) has several distinct advantages over other food processing technologies. For instance, the pressure is transmitted uniformly and instantaneously throughout the system during the operation and, therefore, products are treated evenly without gradient of effectiveness from surface to center, regardless of the shape, size, and composition of products (Farr, 1990; Smelt 1998; Knorr, 1999). It is an energy-efficient and rapid process (Knorr 1995; Patterson and others 2007; Buckow and Heinz 2008) which can be conducted at ambient, chilling or freezing temperature, thereby eliminating thermally induced off-flavors and retaining characteristic of fresh products (Wilkinson and others 2001; Kingsley and others 2005). Therefore, HPP has the potential for being utilized to produce a wide range of heat-sensitive products and for creating ingredients with novel functional properties (Rastogi and others 2007). This novel technology is capable of producing minimally processed food that is preservative-free and safe with extended shelf life to meet the trends in consumer's demands. This non-thermal processing technology has been cited as one of the best innovations in food processing in 50 years (Dunne 2005).

2.2.1 Principles of HPP

According to Le Chatelier's principle, pressure enhances processes and reactions that are accompanied by a decrease in volume and vice-verse (Pauling 1964). The breaking of chemical bonds in the processed food is associated with decrease or increase in volume. HPP does not alter covalent bonds within the ranges of pressures normally used in food processing thus maintaining the primary structure of proteins or

fatty acids. However, it does disrupt the hydrogen, ionic, and hydrophobic bonds that are responsible for maintaining the secondary, tertiary and quaternary structure of proteins (Murchie and others 2005). The secondary structure of proteins is very pressure-resistant and changes in the secondary structure in proteins usually take place at pressure levels above 700 MPa, leading to irreversible protein denaturation (Balny and Masson 1993).

HPP can also modify the secondary structure and function of complex polysaccharides and lipids (Considine and others 2008). For example, starch can aggregate in gel under pressures and, accordingly, pressure may create novel food textures in protein-based or starch-based foods (Okamoto and others 1990). In addition, HPP may reduce the rates of browning reaction (Maillard reaction) (Tamaoka 1991). Furthermore, low molecular weight compounds, such as vitamins, amino acids, flavor molecules, and pigments, are hardly affected by HPP. Therefore, the color, taste, overall appearance and nutritional properties of food products are not significantly changed after treatments (Hayashi 1990; Farkas and Hoover 2000).

2.2.2 HPP inactivation of microorganisms

Microorganisms are inactivated when exposed to conditions that substantially alter their cellular structures or physiological functions. It is generally assumed that HPP can cause DNA strand breakage, cell membrane rupture or mechanical damage, and enzymes denaturation (Hoover and others 1989; Mackey and others 1994) with the cell membrane being the primary site for pressure damage (Paul and Morita 1971).

Pressure-induced membrane damage affects the transport mechanism involved in nutrient uptake and disposal of cell waste (Lado and Yousef 2002). A disruption of the normal function of the mechanism generally leads to the death of a bacterial cell.

The sensitivity to HPP among bacterial species and even strains is variable (Alpas and others 1999; Benito and others 1999; Pag´an and Mackey 2000). Results of experiments conducted by Shigehisa et al. (1991) suggest that the order of sensitivity to HPP to be Gram-negative bacteria > yeasts > Gram-positive bacteria > bacterial spores. Gram-positive bacteria are more resistant to pressure than Gram-negative bacteria due to the rigidity of the teichoic acids in the peptidoglycan layer of the Gram-positive cell wall (Shigehisa and others 1991). Environmental species appear more sensitive than clinical species. Prokaryotic cells tend to be more pressure resistant than eukaryotes (Patterson 1999). Bacteria of small size and cocci in shape are generally more resistant to HPP than the large rod-shaped ones (Arroyo and others 1999).

In general, HPP can be effective in inactivating most vegetative pathogenic and spoilage microorganisms at pressures of 250- 300 MPa for 10 min or 545- 600 MPa for 30-60 s (Patterson 2005; Lau and Turek 2007). The process of 350 MPa, 375 MPa, 450 MPa, 700 MPa, and 700 MPa for 15 min can achieve 5 log reductions in *Salmonella typhimurium, Listeria monocytogenes, Salmonella enteritidis, Escherichia coli* O157:H7, and *Staphylococcus aureus*, respectively (Patterson and others 1995b). *Vibrio* species, which are the predominant shellfish-associated bacterial infections, are relatively sensitive to high pressure (Styles and others 1991; Rippey 1994; Berlin and

others 1999). HPP between 200 to 300 MPa for 5 to 15 min at 25°C inactivated all cells of *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* O:1 and non-O:1, *V. mimicus*, and *V. hollisae* in broth cultures without triggering a viable but nonculturable (VBNC) state (Berlin and others 1999). However, strains of the pandemic O3:K6 serotype of *V. parahaemolyticus* were reported more resistant to pressures than other *Vibrio* strains. Koo et al. (2006) found that it took 11 and 5 min (including a 3-min pressure come-up time) for a process at 241 MPa to achieve a 6-log reduction of *V. parahaemolyticus* O3:K6 and *V. vulnificus*, respectively, in PBS. Both *V. parahaemolyticus* and *V. vulnificus* decreased to non-detectable levels in oysters after being processed at 586 MPa for 8 and 7 min, respectively.

For moulds and yeast, the vegetative forms are relatively sensitive to HPP and can easily be inactivated by pressure treatments between 200 and 300 MPa for a few minutes. However, the ascospores are more resistant to HPP and need a pressure level of higher than 400 MPa for inactivation (Smelt 1998). A 6-log reduction of *Byssochlamys nivea* required a treatment at 800 MPa at 70 °C for 10 min. A pressure of 600 MPa at 10 °C for 10 min was sufficient to eliminate 7-log of *Eupenicillium* spp (Butz and others 1996).

Unlike vegetative cells, bacterial spores are highly resistant to HPP, even at above 1000 MPa at ambient temperature (Sale and others 1970; Cheftel 1992; Smelt 1998). Spores of *Clostridium botulinum* have been identified as the most pressure-resistant bacterial spores (Margosch and others 2006) and may require a HPP be combined with heat and/or low pH as well as pressure cycling treatments for

inactivation and control of outgrowth of the spores (Hayakwa and others 1994; Quested and others 2010).

For virus inactivation, it has been suggested that high pressures can denature the capsid proteins that are essential for attachment to host cells and initiate infection, therefore preventing the binding to host cells (Khadre and Yousef 2002; Kingsley and others 2002; Hogan and others 2005; Buckow and Heinz 2008; Li and others 2009; Tang and others 2010). While some human viruses are known pressure-sensitive, certain viruses such as polio viruses are pressure-resistant. Most viruses can be eliminated by pressure treatments designed for eliminating bacteria of concern. However, resistance of viruses to HPP depends principally on their structure (Man˜as and Paga´n 2005). Whereas viruses show a wide range of pressure sensitivities (Grove and others 2006), the viral capsid coat proteins are in general much less stable to HPP than the assembled icosahedral viral particles (Silva and others 1996).

Limited information is available about the effectiveness of HPP on parasites. The parasitic worms of *Trichinella spiralis* could be killed by a HPP at 200 MPa for 10 min (Ohnishi and others 1993). Based on the study, it is relatively reasonable to assume that parasites are not as pressure-resistant as bacteria. A non-pathogenic bacterium might be used as an indicator organism to judge survival of foodborne parasites after a HPP.

In addition to types of microorganisms (Alpas and others 1999; Benito and others 1999), the degree of microbial inactivation achieved by HPP is dependent on other factors including growth phase or age of bacteria (Pagan and Mackey 2000),

culturing or growth conditions (McClements and others 2001), composition of surrounding matrices such as type, pH, and water activity of the food (Patterson and others 1995a; Simpson and Gilmour 1997), and processing parameters (high pressure level, treatment time and temperature) (Hogan and others 2005). Stationary phase cells appear to be more pressure-resistant than exponential phase cells. There is a synergistic effect of pressure and temperature, and elevated temperatures reduce bacterial resistance to pressure. Mineral salt solutions, nutrient media, and low water activity appear to protect cells from inactivation by HPP (Barbosa-Cánovas and others 1998; Tewari and others 1999). In addition, the more acidic or alkaline the system is, the more effective the HPP is.

2.2.3 Applications of HPP in food industry

Use of HPP in food processing dates back over a century to the research of Hite (1899), who treated milk at 670 MPa for 10 min and detected 5–6 log reduction in total bacterial counts. Later, the process was extended to preserve fruits and vegetables (Hite and others 1914). In 1992, a Japanese company Meidi-Ya introduced the first HPP commercial product, a high-acid fruit-based jam into market (Mertens 1995). Since then, several high pressure processed products have been marketed, such as jellies, rice cakes and shellfish in Japan, oysters and guacamole in the USA, and fruit juices in France, Portugal, Mexico and the United Kingdom (Smelt 1998; Hugas and others 2002; Torres and Velazquez 2005). More recently, HPP has been extended to food products including salsa, rice products, fish, meal kits (containing HP-treated

cooked meats and vegetables), fruit smoothies, apple sauce fruit blends, chicken strips, ham, poultry products, and sliced ready-to-eat meats (Murchie and others 2005; Goh and others 2007).

For a practical HPP, the product is loaded into a stainless steel chamber filled with a pressure-transmitting fluid (normally water) for high hydrostatic pressurization at refrigeration, ambient or moderate heating temperature for seconds to minutes. Processing of solid foods is carried out in a batch mode whereas liquid products can also be treated with a continuous or semi-continuous process (Hogan and others 2005). Typically, technology barriers in HPP define low cost operations such as oyster shucking at below 420 MPa, higher cost operations such as guacamole salsa production at approximately 600 MPa, and current limit for commercial size vessels at approximately 680 MPa.

An example of HPP of food at an industrial level is Avomex Inc., which began HPP-treating avocado using a 25 L batch processing unit in 1996 and expanded to a semi-continuous unit and a larger 215 L batch processing vessel by 2000 (Torres and Velazquez 2005).

HPP can be used to process both liquid and solid foods, even though it cannot be universally applied to all types of foods. This non-thermal processing technology is especially beneficial for heat sensitive products and foods with a high acid content are particularly good candidates for its application. Pressure depresses the freezing point of water and the melting point of ice, as well as enabling various high-density forms of ice to be formed. Therefore, HPP has potential applications in food technology,

including pressure-assisted freezing (Kalichevsky and others 1995), pressure-assisted thawing (Murakami and others 1992; Schubring and others 2003) and non-frozen storage under pressure (Deuchi and Hayashi 1992) of foods.

2.2.4 Effects of HPP on texture, color, and flavor of food products

The appearance and textural properties of foods can have significant impact on product sales. For instance, soft or spongy foods could be perceived 'going off' or decaying. Although HPP processed foods generally have quality similar to that of fresh foods, the applicability of HPP in food processing depends on not only the target microflora but also the suitability of a product. HPP at various pressure and temperature combinations can be applied to achieve desired effects on texture, color, and flavor of foods. The process can also alter the food rheological properties (Patterson and others 2007). For food products containing high moisture contents, the physical structure usually remains unchanged after HPP. However, color and texture may change in gas-containing products after HPP due to gas displacement and liquid infiltration, leading to shape distortion and physical shrinkage and finally irreversible compression of whole foods (Hogan and others 2005). Indeed, those modifications of color and texture are dependent on types of products and treatment conditions (temperature, time and pressure).

HPP can enhance the action of pectinmethylesterase (PME), lower the polygalacturonase (PG) activity, and retard beta-elimination. Basak and Ramaswamy (1998) investigated the effect of HPP (100 - 400 MPa for 5-60 min at ambient

temperature) on the firmness of fruits and vegetables and observed a rapid firmness loss during compression, followed by either a further loss or gradual recovery during the pressure holding period (100 and 200 MPa for 30 - 60 min).The flavor of fruits and vegetables is generally not altered under high pressures. However, HPP might affect the content of flavoring compounds and disturb the balance of flavoring composition of processed food. Similarly, the color of fruits and vegetables at HPP is generally preserved because the process at low and moderate temperatures has minor influence on pigments (e.g. chlorophyll, carotenoids, anthocyanins, etc.). A study carried out by Rodrigo et al. (2007) observed no color degradation of tomato and a maximum increase of 8.8% in L*a */b* parameter for strawberry under combined thermal and high pressure treatments (300 - 700 MPa, 60 min, 65 ºC).

HPP may alter the properties of milk and cheese products. During the process, casein micelle disintegrates into casein particles of smaller diameter, resulting in increase of viscosity of the milk (Johnston and others 1992). A HPP of 100-500 MPa for 30 min could enhance the rennet coagulation properties (Buffa and others 2001). Cheeses made from pressure-treated milk have higher moisture, salt, and total free amino acids contents than raw or pasteurized milk cheeses (Trujillo and others 1999a, 1999b). However, HPP between 100 - 500 MPa at 4, 25 and 50 ºC did not increase free fatty acids (FFA) content in ewe's milk (Gervilla and others 2001). Buffa et al. (2001) investigated cheese texture and microstructure using uniaxial compression and stress relaxation tests, and confocal laser scanning microscopy. Results indicated that cheeses made from raw or pressure-treated milk were firmer and less fracturable than

cheeses made from pasteurized milk. However, the differences became less notable toward the end of ripening. Cheeses made from pasteurized and pressure-treated milk were less cohesive than those made from raw milk. HPP can also be used to induce beneficial changes in product texture and structure such as melting of Mozzarella cheese during processing (O'Reilly and others 2002).

In fresh meat and poultry, pressure-induced color change resulting from changes in myoglobin, heme displacement/release or ferrous atom oxidation may occur and produces a cooked-like appearance (Hugas and others 2002). In addition, protein denaturation can take place during pressure treatments. However, the changes in physical functionality and/or color of raw products are significantly less in pressure-treated products than those in products receiving conventional thermal treatments (Hogan and others 2005). Suzuki et al. (1994) reported that the amounts of certain water-soluble compounds, such as peptides and amino acids, responsible for meaty flavor increased apparently with increased pressure treatments for up to 300 MPa for 5 min at 2 ºC.

HPP may produce harder textures or higher shear strengths in processed seafood products. This has been observed for cod (Angsupanich and Ledward 1998; Angsupanich and others 1999; Matser and others 2000), octopus (Hurtado and others 2001), pacific mackerel (Yoshioka and others 1992), prawn (Lo´pez-Caballero and others 2000a), and salmon (Amanatidou and others 2000) processed at pressures of 150 - 600 MPa at 1-40 ºC. However, the texture of bluefish (Ashie and others 1997) and carp (Yoshioka and Yamamoto 1998) became softer following HPP. HPP may

also change the appearance of seafood. Seafood products processed at pressures above 300 MPa become opaque, which was similar to that observed for the products after mild heating (Hoover and others 1989). A study reported that cod and mackerel muscle had cooked appearance and higher L-values (an index of visual lightness) after HPP (Ohshima and others 1993). Similar results have been reported for salmon (Amanatidou and others 2000), sheephead (Ashie and Simpson 1996), bluefish (Matser and others 2000), hake (Hurtado and others 2000), carp (Yoshioka and Yamamoto 1998), plaice (Mater and others 2000), Pollack (Mater and others 2000), sardine (Wada 1992), and turbot (Chevalier and others 2001). HPP can also make fish gelatin transparent and soft with a smoother and more uniform texture than gelatin processed by heat treatment. In addition, HPP can induce gelation of sarcoplasmic proteins in fish muscle that are usually removed from fish mince during the traditional production of surimi (Ohshima and others 1993).

2.2.5 Effects of HPP on oyster processing

Seafood is more perishable than other food due to high water activity, neutral pH, and presence of autolytic enzymes. The deterioration is primarily a consequence of bacterial action (Sivertsvik and others 2002). Major bacteria responsible for spoilage of seafood are indigenous bacteria naturally present in the marine environments and fecal bacteria introduced into the marine environments by human or animal (Reilly and Kaferstein 1997). Many of these spoilage bacteria are more resistant than human pathogens to high pressures and will survive a HPP intended to

inactivate pathogens in seafood, leading to subsequent spoilage of products during storage (Feldhusen 2000).

Several studies have shown that HPP can reduce bacterial loads and delay microbial growth in raw oysters. He et al. (2002) reported that the total aerobic plate counts (APC) of oysters treated at 310 MPa and stored at 2-4 °C required 20 days of storage to reach 6-log CFU/g while the APC of untreated oysters exceeded this limit of acceptability after 9 days. Linton et al. (2003) observed that shucked oysters treated at 300-600 MPa for 120 sec and stored at 2 °C had APC of less than 4-log CFU/g on the 28th day. It has been reported that oysters processed with 500 MPa for 5 min at 2 °C had a shelf life of 17 days when stored at 2 °C while oysters processed with 400 MPa for 5 min at 2 °C and stored in ice had a shelf life of 21 days (Cruz-Romero and others 2008a, 2008b).

In addition to inactivation of pathogenic and spoilage bacteria, HPP can inactivate harmful viruses in oysters. Nearly a 6-log reduction of Hepatitis A virus in raw oysters was observed by the treatment at 350-400 MPa, 8.7-10.3 °C for 60 s (Calci and others 2005). The research conducted by Kingsley et al. (2007) suggested that exposing oysters at 400 MPa for 5 min at 5 °C might be a viable strategy for a 4-log reduction of norovirus.

It has been reported that oysters became more voluminous and slightly juicier following HPP (Lo´pez-Caballero and others 2000b; Cruz and others 2004; Cruz-Romero and others 2003), and were judged more acceptable in appearance than untreated oysters in a sensory study (Johnston and others 2003) and retained a raw

taste (Hayashi 1992). Hoover et al. (1989) reported an enhancement in flavor of raw oysters after HPP, possibly by pressure infusion of the salty liquor within the oyster shell into the flesh.

Large proportions of oysters are sold on the half shell or shucked meats. The traditional oyster shucking/opening by hand requires a skilled workforce because inexperienced workers can damage the oyster meat, impairing the quality and appearance of products. An important added advantage of HPP for oyster processing is oyster shucking due to the adductor muscle of oysters detached from the shell during the processing. This process reduces labor costs and worker safety concerns, and increases shelf-life and yields of oysters. He et al. (2002) showed that a HPP at 241 MPa for 2 min caused detachment of adductor muscle in 88% of oysters and a treatment at 310 MPa with immediate pressure release resulted in 100% of shucking. Other than shucking of oysters, HPP can also facilitate the removal of the shell of other crustacean shellfish such as lobster, crab and shrimp (Terio and others 2010).

The shellfish industry in the Pacific Northwest has been using HPP for shucking oysters. It would be beneficial to the industry to identify a HPP at the commercial scale to eliminate *V. parahaemolyticus* contamination in oysters. The main objective of this study was to identify a HPP condition capable of achieving greater than 3.52-log reductions of *V. parahaemolyticus* in raw oysters for commercial application and validate the process at a commercial scale according to the FDA's National Shellfish Sanitation Program (NSSP) Post Harvest Processing (PHP) Validation/Verification Interim Guidance for *Vibrio vulnificus* and *Vibrio*

parahaemolyticus (FDA 2007). The identification and validation of such a HPP will allow the shellfish industry to apply the HPP as a post harvest process to minimize *V. parahaemolyticus* infection associated with raw oyster consumption.

Chapter 3

Validation of High Pressure Processing for Inactivating *Vibrio parahaemolyticus* in Pacific Oysters (*Crassostrea gigas*)

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3.1 Abstract

This study identified and validated high hydrostatic pressure processing (HPP) for achieving greater than 3.52-log reductions of *Vibrio parahaemolyticus* in the Pacific oysters (*Crassostrea gigas*) and determined shelf life of processed oysters stored at 5 °C or in ice. Raw Pacific oysters were inoculated with a clinical strain of *V. parahaemolyticus* 10293 (O1:K56) to levels of 10^{4-5} cells per gram and processed at 293 MPa (43K PSI) for 90, 120, 150, 180 and 210 s. Populations of *V. parahaemolyticus* in oysters after processes were analyzed with the 5-tube most probable number (MPN) method. Negative results obtained by the MPN method were confirmed with a multiplex PCR detecting genes encoding thermolabile hemolysin (*tl*), thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*). A HPP of 293 MPa for 120 sec at groundwater temperature (8±1 °C) was identified capable of achieving greater than 3.52-log reductions of *V. parahaemolyticus* in Pacific oysters. Oysters processed at 293 MPa for 120 sec had a shelf life of 6-8 days when stored at 5 °C or 16-18 days when stored in ice. This HPP can be adopted by the shellfish industry as a post harvest process to eliminate *V. parahaemolyticus* in raw oysters.

Keywords: *Vibrio parahaemolyticus*, high pressure processing, oysters, process validation, seafood safety.

3.2 Introduction

High hydrostatic pressure processing (HPP) can be applied to food processing for inactivating microorganisms without apparent changes in flavor, color, and nutritional constituents (Chen and others 2006; Considine and others 2008; Corbo and others 2009; Grove and others 2006). The application of HPP in food processing was first investigated in 1899 and the process has been recognized as a non-thermal processing technique for preservation of foods for over a century (Hite 1899). Through the advancement of the technology and equipment design in the last decade, HPP has become an economically feasible means for preserving quality and inactivating pathogens of a number of foods such as oysters, fish, meat, jams, fruit juices, salsa, guacamole, rice products and milk (Murchie and others 2005; Torres and Velazquez 2005).

Vibrio parahaemolyticus is a human pathogen which is widely distributed in coastal and estuarine environments. This pathogen is frequently isolated from shellfish and can cause acute gastroenteritis resulting from consumption of raw shellfish, particularly oysters. Outbreaks of *V. parahaemolyticus* infections linked to the consumption of contaminated raw oysters have been reported in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions of the United States between 1997 and 1998, in Alaska in 2004, and in New York, Oregon and Washington in 2006 (CDC 1998, 1999, 2006; McLaughlin and others 2005). The United States Centers for Disease Control and Prevention (CDC) reported a 78% increase in the incidence of *Vibrio*-associated infections in 2006 from the 1996-1998 baselines and estimated that

4,500 cases of *V. parahaemolyticus* infection occur each year in the U.S. (CDC 2007, 2008). The increased incidence of *V. parahaemolyticus* infection is a public health concern, particularly for people who eat raw or undercooked oysters.

The U.S. produces more than 27 million pounds of oysters each year (Hardesty 2001). Most of them are stored at refrigeration temperatures and sold live or shucked without further processing. It is estimated that 20 million Americans consume raw shellfish, making raw shellfish the biggest seafood hazard in the U.S. (Rippey 1994). To minimize *V. parahaemolyticus* infection associated with shellfish consumption, the National Shellfish Sanitation Program (NSSP) established a guideline for post harvest processing, which requires a process to reduce *V. parahaemolyticus* in shellfish to an end point of less than 30 per gram with a minimum 3.52-log reduction (FDA 2007). In Japan, oysters intended to be eaten raw should not contain more than 100 MPN/g of *V. parahaemolyticus* (JETRO 2009). However, the European Union Commission Regulation (EC) No. 2073/2005, which sets microbiological criteria for foodstuffs, sets no specific criteria for pathogenic *V. vulnificus* and *V. parahaemolyticus* in seafood traded within the European Community (EC 2005).

Several studies have reported that HPP could be applied as a post harvest treatment of raw oysters to inactivate *V. parahaemolyticus* without adverse affects of sensory properties with an added benefit of shucking oysters during the process (Cruz-Romero and others 2008a, 2008b; Prapaiwong 2009). Styles et al. (1991) reported that a process of 170 MPa for 10 and 30 min eliminated *V. parahaemolyticus* (10^6 CFU/mL) in clam juice and phosphate buffer, respectively. Berlin et al. (1999) showed

that a treatment of 200 MPa for 10 min at 25 °C achieved greater than 6-log reductions of *V. parahaemolyticus* in homogenized raw oysters. Cook (2003) reported that a treatment of 300 MPa for 180 sec at 28 °C could achieve a 5-log reduction of *V. parahaemolyticus* in oysters. Kural et al. (2008) studied effects of temperature and pressure levels on *V. parahaemolyticus* inactivation and reported treatments of 350 MPa or higher pressures for 120 sec at temperatures between 1 and 35 °C and of 300 MPa or higher pressures for 120 sec at 40 °C could achieve a 5-log reduction of *V. parahaemolyticus* in oysters. A treatment of 345 MPa for 7.7 min was also reported capable of reducing *V. parahaemolyticus* in oysters by 5.4-log (Koo and others 2006).

While numerous studies have reported inactivation of *V. parahaemolyticus* in oysters by HPP under various conditions, no study has been conducted to identify a condition for achieving greater than 3.52-log reductions of *V. parahaemolyticus* in raw oysters for commercial application and validate the process at a commercial scale according to the NSSP's post harvest processing (PHP) validation/verification interim guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus* (FDA 2007).

The shellfish industry in the Pacific Northwest has been using HPP of 293 MPa (43K PSI) at groundwater temperature for shucking oysters. It would be beneficial to the industry to identify a processing condition at such a pressure level to eliminate *V. parahaemolyticus* contamination in oysters. This study was conducted to identify and validate a HPP for commercial application of achieving greater than 3.52-log reductions of *V. parahaemolyticus* in the Pacific oysters (*Crassostrea gigas*), and determine the shelf life of oysters stored at 5 °C or in ice after the process.

3.3 Materials and methods

3.3.1 *Vibrio* culture inoculum

Clinical strain of *V. parahaemolyticus* 10293 (O1:K56), a 1997 Washington outbreak strain, obtained from the collection of the Food and Drug Administration Pacific Regional Laboratory Northwest (Bothell, Wash.) was used in this study. The culture was grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Spark, Md.) containing 1.5% NaCl (TSB-Salt) at 37 °C for 18 to 24 h. Enriched culture was streaked to individual plates of tryptic soy agar (TSA; Difco, Becton Dickinson) containing 1.5% NaCl (TSA-Salt) and incubated at 37 °C for 18 to 24 h. After incubation, a single colony was picked from a plate and transferred to a tube of TSB-Salt broth for incubation at 37 °C for 4 h. The enriched culture was harvested by centrifugation at 3,000×g (Sorvall RC-5B, Kendro Laboratory Products, Newtown, Conn.) at 5 °C for 15 min. Pelleted cells were resuspended in 50 mL of sterile salt solution (2%) to produce a culture of approximately 10^{8-9} CFU/mL as determined by the pour-plate method using TSA-Salt.

3.3.2 Oyster preparation

Pacific oysters (*Crassostrea gigas*) were obtained from a local shellfish farm and delivered to the laboratory in a cooler on the day of harvest. The oysters were placed in a rectangular high-density polyethlylene (HDPE) tank (45.72 by 30.48 by 30.48 cm; Nalgene, Rochester, N.Y.) containing artificial seawater (ASW) at room

temperature (20-22 ˚C) for 2 to 4 h before being inoculated with *V. parahaemolyticus*. The ASW (salinity: 30 ppt) was prepared by dissolving Instant Ocean Salts (Aquatic Eco-System, Inc., Apopka, Fla) in deionized water according to manufacturer's instructions.

3.3.3 Accumulation of *V. parahaemolyticus* in oysters

Oysters were transferred to another HDPE tank containing fresh ASW with *V. parahaemolyticus* culture at a level of approximately 10^{4-5} CFU/ml. Accumulation of *V. parahaemolyticus* in oysters was conducted at room temperature overnight (16 to 18 h) with water being circulated at a flow rate of approximately 12 L/h. Air was pumped into the solution to keep dissolved oxygen (DO) levels favorable for oyster pumping and uptake of *V. parahaemolyticus*.

3.3.4 High pressure processing of oysters

Inoculated oysters were individually banded with plastic strips, placed in a cylinder filled with groundwater (8±1 °C), and processed at 293 MPa (43K PSI) for 90, 120, 150, 180 or 210 s in a Flow International Twin 45L high pressure unit equipped with a 25× Waterknife pump. Each process included pressure build-up time (20 s), holding time (90 - 210 s) and pressure release time (25 s). Initial levels of *V. parahaemolyticus* in oysters before HPP were determined with five oysters. *V. parahaemolyticus* in oysters after HPP were analyzed with five samples each consisted of a composite of 12 oysters (totally 60 oysters).

3.3.5 Validation of high pressure processing of oysters

A minimum HPP (293 MPa at 8±1 °C for 120 s) to produce greater than 3.52-log reductions of *V. parahaemolyticus* in oysters was selected for process validation to verify the efficacy of the process in reducing *V. parahaemolyticus* in oyster according to the NSSP's post harvest processing (PHP) validation/verification interim guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus*. The validation was conducted through three studies with each separated by one week. Oysters were inoculated with *V. parahaemolyticus* to a level of at least 10^4 MPN/g as previously described. For each validation, initial levels of *V. parahaemolyticus* in oysters before HPP were calculated based on the adjusted geometric mean (AGM) of the MPN/g of four samples each consisted of a composite of 12 oysters (totally 48 oysters). The AGM was given by: AGM = the geometric mean (MPN/g) of the four samples multiplied by an adjustment factor of 1.3. Ten samples each consisted of a composite of 12 oysters (totally 120 oysters) were used for determination of *V. parahaemolyticus* in oysters after HPP.

3.3.6 Shelf life of high pressure processed oyster

Oysters processed at 293 MPa for 120 s were placed in plastic bags and stored at 5±1 °C or held in a cooler covered with ice (2.54 cm thick) for 7 days followed by storage in plastic bags at 5±1 °C. Oysters not processed by high pressure were stored in plastic bags at 5±1 °C as controls. To determine if cleaning of oysters before HPP would increase shelf life of oysters after HPP, a second batch of oysters were scrubbed

under running water to remove visible mud and debris on shell before being processed at 293 MPa for 120 sec. The oysters were placed in plastic bags after HPP and stored in a cooler covered with ice (2.54 cm thick). Oysters without the cleaning process before HPP were used as controls. Aerobic plate counts (APC) and psychrotrophic plate counts (PPC) in oysters during storage were determined every other day for up to 24 days. Total and fecal coliforms in oysters were determined every six days for up to 18 days. Four oysters were analyzed at each testing point and results were reported as means of four determinations. Shelf life of oyster was determined when APC increased to 10^7 CFU/g (Kim and others 2002).

3.3.7 Microbiological tests

3.3.7.1 Detection of *V. parahaemolyticus*

V. parahaemolyticus analysis was conducted by the five-tube most probable number (MPN) method described in the Food and Drug Administration's Bacteriological Analytical Manual (FDA 2001). Oyster meat was placed in a sterile blender jar and blended with an equal volume of sterile Phosphate Buffered Saline Solution (PBS, Brisbane, Calif.) at high speed for 90 sec using a two-speed laboratory blender (Waring Laboratory, Torrington, Conn.) to prepare a 1:2 dilution sample suspension. Twenty grams of the homogenized oyster were mixed with 80 ml of phosphate buffer saline (PBS) to prepare a 1:10 dilution. Additional 10-fold dilutions of samples were prepared with PBS. All sample dilutions were individually inoculated into five tubes of alkaline peptone water (APW). Inoculated APW tubes were

incubated at 35 to 37 °C for 16 to 18 h and one loopful (3mm) of enriched APW from a turbid tube was streaked onto individual thiosulfate-citrate-bile salts-sucrose agar plates (TCBS). The TCBS plates were incubated at 35 to 37 °C for 18 to 24 h. Formation of colonies that are round (2- to 3- mm diameter) and green or bluish on TCBS were considered positive for *V. parahaemolyticus*. Total populations of *V. parahaemolyticus* in oysters were determined by converting the numbers of APW tubes that were positive for *V. parahaemolyticus* to MPN/g using an MPN table.

3.3.7.2 Detection of *V. parahaemolyticus* with polymerase chain reaction

A multiplex polymerase chain reaction (PCR) detecting *tl*, *tdh*, and *trh* genes of *V. parahaemolyticus* was used to confirm the negative results for *V. parahaemolyticus* obtained from the MPN method by analyzing APW enrichments of oysters after HPP according to the method of Kaysner and DePaola (2001) and Bej et al. (1999) as previously reported (Duan and Su 2005). The oligonucleotide primers for the *tl* (L-*tl*: 5'-aaa gcg gat tat gca gaa gca ctg-3' and R-*tl*: 5'-gct act ttc tag cat ttt ctc tgc-3'), the *tdh* (L-*tdh*: 5'-gta aag gtc tct gac ttt tgg ac-3' and R-*tdh*: 5'-tgg aat aga acc ttc atc ttc acc-3') and the *trh* (L-*trh*: 5'-ttg gct tcg ata ttt tca gta tct-3' and R-*trh*: 5'-cat aac aaa cat atg ccc att tcc g-3') genes were commercially synthesized (Integrated DNA Technologies, Coralville, Iowa) according to the nucleotide sequences reported in previous studies (Taniguchi and others 1985; Nishibuchi and Kaper 1985; Honda and Iida 1993). Presence of *V. parahaemolyticus* cells in a sample resulted in occurrence of a band of 450 bp (*tl*), 269 bp (*tdh*), or 500 bp (*trh*) of PCR products on the gel.

3.3.7.3 Aerobic plate counts and psychrotrophic plate counts

Total aerobic plate counts (APC) of oysters were determined by the pour-plate method using tryptic soy agar (TSA) with incubation at 35 °C for 48 ± 2 h. Psychrotrophic plate counts (PPC) were also determined by the pour-plate method using TSA and incubation at 7±1 °C for 10 days (Cousin and others 2001). Results (CFU/g) were reported as means of four determinations. Oysters were considered spoiled when APC increased to greater than 10^7 CFU/g (Kim and others 2002).

3.3.7.4 Coliforms

Total and fecal coliforms were analyzed with the five-tube MPN method described in FDA's Bacteriological Analytical Manual (FDA 2002). Dilutions of samples were first inoculated to lauryl tryptose (LST) broth with incubation at 35ºC for 24 ± 2 h. Each LST tube that turned turbid with gas production was then inoculated into brilliant green lactose bile (BGLB) broth and incubated at 35 ºC for up to 48 h. Production of gas in a BGLB tube confirmed presence of coliform in samples. Presence of fecal coliform in a sample was determined by transferring BGLB tubes that were positive for coliform to EC broth and incubating at 44.5±0.2 ºC for up to 48 h. An EC tube that showed sign of growth with gas production was concluded positive of fecal coliform.

3.3.8 Data analysis

Results of microbiological tests were transformed into log values for analysis. Reductions of *V. parahaemolyticus* in oysters after HPP were calculated by subtracting mean counts of *V. parahaemolyticus* in oysters after HPP from the mean counts of *V. parahaemolyticus* in oysters before HPP. Statistical analysis was conducted to determine differences in shelf life between oysters with or without scrubbing before HPP and stored in ice using *t*-test (S-plus, Insightful Corp., Seattle, Wash.). Significant differences between means of treatments were determined at a level of $p<0.05$.

3.4 Results

3.4.1 Effects of HPP on inactivating *V. parahaemolyticus* in oysters

V. parahaemolyticus in oysters were reduced by 2.70-, >3.53-, >3.74-, >4.71-, and >4.83- log MPN/g after HPP at 293 MPa at ambient temperature (8±1 °C) for 90, 120, 150, 180 and 210 s, respectively (Table 3.1). A HPP at 293 MPa for 120 s or longer was capable of inactivating *V. parahaemolyticus* in oysters by greater than 3.52-log MPN/g. Therefore, the process of 293 MPa for 120 s at 8±1 °C was considered a minimum treatment for achieving greater than 3.52-log reductions of *V. parahaemolyticus* in Pacific oysters.

Table 3.1 Effects of high pressure processing (293 MPa at 8±1 ºC) on *Vibrio parahaemolyticus* (Vp) in Pacific oysters.

Processing time (s)	Vp before HPP [a] (log MPN/g)	Vp after HPP [b] (log MPN/g)	Reduction Levels [c] (log MPN/g)
90	5.29±0.43	2.42±0.08	2.70
120	3.79±0.18	<0.26[d]	> 3.53
150	4.00±0.26	<0.26	> 3.74
180	4.97±0.44	<0.26	> 4.71
210	5.09±0.39	<0.26	> 4.83

[a] Data are means of five determinations ± standard deviation.
[b] Data are means of five determinations ± standard deviation.
[c] Reductions of Vp in oysters after HPP – mean counts of "Vp before HPP" minus mean counts of "Vp after HPP".
[d] Non-detectable based on a detection limit of 1.8 MPN/g.

3.4.2 Validation of high pressure processing of oysters

For process validation, oysters were inoculated with *V. parahaemolyticus* to a mean value of 42,900 (log 4.63), 19,825 (log 4.30) or 90,025 (log 4.95) MPN/g in three studies. All three studies demonstrated that HPP of 293 MPa for 120 s at 8±1 °C reduced *V. parahaemolyticus* in Pacific oysters to smaller than 30 MPN/g with greater than 3.52-log MPN/g reductions (Table 3.2). The reductions of *V. parahaemolyticus* in oysters ranged from 3.98- to greater than 4.69-log MPN/g, except one sample yielding 3.52-log reduction in the first study. Negative (non-detectable) results obtained from the MPN method were validated by the multiplex PCR assay targeting *tl*, *tdh*, and *trh* genes of *V. parahaemolyticus*. No *V. parahaemolyticus* was detected by the PCR in any of the enriched cultures yielding negative results in the MPN method (results not shown). No apparent difference in the appearance and smell of oysters was noted before and after HPP.

Table 3.2 Validations of HPP (293 MPa at 8±1 °C for 120 s) for inactivating *V. parahaemolyticus* (Vp) in Pacific oysters.

Sample	Study 1		Study 2		Study 3	
	Vp in oyster after HPP MPN/g (Log MPN/g)	Reductions [a] (Log MPN/g)	Vp in oyster after HPP MPN/g (Log MPN/g)	Reductions [b] (Log MPN/g)	Vp in oyster after HPP MPN/g (Log MPN/g)	Reductions [c] (Log MPN/g)
1	2.0 (0.30)	4.33	<1.8 (<0.26)	>4.04	<1.8 (<0.26)	>4.69
2	13 (1.11)	3.52	2.0 (0.30)	4.00	<1.8 (<0.26)	>4.69
3	2.0 (0.30)	4.33	<1.8 (<0.26)	>4.04	2.0 (0.30)	4.65
4	2.0 (0.30)	4.33	<1.8 (<0.26)	>4.04	2.0 (0.30)	4.65
5	<1.8 (<0.26)	>4.37	<1.8 (<0.26)	>4.04	4.5 (0.65)	4.30
6	4.5 (0.65)	3.98	<1.8 (<0.26)	>4.04	<1.8 (<0.26)	>4.69
7	<1.8 (<0.26)	>4.37	<1.8 (<0.26)	>4.04	2.0 (0.30)	4.65
8	<1.8 (<0.26)	>4.37	<1.8 (<0.26)	>4.04	<1.8 (<0.26)	>4.69
9	<1.8 (<0.26)	>4.37	<1.8 (<0.26)	>4.04	7.8 (0.89)	4.06
10	<1.8 (<0.26)	>4.37	<1.8 (<0.26)	>4.04	2.0 (0.30)	4.65

[a] Reductions from initial populations of *V. parahaemolyticus* (4.63-log MPN/g) in oysters.
[b] Reductions from initial populations of *V. parahaemolyticus* (4.30-log MPN/g) in oysters.
[c] Reductions from initial populations of *V. parahaemolyticus* (4.95-log MPN/g) in oysters.

3.4.3 Changes of bacterial counts in oysters during storage

Changes in APC of oysters processed by 293 MPa for 120 s at 8±1 °C during storage at 5 °C are shown in Figure 3.1. Oysters had initial APC of 3.06-log CFU/g and very low levels of total coliforms (no more than 7.8 MPN/g) and fecal coliforms (smaller than 1.8 MPN/g) (data not shown). The APC in oysters were reduced to 2.52-log CFU/g after the pressure treatment but increased gradually during the storage. Oysters processed with high pressure had a shelf life of about 8 days at 5 °C, which was much shorter than that (18 days) of live oysters stored at the same temperature. Storing pressure-processed oysters in ice for one week followed by at 5 °C increased the shelf life of products to 14 days. Multiplication of bacteria in pressure-processed oysters was retarded when oysters were kept in ice. However, the APC increased rapidly in oysters after they were moved to 5 °C for storage.

3.4.4 Effect of oyster cleaning before HPP on shelf life of oysters stored in ice

Washing oysters before HPP did not result in significantly lower APC in oysters after the pressure treatment when compared with those of oysters not washed before HPP. The oysters had initial APC of 3.19-log CFU/g, which were reduced to 2.53-log CFU/g in washed oysters and to 2.58-log CFU/g in non-washed oysters after HPP (Figure 3.2). However, cleaning of oysters before HPP resulted in significantly lower psychrotrophic bacteria in oysters after the pressure treatment. PPC in oysters were reduced from initial levels of 3.99-log CFU/g to 2.50-log CFU/g in washed oysters and to 2.98-log CFU/g in non-washed oysters after the pressure treatment

(Figure 3.2). Both APC and PPC of oysters washed before HPP were always lower than those of oysters not washed before the treatment during the storage study. Results of total coliforms were all less than 12.0 MPN/g in all oysters (data not shown). Washing oysters before HPP increased the shelf life of oysters stored in ice to 17 days.

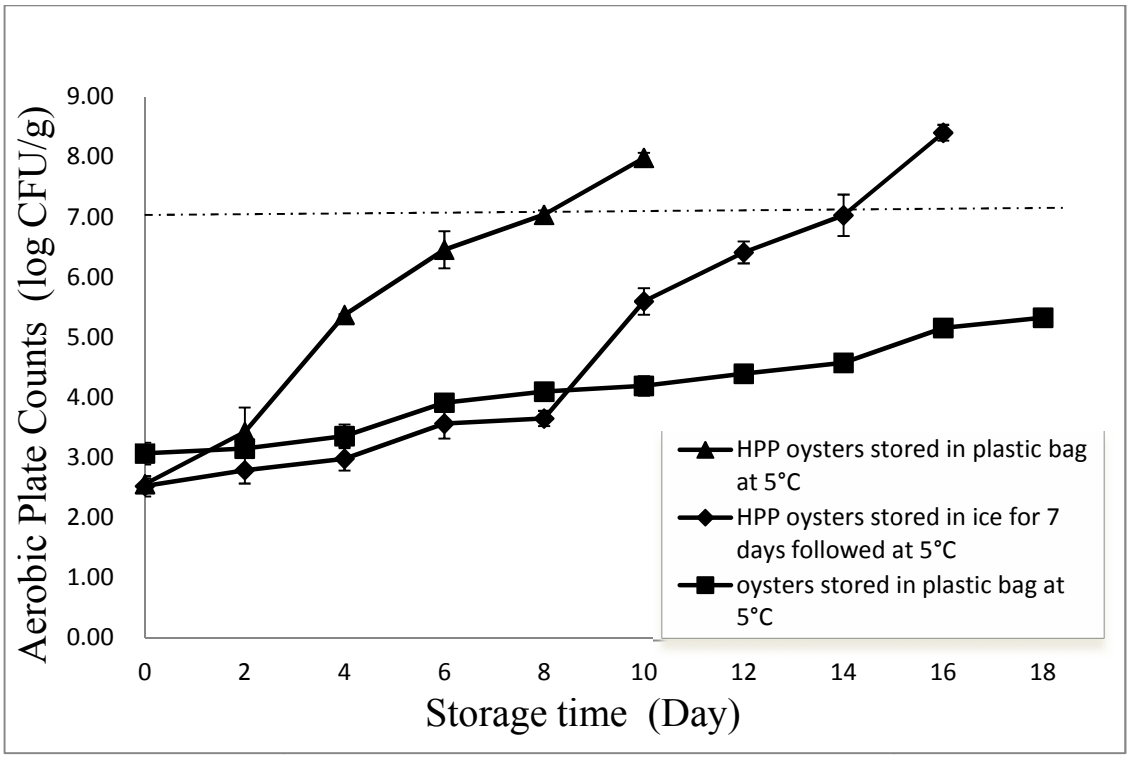

Figure 3.1 Changes of aerobic plate counts in live and high pressure (293 MPa at 8±1 ºC for 120 s) treated oysters stored at 5 °C. Data are mean values of four determinations ± standard deviation. The dotted line indicates spoilage of products.

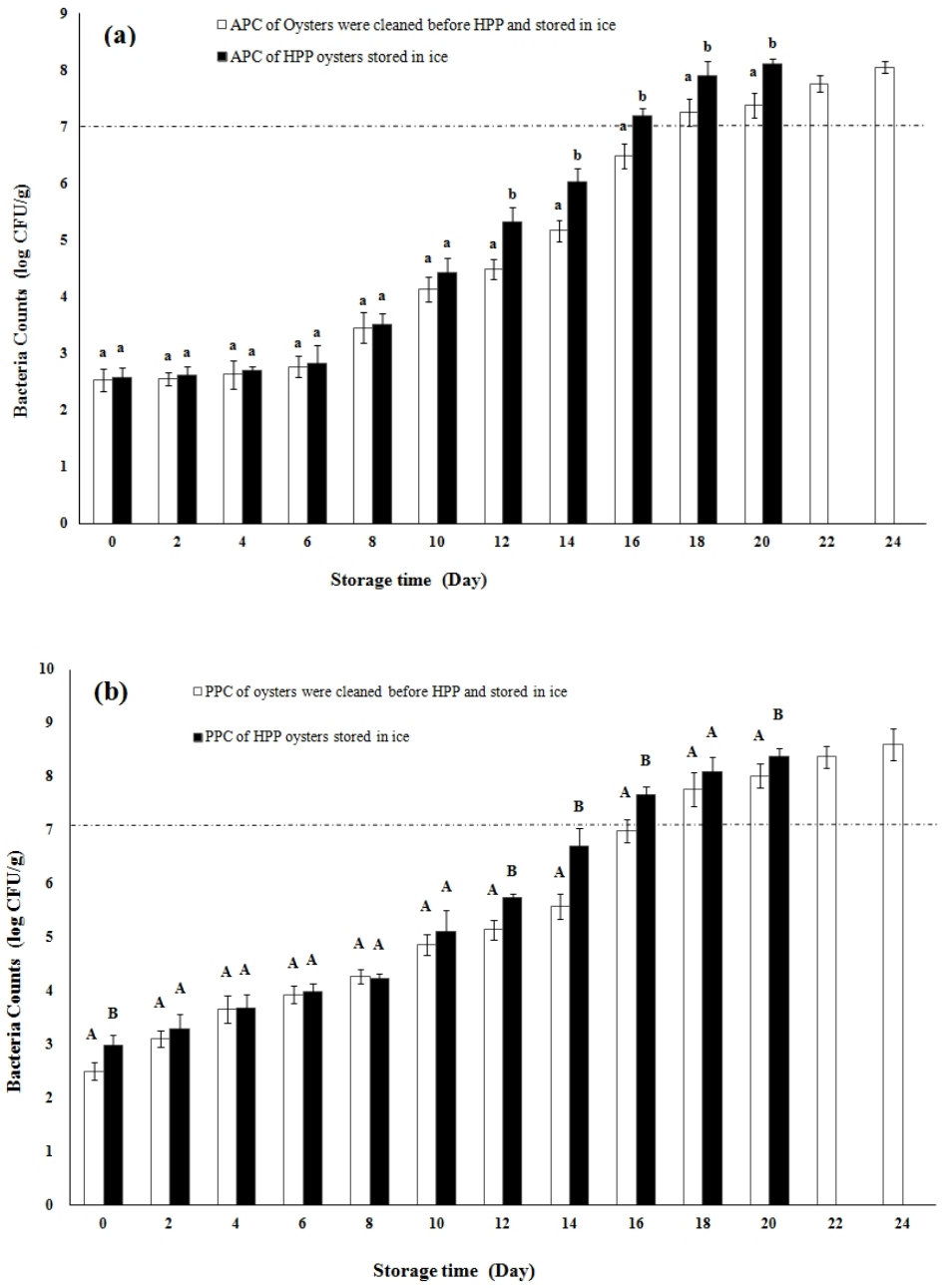

Figure 3.2 Changes of (a) aerobic plate counts and (b) psychrotrophic plate counts in high pressure (293 MPa at 8±1 ºC for 120 s) treated oysters stored in ice. Data are mean values of four determinations ± standard deviation. Initial levels of aerobic plate counts and psychrotrophic plate counts in oysters before HPP were 3.19 and 3.99 log CFU/g, respectively. Bars with different letter are significantly different (p<0.05). The dotted line indicates spoilage of products.

3.5 Discussion

The effectiveness of HPP on inactivating bacteria depends on processing parameters such as pressure level, treatment time, temperature, and physiological state of a microorganism (Grove and others 2006). It has been reported that *Vibrio* species are relatively sensitive to pressure and can be inactivated by treatments at lower than 350 MPa (Chen and others 2006). Kural et al. (2008) reported that a treatment at 300 MPa for 120 sec between 1 and 20 ºC reduced the levels of *V. parahaemolyticus* inoculated into shucked oyster meat by 3.5-3.7 log MPN/g. Cook (2003) showed that *V. parahaemolyticus* O3:K6 inoculated into Pacific oysters could be reduced by 4 log units at 300 MPa for 120 sec or by 3.52 log units at 275 MPa for 240 sec. In addition, moderate pressure treatments between 240 and 350 MPa can also be utilized to destroy the adductor muscle of oysters and thus replace the laborious hand-shucking (Torres and Velazquez 2005). However, no studies have been conducted to validate a HPP for reducing *V. parahaemolyticus* in oysters to an end point of less than 30 per gram with a minimum 3.52-log reduction. This study identified a HPP of 293 MPa for 120 sec at 8±1 °C could deliver a reduction of *V. parahaemolyticus* in Pacific oysters by greater than 3.52 log MPN/g and validated the process according to the NSSP's post harvest processing (PHP) validation/verification interim guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus* (FDA 2007).

The efficacy of HPP in inactivating microorganisms can also be affected by the processing temperature. Kural and Chen (2008) reported that temperatures at lower than 20 °C or higher than 30 °C substantially increased pressure inactivation of *Vibrio*

vulnificus. Similarly, enhanced pressure inactivation of *V. parahaemolyticus* was observed at temperatures no less than 30 °C (Kural and others 2008). While processing of oysters under pressures at elevated temperatures increases the efficacy in inactivating *V. parahaemolyticus*, it also increases the costs of operation and may not be economically feasible to the shellfish industry. This study demonstrated that a HPP (293 MPa for 120 sec) at groundwater temperature (8±1 °C) can be adopted by the shellfish industry to reduce *V. parahaemolyticus* in oysters to less than 30 per gram with greater than 3.52-log reductions. However, the process only reduced total bacterial populations in oysters from 3.0- to 2.5- log CFU/g (Figure 3.1). Therefore, shelf life of oysters after the process is highly dependent on the storage condition. The bacteria, which survived the pressure treatment, were able to multiply in dead oysters stored at 5 °C and caused product spoilage in 8 days (Figure 3.1). This is similar to the shelf life (8 days) of shucked oysters stored at 5 °C observed by Cao et al. (2009). Since many psychrotrophic bacteria survived the process of 293 MPa for 120 sec, oysters need to be stored below 5 °C to retard growth of psychrotrophic bacteria after the process. Shelf life of the oysters was increased to 14 days by storing oysters in ice for one week followed by at 5 °C (Figure 3.1). An alternative to increase the shelf life of oysters is to process oysters at pressures higher than 293 MPa or at 293 MPa for a longer time. It has been reported that oysters processed with 400 MPa for 5 min at 20 °C and stored on ice had a shelf life of 21 days, while oysters processed with 500 MPa for 5 min at 20 °C has a shelf life of 17 days stored at 2 °C (Cruz-Romero and others 2008a, 2008b). However, processing oysters at higher pressures might cause

adverse effects to sensory characteristics of oysters. A longer processing time will reduce the processing efficacy and add costs to production.

In addition to processing oysters at higher pressures and for a longer treatment time, removing visible mud and debris on oyster shell before pressure treatments might also help reduce total bacterial counts of oysters after HPP. It is known that the adductor muscle of oysters can be destroyed by HPP at 240–275 MPa (He and others 2002). Therefore, oysters processed at 293 MPa could no longer hold shell tightly despite banding before processing. Oyster meat could be contaminated by bacteria discharged from shell into water in the pressure chamber during HPP. Although we did not observe a significant reduction of total bacterial counts between washed and non-washed oysters immediately after the HPP, cleaning of oysters before HPP resulted in significantly lower psychrotrophic bacteria in oysters after the pressure treatment and extended the shelf life of oysters stored in ice to 17 days (Figure 3.2).

In conclusion, a HPP of 293 MPa for 120 s at 8±1 °C was identified and validated for reducing *V. parahaemolyticus* in Pacific oysters to smaller than 30 per gram with greater than 3.52-log reductions. Oysters processed under such a condition and stored in plastic bags covered in ice had a shelf life of approximate 17 days. This HPP can be adopted by the shellfish industry as a post harvest processing to minimize *V. parahaemolyticus* infection associated with raw oyster consumption.

3.6 Acknowledgements

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Chapter 4

General Discussion

Recent advancement in the development of high pressure processing (HPP) technology has made HPP a means of non-thermal processing for the food industry. Although a number of products have been commercially produced with HPP with extended shelf life and without significant changes in quality or sensory characteristics, the application of HPP in food processing is restricted by a numbers of issues such as the capital cost of the pressure unit and the resistance to pressure of certain bacteria and microbial spores.

The most critical factor limiting the application of HPP in food processing is the high costs of the initial investment of a high pressure system, which may not be affordable by most small food producers/processor including most oyster producers. The capital costs of a commercial high pressure system are typically around $500,000 to 2.5 million, depending on the capacity and extent of automation of the equipment (Balasubramaniam and Farkas 2008). However, HPP has a relatively low operating cost (roughly estimated at $ 0.05–0.5 per liter or kilogram), depending on the operating parameters and the scale of operation, when compared with thermal processes (Thakur and Nelson 1998; Balasubramaniam 2003). Even though, the large amounts of capital investment may be offset by running the HPP operation at the full capacity and by managing the pressure and time combination used to minimize processing costs (Rogers 1999). It is expected that the cost of HPP will go down as a

consequence of further development in the technology, making HPP technology accessible to more and more food producers.

While HPP can be utilized to inactivate bacteria, including pathogens, in food products, its efficacy in microbial inactivation depends on the processing conditions and a process may not always cause death of bacterial cells resulted from damage to the cell membrane. Sub-lethally injured cells formed during HPP may recover and multiply through repair of outer and cytoplasmic membrane damage when biosynthetic requirements are met during subsequent storage. This phenomenon often leads to an over-estimation of microbial inactivation because the counts in a food determined immediately after HPP will be lower than those observed after the recovery of injured cells (Cheftel 1995). Therefore, it is a challenge for the food industries to achieve a pasteurization or sterilization outcome using HPP alone (Chilton and others 2001).

HPP can be combined with other processes, such as thermal treatment, ultrasound, and antimicrobial agents including lacticin 3147 (Ross and others 2000), lactoperoxidase (Garcia-Graells and others 2003), and nisin (Ponce and others 1998; Black and others 2005), to increase the bacteriocidal effects of the process at the same pressure levels (Rastogi and others 2007). In another way, HPP can be performed at a lower pressure to reduce levels of pathogens and the antimicrobials can prevent the recovery or inhibit growth of pressure-injured bacteria during storage. Meanwhile, HPP may make antimicrobials easier to enter the cells and perform preservation action. Therefore, combining HPP with those antimicrobial compounds has worked

synergistically to increase the inactivation efficacy. The combined effect of high pressure (500 MPa, 15 min, 20 °C) and natural antimicrobial peptides (lysozyme, 400 μg/ml and nisin, 400 μg/ml) resulted in increased lethality for *Escherichia coli* in milk (Garcia and others 1999).

To achieve the balance between food safety and food quality through HPP application, it is critical to optimize conditions for a HPP applicable to a particular food. For oyster processing, many factors including species of oysters, pretreatment method (e.g. shelled or shucked oyster), processing temperatures, and variations among different equipments in pressure come-up or release time have been responsible for controversial results observed from different studies for the efficacies of HPP in eliminating *V. parahaemolyticus*. Therefore, optimal conditions for HPP of shellfish to achieve target reductions of *Vibrio* spp. without a significant change in the quality need to be identified and validated. The development of mathematical models for prediction of bacteria inactivation by HPP would be a useful tool in optimizing process conditions and constructing hazard analysis critical control point programs to guarantee food safety (Torres and Velazquez 2005; Rastogi and others 2007).

While HPP alone or in combination with other processes can be used to preserve quality and ensure safety of food, the quality or processed food can be greatly influenced by subsequent storage and distribution conditions due to growth of bacteria surviving the process and activities of chemical reactions such as oxidation and biochemical reactions. High pressure processed oysters are usually distributed and

stored at refrigeration temperatures, which need to be carefully monitored in order to give a maximum shelf life of the processed oysters.

Chapter 5

Conclusion

Oyster is a nutrient-rich seafood, but can be contaminated with *Vibrio parahaemolyticus* which occurs naturally in the growing environments. Therefore, there is a risk of developing gastroenteritis linked to consumption of raw oysters. Multiple outbreaks of *V. parahaemolyticus* infections caused by consumption of raw or undercooked shellfish, especially oysters, have been documented over the past years. The threat of *V. parahaemolyticus* infection associated with consumption of raw or undercooked oysters is a major concern for public health and can cause substantial economic losses to the shellfish industry.

High pressure processing (HPP) has been recognized as a non-thermal process for inactivating pathogenic and spoilage microorganisms without apparent changes in flavor, color, and nutritional constituents of foods. While several studies have reported application of HPP for inactivation of *V. parahaemolyticus* in oysters under various conditions, no study has been conducted to identify a HPP condition for commercial application to achieve greater than 3.52-log reductions of *V. parahaemolyticus* in raw oysters and validate the process at a commercial scale according to the National Shellfish Sanitation Program (NSSP)'s post harvest processing (PHP) validation/verification interim guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus* (FDA 2007).

In this study, we identified and validated a HPP at 293 MPa for 120 s at 8±1 °C capable of reducing the levels of *V. parahaemolyticus* in Pacific oysters to smaller than 30 per gram with greater than 3.52-log reductions for commercial operation. Oysters processed under such a condition and stored in plastic bags had a shelf life of approximate 8 days when stored at 5 °C or 16-18 days when stored in ice. This HPP was approved by the FDA as a post harvest process to minimize *V. parahaemolyticus* infection associated with raw oyster consumption.

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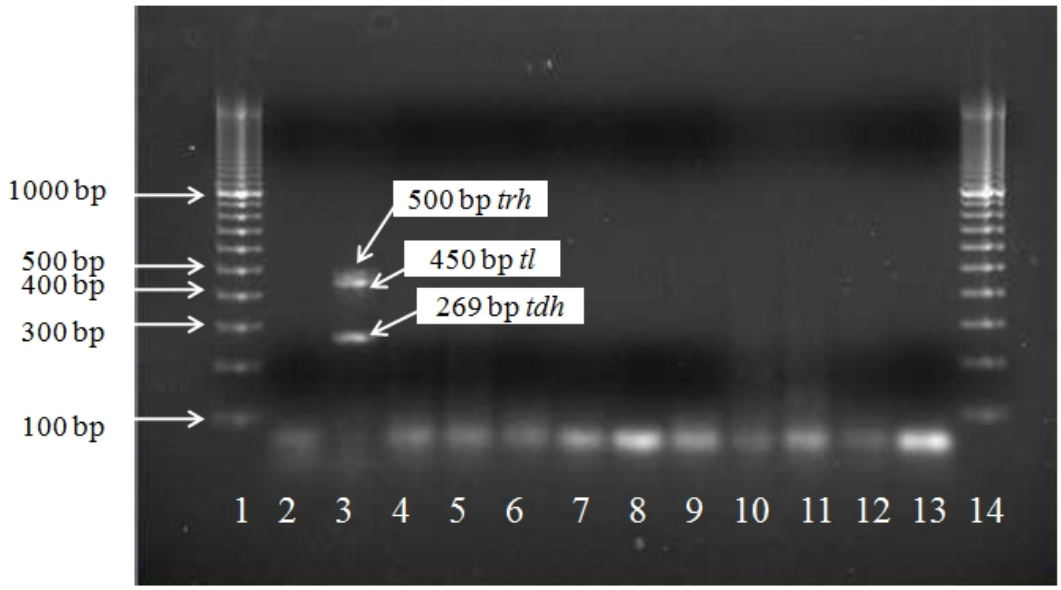
Appendices

Appendix A

Agarose gel electrophoresis of multiplex PCR analyses of *Vibrio parahaemolyticus* in samples yielding negative results by the MPN procedure in validations of HPP (293 MPa at 8±1 ºC for 120 s) for inactivating *V. parahaemolyticus* (Vp) in Pacific oysters described in Chapter 3.



Lanes 1 and 14: PCR molecular ruler;
Lane 2: Negative control;
Lane 3: Positive control (*V. parahaemolyticus strain* 10293);
Lanes 4 – 13: Enriched samples (1-10 from the left) yielding negative results for *V. parahaemolyticus* by the MPN procedure.

Appendix B

Explanation of the storage conditions used in the shelf life study described in Chapter 3.

The purpose of holding high pressure processed oysters in a cooler covered with ice (2.54 cm thick) for 7 days followed by storage in plastic bags at 5±1 °C was to compare the process with the traditionally used storage condition at 5±1 °C on increasing shelf life of the products.